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Osteoblasts increase proliferation and release of pro-angiogenic interleukin 8 by native human acute myelogenous leukemia blasts

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A B S T R A C T

Background and Objectives. Interactions between acute myelogenous leukemia (AML) blasts and non-leukemic cells in the bone marrow seem to be important for both disease development and susceptibility to chemotherapy. Recent studies have focused on the endothelial cells, but other non-leukemic cells may also be involved. In the present study we investigated how osteoblasts affect native human AML blasts.

Design and Methods. AML cells were derived from a large group of consecutive patients. The AML blasts and osteoblastic sarcoma cell lines (Cal72, SJSA-1) were incubated together in different chambers separated by a semipermeable membrane. We investigated effects of co-culture on proliferation, apoptosis and cytokine release.

Results. The cross-talk between these two cell populations, achieved via release of soluble mediators, resulted in increased AML blast proliferation, including increased proliferation of clonogenic progenitors, but did not affect spontaneous *in vitro* apoptosis. Both interleukin (IL) 1-β and granulocyte-macrophage colony-stimulating factor were involved in this growth-enhancing cross-talk, and normal osteoblasts could also increase the AML blast proliferation. Furthermore, co-culture of AML blasts with osteoblastic sarcoma cells as well as normal osteoblasts increased the levels of the pro-angiogenic mediator IL8.

Interpretation and Conclusions. Our *in vitro* results suggest that the release of soluble mediators by osteoblasts supports leukemic hematopoiesis through two major mechanisms: (i) direct enhancement of AML blast proliferation; and (ii) enhanced angiogenesis caused by increased IL8 levels.

Key words: acute myelogenous leukemia, osteoblasts, apoptosis, interleukin 8.

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Acute myelogenous leukemia (AML) is an aggressive disorder characterized by accumulation of immature malignant cells in the bone marrow.[1] Leukemia relapse is an important cause of death both in patients receiving conventional therapy and in those managed with allogeneic stem cell transplantation, but the relapse risk differs considerably between patients and depends on genetic abnormalities (especially cytogenetic abnormalities and mutations of the Flt3 gene)[2-6] as well as the *in vivo* susceptibility of AML blasts to chemotherapy.[2] Recent clinical studies suggest that local angiogenesis with increased bone marrow vessel density is important both for disease development and chemosensitivity in AML.[7-10]

Thus, the development of AML depends on (i) malignant transformation to leukemic stem cells with high proliferative capacity; and (ii) bone marrow angiogenesis, which supports the progression from microscopic to clinical disease. The observation of decreased spontaneous as well as chemotherapy-induced apoptosis of native human AML blasts after co-culture with a virus-transformed stromal cell line further supports the hypothesis that interactions between AML blasts and other neighboring non-leukemic cells are important for both leukemic hematopoiesis and chemosensitivity.[11] However, the stromal cell population is heterogeneous and includes fibroblasts, fat cells, macrophages and endothelial cells.[11,12] The possible involvement of these various non-leukemic cells in leukemic hematopoiesis should be further explored.

Various types of non-leukemic cells contribute to the bone marrow microenvironment in AML,[12] one of these being osteoblasts, which have many characteristics in common with fibroblasts.[13] Previous

studies suggest that osteoblasts can support normal hematopoiesis,[14-17] but the possible importance of these cells in leukemic hematopoiesis has not been examined. In the present study we used *in vitro* models to investigate the effects of osteoblasts on proliferation, apoptosis and release of pro-angiogenic interleukin-8 (IL8) by native human AML blasts.

Design and Methods

Patients

AML patients. AML blasts were derived from 50 consecutive patients with high peripheral blood blast counts. All patients had at least 80% blasts among their circulating leukocytes. The characteristics of the patients are presented in Table 1.

Patients with acute lymphoblastic leukemia (ALL). Leukemia blasts were also derived from 8 consecutive patients (Table 2). Seven patients had B-lymphocyte disease.[18]

Preparation of leukemia 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 leukemia blasts among their blood leukocytes (Table 1). The percentage of blasts among leukemic PBMC exceeded 95% for all patients judged by light microscopy, and this high percentage of leukemia blasts was also verified by flow cytometry for 32 randomly selected patients. The cells were stored frozen in liquid nitrogen.[19-21] Our methods for freezing and thawing AML cells have been previously characterized, and more than 70% of the thawed cells are viable as judged by trypan blue exclusion.[21] The purity of the ALL blasts was similar to that of the AML cells.

Non-leukemic cells

Human osteosarcoma cell lines. The cell line Cal72 (Deutsche Sammlung von Zellkulturen und Mikroorganismen; Braunschwaig, Germany) has previously been characterized in detail.[14,22] It has a phenotype close to that of normal osteoblasts with an adherent growth pattern and a broad cytokine release profile.[22] Pilot experiments demonstrated that this cell line releases high levels of IL6, VEGF, HGF and CCL2. The SJSA-1 cell line (American Type Culture Collection, ATCC, Vanassas, VA, USA; ATCC no. CRL-2098) has a similar growth pattern and shows similarities in its cytokine release profile (detectable release of IL1β, IL6, IL8, VEGF and CCL2 during *in vitro* culture). The cell line U2OS (ATCC no HTB96) is also regarded to have an osteoblastic phenotype[15] and showed only minor differences in cytokine release, whereas the lines Saos-2 (ATCC no. HTB-85), SK-ES-1 (ATTC no. HTB-86), 143.98.2 (ATCC no. CCL-11226), and KHOS-32IH (ATCC no. CRL-1546) all showed an epithelial growth pattern or a cytokine release profile different from those of Cal72 and SJSA-1 (*see above*).

Normal human osteoblasts. These cells were obtained frozen in vials (Clonetics-BioWhittaker; Walkersville, MA, USA) and stored frozen in liquid nitrogen until used. The cells were then thawed and used directly in the co-culture assay. The osteoblasts were derived from a healthy 16-year old male Caucasian by an explantation technique. After *in vitro* redifferentiation the population was characterized by (i) positivity when stained with alkaline phosphatase and bone mineralization (von Kossa stain); (ii) 95% purity; and (iii) negativity when tested for mycoplasma, human immunodeficiency virus 1, hepatitis B and hepatitis C (polymerase chain reactions) (distributor's information).

