Leptin in human acute myelogenous leukemia: studies of *in vivo* levels and *in vitro* effects on native functional leukemic blasts

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Background and Objectives. Leptin receptors can be expressed by acute myelogenous leukemia (AML) cells, but the functional effects of leptin on native AML blasts have not been characterized in detail. We investigated systemic leptin levels in AML patients and *in vitro* effects of leptin on cultured AML blasts.

Design and Methods. Serum leptin levels were compared for patients with untreated AML and healthy controls. Native AML blasts were derived from a large group of consecutive patients, and effects of leptin on proliferation (suspension cultures and colony formation), constitutive cytokine secretion, differentiation and apoptosis regulation were assayed *in vitro*.

Results. Systemic leptin levels were decreased in patients with untreated AML, and leptin levels in acute leukemia patients were not altered during severe chemotherapy-induced cytopenia and complicating febrile neutropenia. *In vitro* studies demonstrated that leptin increased AML blast release of interleukin (IL) 1β, IL6, tumor necrosis factor (TNF) α and granulocyte-macrophage colony-stimulating factor (GM-CSF). This enhancing effect showed no correlation with CD34 expression and was not dependent on the presence of serum, induction of differentiation or alteration of caspase 3 activity with decreased *in vitro* apoptosis. Leptin also increased spontaneous AML blast proliferation, whereas divergent effects on blast proliferation were observed in the presence of exogenous cytokines. The *in vitro* effects were usually observed at concentrations exceeding the systemic levels.

Interpretation and Conclusions. Our results suggest that systemic leptin levels alone do not have a major influence on native AML blasts, but the systemic levels in combination with local leptin release in the bone marrow may affect the functional characteristics of these cells.
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Key words: leptin, serum levels, *in vitro* effects, acute myelogenous leukemia, cytokines.

Acute myelogenous leukemia (AML) is characterized by clonal proliferation and accumulation of immature myeloid cells.[1] The overall long-term AML-free survival after intensive chemotherapy is less than 50%, but the prognosis can be improved by allogeneic stem cell transplantation for subsets of younger patients.[1] However, allotransplantation has a high treatment-related mortality, and other therapeutic approaches with lower risks of fatal complications are now considered.[1-4] The further investigation of these new treatment strategies will require detailed knowledge about the regulation of leukemic hematopoiesis by the cytokine network in the bone marrow.

Leptin is a regulator of fat metabolism that is synthesized in adipocytes and released into the circulation as a non-glycosylated 16 kD peptide.[5] The serum levels of leptin are therefore correlated with body fat mass and show a wide variation in healthy individuals.[5-7] Leptin is, in addition, released locally in the bone marrow by adipocytes and stromal cells.[8,9] The human leptin receptor exists in several isoforms that are expressed by normal as well as leukemic hematopoietic cells, including native AML cells.[10-12] Furthermore, angiogenesis with increased blood vessel density in the bone marrow seems to be involved in the pathogenesis of AML and in blast susceptibility to systemic chemotherapy,[13,14] and leptin may thereby have an additional indirect effect on leukemic hematopoiesis through its effects in angiogenesis.[15-17] Several authors have, therefore, suggested that leptin has a role in leukemic hematopoiesis,[12,18,19] even though a detailed characterization of leptin's effects on native human AML cells has never been made. In this context we have, therefore, characterized (i) systemic levels of leptin in acute leukemia patients compared with those of a group of healthy controls matched for age and sex; (ii) functional effects of leptin on pro-

liferation, cytokine release and differentiation of native AML blasts derived from a large group of consecutive patients; and (iii) effects of leptin on regulation of apoptosis in native AML blasts and not only AML cell lines.[12]

Design and Methods

Patients

All studies were approved by the local Ethics Committee. Samples were collected after informed consent from patients following routine blood sampling without any additional venipuncture.

AML blast donors

Eighty-nine patients with AML were admitted to our institution in the period 1991-2000. The patients included in the present study represent the 48 patients (all Caucasians) with high peripheral blood blast counts. The patients were classified as having AML-M0/M1 (16 patients), AML-M2 (15 patients) and AML-M4/M5 (17 patients). This classification was based on light microscopy of leukemia cells after May-Grünwald-Giemsa as well as histochemical staining (Sudan Black B, chloroacetate esterase, naphthol AS acetate esterase, Periodic Acid-Schiff reaction), and flow cytometric analysis of membrane molecule expression. Leukemia cells from the last 18 consecutive patients were included in a majority of the experiments, and the characteristics of these patients are presented in Table 1.

Flow cytometric analysis of membrane molecule expression by AML blasts was performed for all patients, and for 29 patients AML blasts derived both from bone marrow and peripheral blood were analyzed. Only minor quantitative differences in molecule expression (variation corresponding to ± 5% of positively stained cells) were observed for most of these patients, but for 6 patients the percentage of positively stained cells differed by 6-15% for one or two of the investigated molecules (CD2, CD3, CD11c, CD13, CD14, CD15, CD19, CD20, CD33, CD34, CD45, CD34, HLA-DR).

Analysis of leptin serum levels

Acute leukemia patients. Serum samples were derived from 20 consecutive untreated AML patients (median age 49 years, range 19-84 years, 11 women and 9 men). The levels were compared with those in 20 healthy controls matched for age and sex (all patients and controls being Caucasians). Body mass index did not differ between patients and controls. All samples were collected in the morning (8-10 a.m.)

Serum samples were also derived from 16 acute

leukemia patients receiving intensive chemotherapy (median age 31 years, range 18-60 years; 12 men and 4 women; 13 AML patients and 3 patients with acute lymphoblastic leukemia, ALL). Twelve patients received consolidation therapy. All samples were collected in the morning (8-10 a.m.). A complicating bacterial infection was defined as (i) fever >38°C lasting for at least 4 hours; (ii) serum C-reactive protein >100 mg/L; (iii) bacterial growth in blood cultures or local signs of bacterial infection. Nine patients had septicemia.

Patients investigated during stem cell mobilization. Plasma samples were collected from PBSC grafts and from venous blood before and after apheresis.[20] All patients were Caucasians (median age 56 years, range 21-63 years; 7 males and 7 females). The patients were compared with healthy controls matched for age and sex, and body mass index did not differ between the two groups.

Reagents

Recombinant human leptin was purchased from R&D Systems (Abingdon, UK) and from Peprotech (Rocky Hill, NJ, USA). Recombinant human interleukin 3 (IL3), stem cell factor (SCF) and Flt-3 ligand (Flt3-L) were used at 20 ng/mL (Peprotech); GM-CSF (Sandoz; Basel, Switzerland) and G-CSF (Roche; Basel, Switzerland) were used at 100 ng/mL. Unless otherwise stated the culture medium was RPMI 1640 with hepes and glutamine (Bio-Whitacker; Walkersville, MD, USA) and supplemented with 10% inactivated fetal calf serum (FCS; Bio Whitacker).[21] The serum-free media X-vivo 10®, X-vivo 15® (Bio-Whitacker) and StemSpan SFEM™ (Stem Cell Technologies, Vancouver, BC, Canada) were used in certain experiments.[22] All media contained 100 µg/mL of gentamicin.

Preparation of AML blasts

Native AML blasts. Leukemic peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation (Ficoll-Hypaque; NyCoMed, Oslo, Norway; specific density 1.077) from peripheral blood. Cells were stored frozen in liquid nitrogen.[23] The percentage of blasts among leukemic PBMC exceeded 95% for all patients judged by light microscopy of May-Grünwald-Giemsa stained cytospin smears.[24,25] This staining method was also used to investigate morphologic signs of differentiation in cultured cells. For 16 patients flow cytometric analysis after separation confirmed that the isolated blasts were representative of the original leukemia cell population.

Enriched AML blasts. Immunomagnetic beads coated with anti-CD2 and anti-CD19 specific mon-

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

Patient	Sex	Age	Previous malignant or premalignant diseas	FAB classification	Membrane molecule expression[1]					Cytogenetic analysis	WBC counts[2]
					CD13	CD14	CD15	CD33	CD34		
1.	M	44		AML-M5	+	−	+	+	+	inv(16)	351
2.	F	36	Neurofibromatosis, malignant schwannoma	AML-M5	+	−	+	+	−	t(9;11)	37.6
3.	M	49		AML-M4	−	−	+	+	−	nt[3]	78
4.	M	69		AML-M2	+	−	nt	+	−	inv(16)	89
5.	F	87		AML-M1	−	−	nt	+	−	nt	51.2
6.	M	83		AML-M1	−	−	-	+	+	nt	80
7.	M	72		AML-M4	+	+	+	+	−	+11	290
8.	M	49		AML-M5	+	+	nt	+	−	Normal	63.5
9.	F	58		AML-M2	+	−	+	+	−	Normal	40.7
10.	F	56		AML-M2	+	−	−	+	−	+21	69.2
11.	F	38		AML-M5	+	+	+	+	+	Normal	182
12.	F	55		AML-M0	+	−	−	−	+	Normal	43.6
13.	M	51		AML-M4	+	+	+	+	−	Normal	31.4
14.	F	49		AML-M1	−	−	−	+	+	47,XX,+21	121
15.	M	65		AML-M1	+	−	−	+	−	Normal	166
16.	M	64		AML-M2	+	−	+	+	+	nt	23.5
17.	F	63		AML-M5	−	+	+	+	−	t(2;3), (q37;q21), (q13;q21;q21) der(11q), 19q+	57.8
18.	F	36		AML-M5	+	+	+	+	−	Normal	88.6