Reagents for tissue culture

Culture medium. The Stem Span SFEM™ medium (referred to as StemSpan™; Stem Cell Technologies; Vancouver, BC, Canada), supplemented with 10% heat-inactivated fetal calf serum (FCS; BioWhitacker) and 100 μg/mL of gentamicin, was used in all co-culture experiments except for the cultures including normal osteoblasts which were prepared in osteoblast growth medium with FCS (Clonetics).

The following media were used for expansion of the cell lines (see above): Ham's F12K medium (HFL1 fibroblasts), McCoy's medium (Saos-2, SK-ES-1 and U2OS sarcoma cell lines), minimum essential medium in Earle's buffered salt solution (KHOS-321H sarcoma cell line) (all from ATCC), RPMI 1640 (Gibco; Paisley, UK; SJSA-1 sarcoma cell line) and Dulbecco's modified Eagle's medium (referred to as DMEM, Gibco; Hs27 fibroblasts, Cal72 and 143.98.2 sarcoma cell lines). All the media were supplemented with 10% FCS and gentamicin 100 μg/mL.

Reagents for cytokine inhibition. Recombinant human IL1 receptor antagonist (IL1RA, R&D Systems, Abingdon, UK) was used at 50 ng/mL. The following cytokine-specific neutralizing antibodies (R&D Systems; neutralization data reported by the distributor) were used: (i) anti-human granulocyte-macrophage colony-stimulating factor (GM-CSF): monoclonal mouse IgG_1 antibody 3209.01, 0.3-0.5 μg/mL of this antibody neutralizing 50% of the biological activity of rhGM-CSF 0.5 ng/mL; (ii) anti-human hepatocyte growth factor (HGF): mouse monoclonal IgG_1 antibody 24612.111; 0.1-0.3 μg/mL neutralizing 50% of the biological activity of rhHGF 100 ng/mL; (iii) anti-human stem cell factor (SCF): polyclonal goat antiserum, 0.05-0.1 μg/mL neutralizing 50% of the biological activity of rhSCF 10 ng/mL. Anti-

Table 1. Clinical and biological characteristics of acute myeloid leukemia patients.

Patient	Sex	Age	Previous malignant or premalignant disease	FAB classification	Membrane molecule[1] expression					Cytogenetic abnormality	Flt3 abnormality[2]	Peripheral blood blast counts[3]
					CD13	CD14	CD15	CD33	CD34			
1.	F	66		AML-M1	+	+	nt	–	nt	nt	–	59.4
2.	M	56		AML-M4	+	–	+	+	–	Normal	–	10.7
3.	F	55		AML-M0	+	–	–	–	+	Normal	ITD	37.4
4.	F	54	Breast cancer	AML-M1	+	–	nt	+	nt	nt	ITD	34.9
5.	F	36		AML-M5	+	–	+	+	–	t(9;11)(p21;q23)	–	12.7
6.	F	45	Ovarian carcinoma	AML-M4	+	–	+	+	–	Normal	–	67.2
7.	F	55	MDS	AML-M0	+	–	–	+	+	t(3;3)(q21;q26)	D835	41.3
8.	M	65	CML	AML-M2	+	–	–	–	+	t(13;15)(q10;q10) -17,+21?,+22?	–	46.6
9.	M	69	MDS	AML-M1	+	nt	+	+	+	nt	–	94
10.	F	64		AML-M1	+	–	+	+	+	(5)t(5;?8)(q14;q21),-7	–	13.2
11.	F	58		AML-M2	+	–	+	+	–	Normal	ITD,wt-	39.4
12.	M	36	CML	AML-M2	+	nt	nt	+	+	(9)(ins22q12;q34)	–	202
13.	F	63		AML-M1	+	–	+	–	+	nt	nt	65.4
14.	F	74		AML-M2	nt	nt	nt	nt	nt	nt	–	26.6
15.	M	64	MDS	AML-M1	+	–	+	+	+	37-46XY	D835	11.9
16.	M	83		AML-M2	+	–	nt	+	+	nt	–	45.0
17.	M	40	MDS	AML-M6	+	–	+	+	+	Normal	ITD	25.0
18.	F	78		AML-M1	+	–	–	+	+	nt	nt	72.1
19.	M	43		AML-M5	+	–	+	+	+	inv(16)(p13q22)	D835	351
20.	M	79		AML-M4	–	–	+	+	–	nt	ITD	102
21.	F	45		AML-M2	+	–	–	+	–	Normal	ITD, D835	120
22.	M	81	MDS	AML-M4	–	+	nt	–	–	nt	–	56.1
23.	M	79		AML-M0	+	–	+	–	+	nt	–	54.1
24.	M	79		AML-M1	+	–	–	–	+	Normal	ITD	5.6
25.	M	33		AML-M1	+	–	–	+	+	+4	ITD	37.1
26.	M	79		AML-M1	+	–	–	–	+	Normal	ITD	5,6
27.	F	70	AML	AML-M1	+	–	+	–	+	nt	ITD	142
28.	M	29		AML-M4	+	–	+	+	+	Normal	ITD, D835	18.6
29.	M	82		AML-M1	–	–	nt	+	+	nt	–	73.1
30.	M	75		AML-M0	+	–	nt	nt	+	nt	–	15,4
31.	F	48		AML-M1	+	–	–	+	+	del(7) (q22)	nt	26.9
32.	F	75		AML-M5	+	–	+	+	+	Normal	ITD	104
33.	M	69		AML-M2	+	nt	nt	+	+	inv(16)	–	65.8
34.	F	45		AML-M4	+	+	+	–	–	Normal	D835	60.3
35.	F	51		AML-M2	+	–	+	+	+	Normal	ITD,wt-	154
36.	F	78		AML-M4	+	+	+	+	–	-4,-5,+der(8)T(8;?) q21;?), inv(16), +20,+21	–	86.4
37.	F	63		AML-M4	+	–	–	+	+	Normal	ITD	81.7
38.	M	74	MDS	AML-M5	+	–	–	+	+	nt	ITD	72.4
39.	F	59		AML-M2	+	–	–	+	+	-7	–	39.9
40.	M	56		AML-M2	+	–	+	+	+	Normal	ITD	38.1
41.	M	72		AML-M5	+	+	+	+	–	Normal	–	36.1
42.	M	64		AML-M4	+	–	–	+	+	Normal	ITD, wt-	135
43.	F	48		AML-M2	–	–	–	+	–	t(9;11)	–	29.2
44.	F	61		AML-M4	+	+	+	+	–	Normal	–	27.1
45.	M	63		AML-M1	+	+	nt	+	+	Normal	–	14,5
46.	M	74		AML-M0	–	–	+	+	+	90-94,XXYY,	–	123
47.	M	82		AML-M5	+	+	+	+	–	45X	–	198
48.	F	34		AML-M5	–	–	+	+	–	t(9;11)(p22;q23)	D835	286
49.	F	79		AML-M5	+	–	+	+	nt	del(12)(p11)	D835	201
50.	F	64	MDS	AML-M1	+	–	–	+	+	del(11)(q14), del(20)(q11),+21	–	14.9