[1]Patients were regarded as positive when more than 20 % of blast cells stained positive judged by flow cytometric analysis. All AML populations were negative for T-lymphocyte (CD2, CD3) and B-lymphocyte (CD19, CD20) markers. These analyses were done on circulating AML blasts at the same time as the preparation of native AML blasts for functional studies. [2]White blood cell (WBC) counts in peripheral blood are expressed as $\times 10^9$/L (normal range 3.5-10.5 $\times 10^9$/L). The WBC included at least 80% leukemia blasts. [3]nt: not tested.

oclonal antibodies (Dynabeads; Dynal, Oslo, Norway) were used for depletion of CD2+ and CD19+ cells.[24,25] Depletion was performed in two separate steps before removal of adherent cells, and these populations contained >99% AML cells judged from light microscopy of May-Grünwald-Giemsa stained cytospin smears and flow cytometric analysis of expression of selected monocyte, T- and B-lymphocyte markers.

Assays for AML blast proliferation

Suspension cultures. As described previously,[22, 24,25] 5×10^4 cells/well were cultured in 150 μL medium in flat-bottomed microtiter plates (Costar 3796; Cambridge, MA, USA). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. After six days ³H-thymidine (37 kBq/well; TRA 310, Amersham International, Amersham, UK) was added in 20 μL saline and nuclear radioactivity assayed 18 hours later.

Colony formation assays. AML blasts were cultured in the following methylcellulose-based media: (i) methylcellulose medium (MethoCult H4230; Stem Cell Technologies) supplemented with GM-CSF 100 ng/mL; and (ii) medium with erythropoietin and phytohemagglutinin-leukocyte conditioned medium (MethoCult H4433; Stem Cells Technologies). The cells were cultured in 24-well tissue culture plates (Costar 3524) with 10^5 cells in 0.5 mL medium per well. Cultures were incubated for 14 days before the number of colonies containing at least 20 cells was counted by light microscopy (duplicate determinations). The colonies were classified as erythroid (red color in the whole or a part of the colony) and non-erythroid.

Studies of apoptotic cell death

Apoptotic cell death was investigated as described previously.[24,26] Briefly, AML blasts were incubated for 24 and 48 hours before cells were stained with DNA-specific bisbenzimide H33258 (Hoechst; Basel, Switzerland), and the percentage of cells showing chromatin distribution consistent with apoptosis was determined by fluorescence microscopy.

Caspase activity was measured in cell extracts using a caspase-3 cellular activity assay kit (Cal-

biochem, La Jolla, CA, USA). Briefly, 1×10^6/mL AML blasts (2 mL medium per well of 24-well tissue culture plates Costar 3524) were cultured for 48 hours before cells were harvested and washed twice. The cell concentration was adjusted to 1×10^9/mL in lysis buffer, and the cell extracts stored at - 70°C until analyzed. The activity is presented as pmol/80 μL sample volume (total volume after addition of reagents was 100 μL/well).

Cytokine analysis

Leptin levels in plasma and serum. ELISA analysis (R&D Systems) was used to determine the leptin concentrations in serum and plasma samples (duplicate determinations). The minimal detectable level was 10 pg/mL.

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

Cytokine-specific RNA levels. AML blasts (2×10^6 cells in 2 mL FCS-containing medium per well; Costar 3524 culture plates) were cultured for 48 hours before the cells were harvested and washed in phosphate-buffered saline. The cell pellets were stored frozen at -70°C until total RNA was isolated.[27] For quantification of IL1β– and IL6-specific RNA the samples and calibrators were hybridized with gene-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labeled detection probes in microwells, and cytokine-specific RNA levels then determined in a calorimetric microplate assay (Quantikine RNA assay, R&D Systems). The results are expressed as concentrations of IL1β- and IL6-specific RNA when testing total RNA at the concentration 2.5 μg/mL.

Presentation of the data

[3]H-thymidine incorporation was assayed in triplicate and the mean counts per minute (cpm) used for all calculations. The incremental response was defined as the cpm for cultures with AML blasts minus cpm for negative controls, and significant blast proliferation was defined as an incremental response exceeding 1,000 cpm. Incremental responses were used for all calculations. A significant alteration of a proliferative response was defined as a difference (i) exceeding 2000 cpm, and (ii) the difference in cpm being >20% of the control response. A significant alteration of AML blast colony formation was defined as a difference corresponding to >20% of the control response and with an absolute value >10 per 5×10^4 cells. Cytokine concentrations were transformed to logarithmic values that were used for statistical comparisons. For statistical analysis the Wilcoxon's test for paired samples was used, and differences were regarded as significant when $p<0.05$ after correction for the number of comparisons.