F: female; M: male; AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; CML: chronic myelogenous leukemia. The patients' age is given in years. [1]Patients were regarded as positive when more than 20% of the blasts cells stained positive judged by flow cytometric analysis. [2]Flt3 abnormalities were internal tandem duplications (ITD), Asp(D) 835 mutations (D835), and loss of wild type (wt-); nt: not tested. [3]The leukemia blast counts in peripheral blood are expressed as $\times 10^9$/L. The peripheral blood white blood cells (WBC) included at least 80% leukemia blasts.

Table 2. Clinical and biological characteristics of ALL patients.

Patient	*Sex*	*Age*	*Previous disease or chemotherapy*	*ALL sub-classification*[1]	*Cytogenetic analysis*[2]	*Blast count*[3]
1.	M	82		B-ALL	nt	125
2.	F	23	Previous chemotherapy	Pro-B-ALL	nt (bcr/abl$^+$)	47.1
3.	M	24	Previous chemotherapy	Pro-B-ALL	t(9;22) (bcr/abl$^+$)	89
4.	M	21		B-ALL	46XY,dic(7;9)(p11;p11)	15.2
5.	F	28		T-ALL	nt (bcr/abl-)	68
6.	M	74		Common-B-ALL	nt	78
7.	F	54		Common-B-ALL	t(9;22) (bcr/abl$^+$)	560
8.	F	22		Common-B-ALL	nt (bcr/abl-)	3.6

[1]ALL blasts were regarded as positive for membrane molecules when more than 20% of the cells stained positive judged by flow cytometric analysis. The classification was based on the guidelines given by the European Group for the immunological classification of acute leukemias.[18] According to this classification B lineage ALL blasts are positive for at least two of the three markers CD19, CD22 and CD79a. Patients classified as having pro-B-ALL (also referred to as B-I or null ALL) express no other B-cell differentiation antigens, patients with common ALL (also referred to as c-ALL, pre-pre-B-ALL or B-II) express CD10, those with pre-B-ALL (B-III) express cytoplasmic Ig and those with mature-B-ALL (also referred to as B-ALL or B-IV) express surface membrane Ig.[18] [2]Routine screening for chromosomal abnormalities was done by analysis of cells in mitosis. The abbreviation nt (not tested) means that cells were not available for testing or did not proliferate in vitro, for three of these patients the presence of the bcr/abl translocation (Philadelphia chromosome) was analyzed by a fluorescent in situ hybridizaton technique. [3]The peripheral blood blast counts are expressed as $\times 10^9$/L.

GM-CSF and anti-HGF were used at 10 μg/mL and anti-SCF at 2 μg/mL. Control cultures were prepared with corresponding normal mouse IgG_1 antibodies (anti-GM-CSF, anti-HGF) and normal goat antiserum (anti-SCF) (both supplied by R&D Systems) at the same concentrations as the specific antibodies.

In vitro culture of native human AML blasts

Suspension cultures of AML blasts alone. AML blasts (10^6 cells in 1.5 mL) were cultured in transwell culture plates (see below) for 7 days before supernatants were harvested.

Co-culture with non-leukemic cells. Cultures were prepared in Transwell culture plates (Transwell 3401; Costar, Cambridge, MA, USA) in which cells in the lower large compartment were separated from the cells in the upper small chamber by a semipermeable membrane with a pore diameter of 0.4 μm.[23] Non-leukemic cells were seeded in the lower part of each well (10^4 cells in 1 mL) and incubated for 3 days until they formed a proliferating population of regularly distributed cells. The leukemic blasts (10^6 cells in 0.5 mL) were then added to the upper chamber and the cultures thereafter incubated for a further 7 days. The cultures were ended before the non-leukemic cells were confluent. Alternatively AML blasts and osteosarcoma cells were cultured in direct contact in 24-well culture plates (Costar 3524) in a manner otherwise similar to that of the transwell cultures.

Analysis of AML blast proliferation (^{3}H-thymidine incorporation) during co-culture. AML blasts and non-leukemic cells were cultured together in separate chambers (Transwell 3401 culture plates, see above) for 6 days before ^{3}H-thymidine was added (280 kBq/well added in 150 μL saline; TRA 310, Amersham International, Amersham, UK). Nuclear incorporation in AML blasts was assayed 18 hours later. The leukemic cells were then resuspended in their small chamber and nuclear radioactivity assayed on 50 μL aliquots of this cell suspension transferred to separate wells of a microtiter plate; during harvesting free ^{3}H-thymidine was washed away whereas nuclear material was harvested onto a separate filter for each well. The nuclear radioactivity could thereafter be determined by liquid scintillation counting.

Analysis of AML colony formation. AML blasts and non-leukemic cells were cultured in transwell plates as described above. After 7 days of co-culture AML blasts were resuspended in the upper chamber and 100 μL of this suspension were mixed with 900 μL of methylcellulose medium containing erythropoietin and conditioned medium from phytohemagglutinin-stimulated normal PBMC (MethoCult H4433; Stem Cell Technologies).[19] The AML blasts were thereafter cultured (Costar 3524 24-well culture plates, 0.5 mL medium per well) for 14 days before the colony numbers were determined by light microscopy.

Analysis of IL1β and IL8 levels in culture supernatants. All supernatants were harvested after 7 days of co-culture and stored frozen at –70°C. The IL1β and IL8 levels were determined by ELISA analyses (Quantikine ELISA kits; R&D Systems), which were performed strictly according to the manufacturer's instruction. Standard curves were prepared as recommended, and differences between duplicates were generally less than 10% of the mean. The minimum detectable level of IL1β was 2.4 pg/mL and the minimum detectable level of IL8 was 4 pg/mL.

Analysis of AML blast apoptosis

Native human AML blasts were incubated in transwell cultures with and without non-leukemic cells for 2 days before the percentages of viable/apoptotic/necrotic cells

were determined. Cells were stained with propidium iodide (PI) and with FITC-annexin V for detection of phosphatidyl exposure on the cell surface.[24-26] Flow cytometric analysis demonstrated three different AML cell populations: viable ($PI^-annexin^-$), early apoptotic ($PI^-annexin^+$) and late apoptotic/ necrotic ($PI^+annexin^+$; some patients had one population and other patients had two separate populations with different mean channel fluorescence intensity of the PI staining).

Presentation of the data

Cell proliferation was assayed by ^{3}H-thymidine incorporation and the mean counts per minute (cpm) of triplicate determinations was used in all calculations (three samples derived from the same transwell culture). Detectable ^{3}H-thymidine incorporation was defined as >1000 cpm. A marked alteration of ^{3}H-thymidine incorporation was defined as a difference corresponding to (i) an absolute value of at least 2000 cpm; and (ii) this absolute value being >20% of the corresponding control. The relative IL8 level for an AML cell population was defined as the IL8 level in cultures containing AML blasts + non-leukemic cells relative to the summarized levels for the corresponding AML blasts and non-leukemic cells cultured alone. A relative IL8 level >1.00 was considered to indicate a supra-additive effect. Differences were regarded as statistically significant when $p<0.05$.

Results

AML blast proliferation during co-culture with osteoblastic sarcoma cells

AML blasts derived from 48 consecutive patients (Table 1, patients 1-42, 44-49) were cultured together with the osteosarcoma cell lines Cal72, SJSA-1 (both with an osteoblastic phenotype) and 143.98.2 (epithelial growth pattern). Detectable AML blast proliferation corresponding to >1000 cpm was observed either in medium alone or in the presence of at least one osteoblastic sarcoma cell line for 39 patients (Figure 1).

Co-culture with Cal72 and SJSA-1 osteoblastic sarcoma cells increased AML blast proliferation significantly (Wilcoxon's test for paired samples, $p<0.002$ for both cell lines), but the proliferation differed in the presence of the two cell lines. This growth-enhancing effect was reproduced in one additional experiment for 16 patients and in 2 additional experiments for 7 other patients (*data not shown*). The AML blast proliferation in the presence of osteoblastic sarcoma cells did not differ between patients with and without genetic Flt3 abnormalities (*data not shown*). Furthermore, the epithelial 143.98.2 sarcoma cells did not alter blast proliferation significantly (*data not shown*). Native AML blasts derived from 10 patients (Table 1, patients 32, 36, 37, 40-42, 44, 46-48) were also cultured together with 4 other osteosarcoma cell lines (Saos-2, SK-ES-1, U2OS, KHOS-32IH) in transwell cultures. These lines differed from Cal72 and SJSA-1 by having a non-adherent

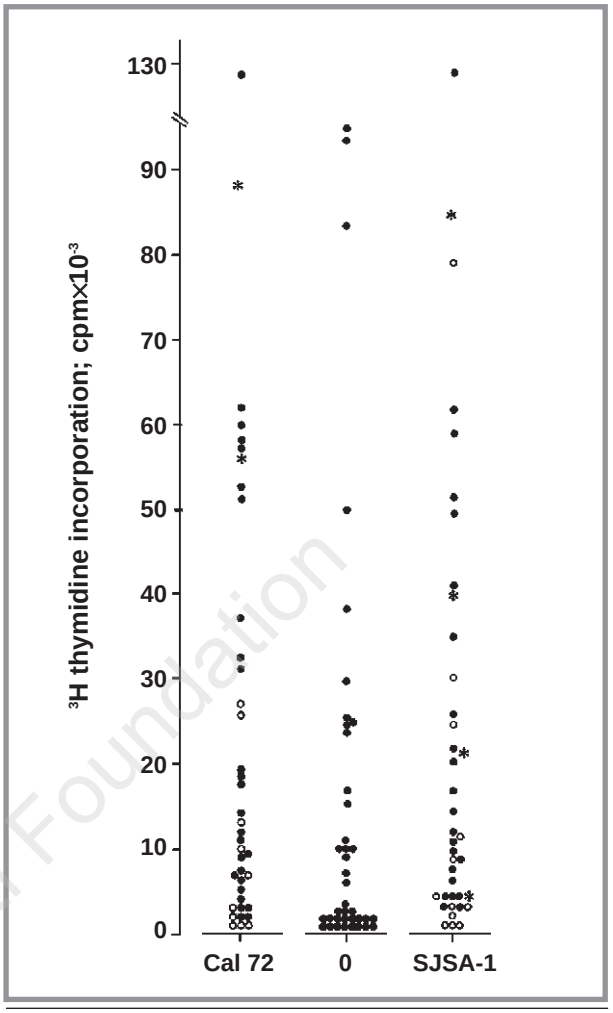

Figure 1. Proliferation of native human AML blasts during co-culture with the osteoblastic sarcoma cell lines Cal72 and SJSA-1. Native human AML blasts derived from 48 patients (Table 1, patients 1-42, 44-49) were cultured in medium alone (middle) and together with Cal72 (left) and SJSA-1 (right) in transwell cultures. Proliferation was assayed as ^{3}H-thymidine incorporation after 7 days of *in vitro* culture, and the results are presented as the mean cpm of triplicate determinations. Detectable proliferation (corresponding to >1000 cpm) either in medium alone or in co-culture with at least one of the cell lines was observed for 39 patients, and the figure presents the results only for these patients. An increase (•) or decrease (*) corresponding to at least 2000 cpm and exceeding the corresponding sarcoma-free control by at least 20% is indicated in the figure for Cal72 and SJSA-1, whereas open symbols represent patients with smaller alterations.

epithelial growth pattern or a different cytokine release profile. All the investigated AML cell populations showed increased proliferation during co-culture with Cal72 and SJSA-1 osteoblastic cells. Increased proliferation was also observed for the osteoblastic U2OS cells, but the other sarcoma lines often had minor and divergent effects on AML cell proliferation (*data not shown*).

AML blasts derived from 16 patients (Table 1, patients 23-32, 36-40, 46) were also cultured in direct contact with Cal72 and SJSA-1 osteoblastic sarcoma cells and their proliferation assayed after 7 days. Increased AML blast proliferation in the presence of Cal72 or SJSA-1 was detected in transwell cultures for all these patients except for 4 patients who showed no detectable proliferation with or without sarcoma cells (*see above*). AML blast proliferation was also observed when the cells were cultured in direct contact with osteoblastic sarcoma cells (*data not shown*).

Effects of osteoblasts on AML colony formation

Native AML blasts derived from 18 consecutive patients (Table 1, patients 28-45) were cultured in transwell cultures together with the Cal72 osteoblastic sarcoma cells for 7 days before the leukemic cells were transferred to the erythropoietin-containing colony assay. Colony formation was detected for 16 patients, but the colonies were erythroid only in a subset of these patients (Table 3). When comparing the overall results, pre-culture with Cal72 cells increased non-erythroid colony formation significantly (Wilcoxon's test for paired samples, p=0.0005), and a marked increase (i.e. an absolute increase >10 and this increase being >10% of the corresponding control) was observed for 10 patients. Increased numbers of erythroid colonies were also observed.

Table 3 The effect of osteoblasts on clonogenic AML cells: a comparison of the frequency of colony-forming cells after *in vitro* culture of native human AML blasts in medium alone and in the presence of Cal72 osteoblastic sarcoma cells.

	Non-erythroid colonies		*Erythroid colonies*	
Patient	*Medium*	*Cal72*	*Medium*	*Cal72*
28.	19.0±4.2	**35±14.2**	0	4±2.3
29.	6±5.2	14±8.3	0	0
30.	3±4.3	**13.0±6.4**	2.0+2.6	2.0±2.6
31.	12.5+4.4	17.5±4.4	0	0
32.	54±8.2	**131±6.2**	0	0
33.	0	4.5±1.3	0	0
34.	35±4.7	**107±32**	1.5±1.7	**22±9.3**
35.	54±5.2	60±12.3	22±0	21±3.9
36.	57±22.3	**90±13.0**	14±12.3	**27±12.3**
37.	26.5±14.8	**43±12.3**	93±15.6	**143.5±9.2**
38.	58±5.3	**119±10.3**	0	0
39.	2.0±3.2	0	0	0
40.	26±6.3	**54.5±7.3**	0	1.5±2.2
41.	27.5±8.3	**38.5±15.5**	45.5±8.2	**149.5±13.5**
42.	11±4.2	**82±3.6**	0	0
43.	0	0	0	1.0±1.3
44.	11.0±9.1	2.0±1.3	0	0
45.	0	0	0	0

Native AML blasts were incubated for 7 days in medium alone or in cultures together with Cal72 osteoblastic sarcoma cells before they were analyzed in the colony formation assay. The results are presented as the number of non-erythroid and erythroid (parts of the colony showing a clear red color) colonies per 50 μL cell suspension. A large difference was defined as a difference corresponding to (i) at least 10% of the control number, and (ii) an absolute value of at least 10; these differences are marked in bold.

Viability and spontaneous in vitro apoptosis of native human AML cells

Previous studies demonstrated that our thawed AML cell populations generally have a viability of >70%.[21] We estimated the fraction of viable cells by flow cytometry for 25 randomly selected patients; using this methodological approach the median number of viable cells was 74% (2 patients had <10% viable cells and 2 other patients had 40-60% viable cells). The percentage of viable cells decreased during *in vitro* culture for all patients examined, and after 65 hours of culture the median percentage of viable cells was 35% (range 1-88%). Cells from 7 patients were also examined after 24 and 48 hours, and the fraction of viable cells was found to decrease gradually during the 65-hour culture period. Cell viability was also examined after 7 days of culture by which time viability had further decreased (median value 25%, range 0.6-47%).

Native AML blasts derived from 9 patients (Table 1, patients 28-36) were cultured together with Cal72 osteoblastic sarcoma cells in transwell cultures as described above. These patients were all selected because their cells had shown increased AML blast proliferation during co-culture with the Cal72 and SJSA-1 osteoblastic sarcoma cells. The percentage and absolute number of AML cells with phosphoserine exposure on the cell surface were analyzed by flow cytometry after 48 hours of co-culture. The number of viable (range 27-60%), apoptotic (range 23-42%) and late apoptotic/necrotic cells (range 9-33%) in control cultures without non-leukemic cells showed a wide variation between patients, and a similar variation was observed after co-culture of AML blasts with Cal72 cells (viable 33-60%, apoptotic 25-48%, necrotic 8-34%). No significant differences were detected when comparing absolute cell numbers (*data not shown).*

Effect of cytokine neutralization on AML blast proliferation during co-culture with Cal72 osteoblastic sarcoma cells

Spontaneous IL1β release was investigated by measuring IL1β concentration in 7-day culture supernatants. Detectable levels (>2.8 pg/mL) were observed for 20 of the AML patients (range 4.8-73 pg/mL). To

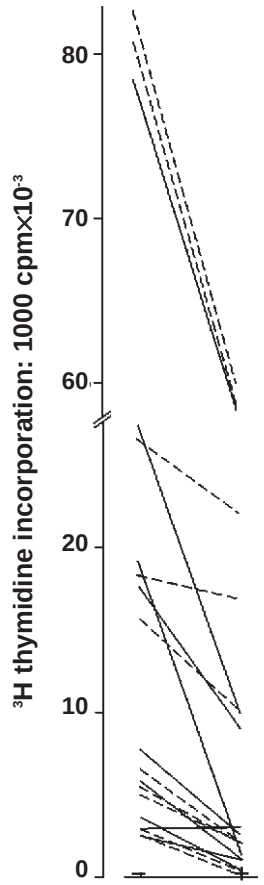

Figure 2. The effect of IL1RA and anti-GM-CSF on AML blast proliferation during co-culture of leukemia cells with Cal72 osteoblastic sarcoma cells in transwell cultures. Native AML blasts derived from 11 patients were tested in the IL1RA experiments (7 patients showing detectable IL1β release: the range of variation for AML blasts cultured alone was 4.8-203 pg/mL, the increased levels in co-cultures for all patients with variation ranged from 8.0-2090 pg/mL). The figure presents the results for the 9 patients who showed detectable *in vitro* proliferation corresponding to >100 cpm (continuous lines). Twelve patients were tested with anti-GM-CSF, and the figure presents the results for the 10 patients showing detectable proliferation (stippled lines). The results are presented as the median cpm of triplicate determinations for co-cultures prepared in medium alone (left part, -) and co-cultures withcytokine-blocking/neutralizing mediators (right part, +). The proliferation in negative controls corresponded to <300 cpm.

investigate the role of IL1β further, native human AML blasts derived from 11 patients (Table 1, patients 32, 36, 37, 40-42, 44, 46-49) were cultured with Cal72 sarcoma cells, and leukemia cell proliferation was then compared for cultures prepared with and without IL1RA 50 ng/mL. Nine of these patients were selected because their cells had shown increased AML blast proliferation in the presence of Cal72, the 2 last patients because detectable AML blast proliferation had not been noted in either medium alone or in the presence of Cal72 cells. Spontaneous IL1β release exceeding 2.4 pg/mL was observed for 7 of these patients, whereas IL1β was not detected in control cultures only containing Cal72 cells. Addition of IL1RA inhibited AML blast proliferation for all the 9 patients with detectable proliferation (Figure 2, Wilcoxon's test for paired samples, p=0.002), the weakest inhibition being observed for cells from a patient without spontaneous IL1β release. IL1RA did not induce proliferation in the cells from the 2 last patients with undetectable ^{3}H-thymidine incorporation in the control cultures.

Native AML blasts derived from 12 patients (Table 1, patients 22, 27, 29-33, 39-43) were co-cultured with Cal72 cells in cultures prepared with either anti-GM-CSF, anti-HGF, anti-SCF or their corresponding control antibodies. Ten of these patients were also selected because their cells had shown increased AML blast proliferation in the presence of Cal72 cells and the two last patients because detectable proliferation did not occur either in medium alone or in the presence of Cal72 cells. When comparing the overall results for these patients only anti-GM-CSF caused a significant decrease in leukemia cell proliferation (Figure 2, p=0.001). However, an inhibition corresponding to an absolute value of >2000 cpm and >20% of the corresponding controls was observed for certain patients when testing anti-HGF (3 patients) and anti-SCF (2 patients, *data not shown*). Detectable proliferation was not observed in any culture for the last 2 patients.

IL8 levels during co-culture of AML blasts and osteosarcoma cells

Native human AML blasts derived from 40 patients (Table 1, patients 1-31, 33-35, 37-39, 45, 46, 50) were cultured together with the sarcoma lines for 7 days before IL8 levels were determined in the supernatants. These patients were a group of consecutive patients (the last patients included in the study). The patients were examined in 6 separate experiments, and IL8 levels in control cultures containing only AML blasts ranged from 15.7 to 810,000 pg/mL (median 7842 pg/mL). In contrast, IL8 levels in control cultures only containing Cal72 osteoblastic sarcoma cells were <56 pg/mL in all experiments. When the overall results were analyzed, the IL8 levels for co-cultured cells were made relative to the summarized levels for corresponding control cultures containing Cal72 cells and AML cells alone. Thus, a relative IL8 level of 1.0 corresponds to an additive effect, whereas a value exceeding 1.0 represents a supra-additive effect. The effect of co-culture differed between AML blast populations with high and low constitutive IL8 release (Figure 3). For AML cells co-cultured with Cal72 cells, a supra-additive effect was significantly more common for patients with low constitutive IL8 release (below the median level of 7842 pg/mL) than for patients with high release (16 versus 5 patients, χ^2 test, p<0.001).

IL8 levels in control cultures containing SJSA-1 cells alone were 930-1387 pg/mL in the six separate experiments (38 patients examined). A supra-additive increase in IL8 levels during co-culture of SJSA-1 cells and AML blasts was especially observed for patients with relatively low constitutive IL8 release (below the median IL8 level of 7842 pg/mL). The median relative IL8 level for patients with low constitutive IL8 release was 2.79 (a supra-additive effect), whereas for patients with high IL8 release the median relative level was only 1.34 (Figure 3). This difference is also illustrated by compar-

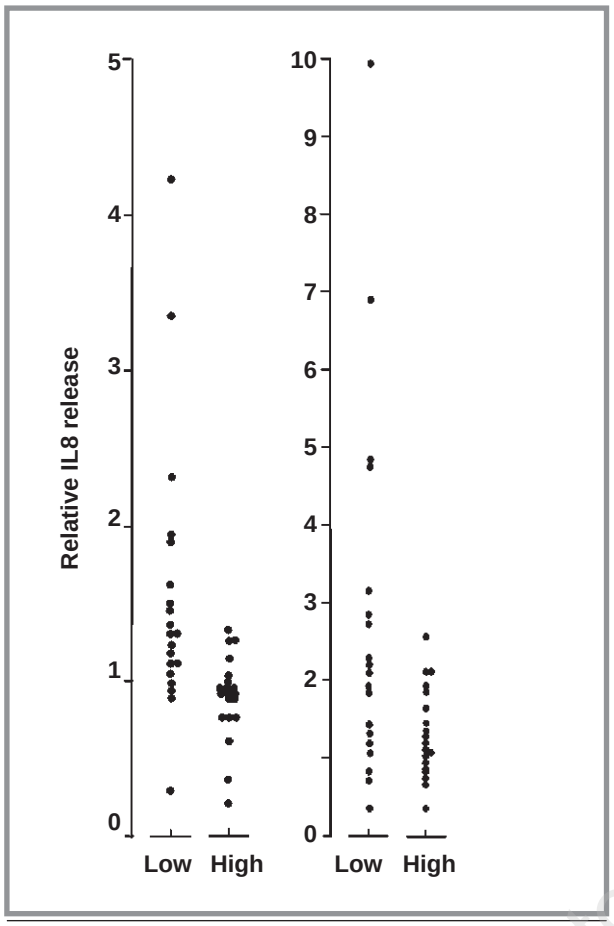

Figure 3. IL8 levels during co-culture of native human AML blasts with osteoblastic sarcoma cells. Native human AML blasts were cultured in transwell cultures together with Cal72 (left, 40 patients examined) and SJSA-1 cells (right, 38 patients examined). The results are presented as the relative IL8 level, i.e. the IL8 concentration for co-cultured cells relative to the summarized IL8 levels for control cultures containing corresponding AML blasts and sarcoma cells alone. The results for patients with high and low constitutive IL8 release (above or below the median level of 7842 pg/mL for constitutive secretion) are presented separately.

ing the number of patients with a relative IL8 level >2.0; this was significantly more common for patients with low constitutive IL8 release (observed for 10 patients) than for patients with high release (only 3 patients; χ^2 test, $p<0.05$).

Co-culturing the AML blasts with 143.98.2 cells did not alter the IL8 levels (*data not shown*). Furthermore, the IL8 released during co-culture with osteoblastic sarcoma cells did not differ between AML cells with and without genetic Flt3 abnormalities (*data not shown*).

The IL8 levels for AML cells cultured alone and in the presence of osteoblastic sarcoma cells (Cal72 and SJSA-1) showed significant correlations (*data not shown*). We also compared the IL8 levels and the AML cell viability when the leukemia cells were cultured alone (16 patients, see above). For 11 of these patients the percentage of viable cells varied between 10 and 40% and the percentage of apoptotic cells between 30 and 78%, but the IL8 levels varied from being undetectable (<4 pg/mL) to >10 ng/mL without these levels showing any correlation with viability. The relatively larger variation range for IL8 levels than for viability/apoptosis further suggests that AML cell viability is not a major determinant of IL8 levels during *in vitro* culture of native human AML cells.

AML blast proliferation and IL8 levels during co-culture of native human AML blasts and normal osteoblasts

Native human AML cells derived from 15 patients (Table 1, patients 23-25, 27-32, 36-40, 46) were co-cultured with normal osteoblasts (all cultures were prepared in osteoblast growth medium). Cells from all these patients showed increased AML blast proliferation in the presence of Cal72 or SJSA-1 except for 4 patients who showed no detectable proliferation either with or without sarcoma cells (*see above*). None of the AML blast populations showed detectable proliferation in the osteoblast growth medium alone, but in the presence of normal osteoblasts detectable proliferation was observed for 8 of the patients (Figure 4, χ^2 test, $p<0.001$). IL8 levels in co-culture supernatants were also determined. The IL8 level in control cultures containing only osteoblasts was 8.3±1.1 pg/mL, whereas the range of IL8 levels in cultures containing only AML blasts was from <8.0 to 96,000 pg/mL. IL8 was not detectable in either control cultures or in co-cultures for 3 patients, whereas detectable release was observed for the other 12 patients during co-culture (Figure 5). Co-culture of AML blasts with osteoblasts significantly increased the IL8 levels compared with levels in cultures only containing AML blasts (Wilcoxon's test for paired samples, $p=0.008$).

ALL blast proliferation during co-culture with osteosarcoma cells

Native human ALL blasts derived from 8 consecutive patients were cultured together with the three osteosarcoma cell lines Cal72, SJSA-1 and 143.98.2. For the first 5 of these patients increased ALL blast proliferation was observed in the presence of Cal72, whereas for the last 3 patients the ALL blasts did not show detectable proliferation when cultured in medium alone or together with the Cal72 sarcoma cells. Divergent results were also observed for co-culture with SJSA-1 and 143.98.2 sarcoma cells (*data not shown*).

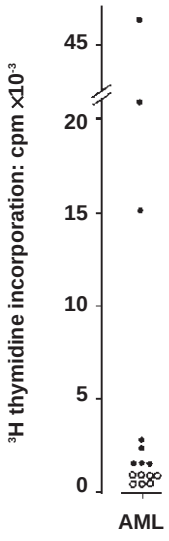

Figure 4. AML blast proliferation after co-culture with normal osteoblasts. Native human AML blasts derived from 15 patients were cultured in transwell cultures either alone or together with normal osteoblasts. The AML blast proliferation was assayed after 7 days of co-culture and none of the AML cell populations then showed detectable spontaneous proliferation (corresponding to <1000 cpm) when cultured in medium alone. The figure presents AML blast proliferation during co-culture with normal osteoblasts, dark symbols represent detectable proliferation and open symbols correspond to undetectable proliferation.

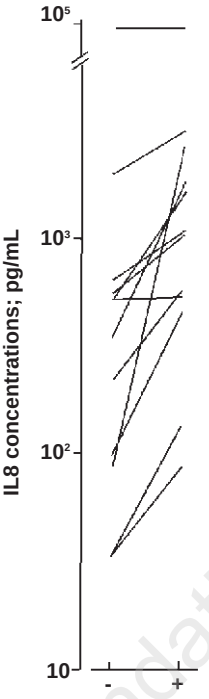

Figure 5. IL8 levels for native human AML blasts co-cultured with normal osteoblasts. AML blasts derived from 15 patients were examined. Detectable IL8 levels were observed only for 12 patients, and the figure includes the results only for these patients. IL8 concentrations in control cultures only containing osteoblasts corresponded to 8.3 pg/mL. The results are presented as the incremental IL8 levels, i.e. concentrations for AML blast-containing co-cultures minus the level in the osteoblast control. The figure compares the results for AML blasts cultured alone (–) and together with normal osteoblasts (+).

Discussion

Our experimental approaches are based on previous methodological studies. Firstly, preparation of enriched AML blasts by density gradient separation alone minimizes the risk of inducing functional alterations, but this strategy requires selection of patients with high peripheral blood blast counts and the results may, therefore, be representative only for this subgroup. This aspect is discussed in our previous papers.[27] Secondly, except for the experiments with normal osteoblasts we used the Stem Span™ medium, which is suitable for serum-free culture of native human AML blasts.[28,29] However, osteosarcoma cells often showed low proliferation in the absence of serum (*Bruserud, unpublished data*), and the medium was therefore supplemented with 10% FCS.[28] Thirdly, by using co-culture with well-characterized cell lines we have a standardized and reproducible experimental model that allows comparisons between patients. However, we also included experiments with normal osteoblasts to further investigate whether our observations are relevant to normal cells. In most experiments AML cells and osteoblasts were co-cultured in different chambers separated by a semipermeable membrane, this experimental approach allowing us to study the cross-talk between the two cell populations via alterations of the local cytokine network. However, it should be emphasized that this experimental approach only allows the study of effects mediated by soluble mediators. Other interactions involving direct contact between AML cells and non-leukemic neighboring cells, extracellular stromal molecules and probably also stromal-bound growth factors will have additional effects on the leukemia cells, modulating the proliferation, apoptosis, survival and migration of native human AML cells.[30-34] However, our studies of AML cells cultured in direct contact with osteoblastic sarcoma cells suggest that the cytokine-induced growth enhancement is maintained when the cells are cultured in direct contact.

AML cell populations have a hierarchical organization including a minor subset of proliferating immature cells and a majority of cells with a limited proliferative capacity.[27] This organization is also reflected in our present results in which the colony-forming cells, as expected, represent a small minority of the AML cells; this subset is probably also responsible for the proliferation in our suspension cultures because an enrichment of this subset is observed when native human AML cells are incubated in suspension cultures for 7 days.[27] Thus, *in vitro* ^{3}H-thymidine incorporation, assayed from day 6 to day 7 of the culture period, probably reflects the characteristics of a minor AML cell subset, whereas the *in vitro* viability/spontaneous apoptosis after 48 hours of culture reflects the characteristics of the majority AML cell population. This hypothesis is further sup-

ported by our present results in which *in vitro* co-culture of AML cells and osteoblasts increased AML cell proliferation but did not alter *in vitro* viability. The wide variation of IL8 levels during *in vitro* culture cannot be explained by the relatively smaller variation in cell viability either.

We selected a panel of well-characterized osteosarcoma cells for our study. The cell line Cal72 has a phenotype very similar to that of normal osteoblasts[14,22] including an adherent growth pattern and a broad cytokine release profile. The SJSA-1 cells showed a similar growth pattern and similarities in their cytokine release profile. The cytokine profile of the osteoblastic U2OS cells showed only minor differences, whereas other cell lines showed an epithelial growth pattern or a cytokine release profile with low/undetectable levels of HGF, IL6 and VEGF (*Bruserud, unpublished data*). Cal72 and SJSA-1 cells supported the proliferation of native human AML blasts in transwell cultures and also when the cells were cultured in direct contact (examined for a subset of patients), but this was not an effect common to all the sarcoma cell lines. Thus, the increased AML blast proliferation seems to be associated with an osteoblastic phenotype and is not a general characteristic of osteosarcoma cells.

The effects of osteoblasts on AML cells were characterized in detail for subsets of patients who showed a typical enhancement of AML blast proliferation in the presence of Cal72 and SJSA-1 cells. Firstly, osteoblasts could enhance the proliferation of colony-forming AML cells. Secondly, the frequencies of cells undergoing spontaneous *in vitro* apoptosis varied widely among patients[17] and were not significantly altered by co-culture with Cal72 cells. Taken together these results suggest that the most important effect of osteoblasts is modulation of local cytokine networks with growth enhancement rather than an anti-apoptotic effect. However, this enhancement is not specific for immature myeloid cells as it was also observed for certain ALL blast populations.

We used cytokine neutralization/blocking to identify soluble mediators involved in the growth-enhancing cross-talk between AML blasts and osteoblastic sarcoma cells. IL1β and GM-CSF can be released by the sarcoma cells as well as by native AML blasts.[14,17,19-22] Both these cytokines can function as growth factors for leukemia cells, and our results suggest that they are involved in the AML-growth enhancing cross-talk. However, native AML blasts derived from different patients are functionally heterogeneous, and our results suggest that other mediators (e.g. HGF, SCF) may also contribute in certain patients. Despite this heterogeneity between AML blasts the final effect of osteoblast-induced growth enhancement seems common to most patients. The aggressive course of untreated AML seems to depend on both (i) malignant cell transformation to leukemic stem cells with a high proliferative capacity; and (ii) bone marrow angiogenesis that supports the development of clinical disease.[1-10,27] IL8 is a quantitatively important pro-angiogenic mediator released by native human AML blasts, and the levels reached during *in vitro* culture of AML cells are sufficient to increase endothelial cell migration but usually not to induce endothelial cell proliferation.[35-37] All our AML cell populations released this mediator. The IL8 levels varied greatly but for most patients the release by AML blasts was considerably higher than the release by osteoblastic sarcoma cells and normal osteoblasts. Angiogenesis is probably important for both disease development and chemosensitivity in AML.[7-10] Our results demonstrated that osteoblasts increased the IL8 levels especially among those patients with relatively low constitutive secretion. Taking into account the low spontaneous IL8 release by both sarcoma cells and normal osteoblasts it seems most likely that the increased levels are caused by increased release by the AML blasts. Based on these observations we therefore suggest that the cross-talk between native human AML blasts and osteoblasts has a pro-angiogenic effect through increased release of IL8, this leading to a decreased variation in local IL8 levels between patients.

Several recent studies have demonstrated that both genetic Flt3 abnormalities as well as pro-angiogenic signaling are associated with decreased AML-free survival after intensive chemotherapy.[4-6] IL8 is a pro-angiogenic mediator that is released by AML cells, and we therefore compared IL8 levels for AML cell populations with and without FLT3 abnormalities to investigate whether increased IL8 levels with increased proangiogenic signaling contribute to the adverse prognostic impact of Flt3 abnormalities. Neither AML blast proliferation nor IL8 levels during co-culture with osteoblastic sarcoma cells differed for patients with and without Flt3 abnormalities. These observations suggest that the adverse prognostic impact of Flt3 abnormalities is not dependent on either growth-enhancing interactions with neighboring osteoblasts or a pro-angiogenic effect due to the increased IL8 levels.

We investigated whether the effects of normal osteoblasts on AML blast proliferation and IL8 levels were similar to those of the osteoblastic sarcoma cell lines. In these experiments we used a culture medium that is optimal for normal human osteoblasts. This medium seems suboptimal for culture of native human AML blasts because none of the blast populations then showed spontaneous *in vitro* proliferation, and the constitutive IL8 release was also slightly decreased. However, increased AML blast proliferation as well as

increased IL8 levels were detected during co-culture of AML blasts with normal osteoblasts. These observations suggest that our results for osteoblastic sarcoma cells are relevant to normal osteoblasts.

Stromal elements can affect AML cells through several mechanisms, including cross-talk via the cytokine network and direct contact between AML cells and stromal cells, extracellular stromal molecules and stromal-bound growth factors.[29-33] Our present results suggest that the cross-talk via the local cytokine network supports leukemic hematopoiesis in human AML through direct growth enhancement (an effect that does not seem to be counteracted when cells are cultured in direct contact) and probably also through indirect pro-angiogenic effects. These osteoblast effects seem to be common to most AML patients, even though patients are heterogeneous with regard to the genotype and phenotype of the leukemic cells.

ØB initiated and planned the study, had a major responsibility for all experiments on proliferation and IL8 release, contributed to the studies of apoptosis; LW contributed to the studies of proliferation, IL8 release and genetic Flt3 abnormalities; NG contributed to the studies of proliferation and IL8 release; AR took the major responsibility for the apoptosis studies; BTG took part in the apoptosis studies. All authors took part in data analysis, data presentation and writing the manuscript. The technical assistance of Kristin Paulsen is gratefully acknowledged. The authors reported no potential conflicts of interest.

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