Results

Serum levels of leptin in acute leukemia patients with chemotherapy-induced cytopenia

Serum levels of leptin were determined in 20 patients with untreated acute myelogenous leukemia, and the results were compared with the levels determined in 20 healthy controls. Patients with acute leukemia showed significantly decreased serum levels of leptin (Figure 1, Wilcoxon's rank sum test, $p=0.0029$). The levels in patients showed a wide variation without any significant correlation with FAB-classification or peripheral blood blast counts.

Serum levels of leptin were also determined in acute leukemia patients receiving intensive chemotherapy (Table 2). The leptin levels in patients in remission did not differ significantly from those in normal controls, and the systemic levels showed a similar wide variation both before the start of therapy, after development of chemotherapy-induced cytopenia, and during febrile neutropenia due to complicating bacterial infections. As a comparison plasma levels of leptin were determined before and after harvesting of autologous PBSC (15 aphereses in 12 patients). These procedures did not seem to affect the systemic levels of leptin either (Table 2), the leptin levels in venous blood and PBSC grafts showed a significant correlation (Kendall's test, $p=0.0026$) and did not differ significantly from the levels in healthy controls.

Effects of leptin on constitutive cytokine secretion by native AML blasts

AML blasts derived from 10 patients (Table 1, patients 1-10) were cultured in FCS-containing medium with and without leptin (Peprotech; 10, 2, 1, 0.2, 0.1, 0.02 and 0.002 μg/mL) for 48 hours before IL1β and IL6 levels were determined. The
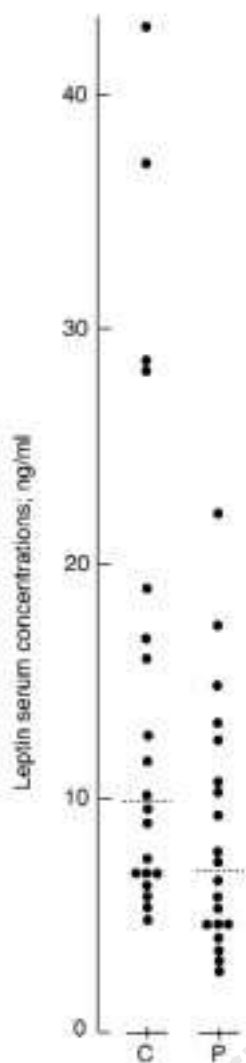
Figure 1. Serum levels of leptin (ng/mL) for 20 patients with AML (P) and 20 healthy individuals matched for age and sex (C). The results are presented as the mean concentration (ng/mL) of duplicate determinations.
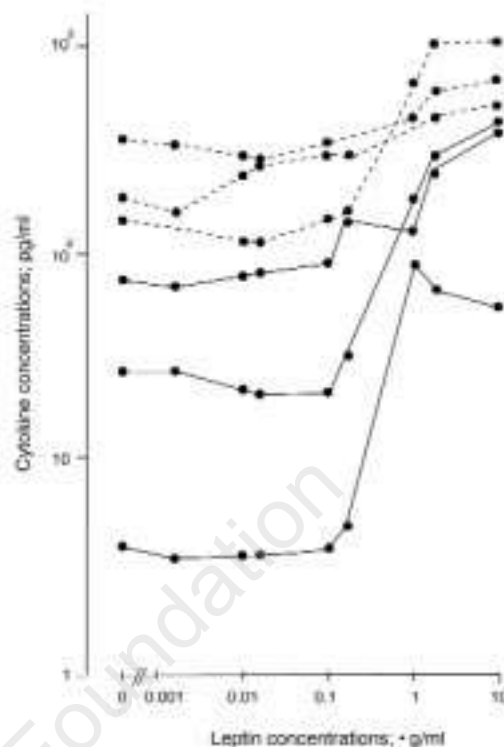

Figure 2. The effect of leptin on IL1β and IL6 levels in supernatants derived from cultures containing native AML blasts. The blasts were derived from three patients (Table 1, patients 4, 7, 10) and cultured at 1×10^6 cells/mL for 48 hours before cytokine levels were determined by ELISA analysis. The figure shows IL1β (—) and IL6 (– – –) levels for AML blasts cultured in medium alone and in medium containing different concentrations of leptin (10, 2, 1, 0.2, 0.1, 0.02 and 0.002 μg/mL). The results are presented as the median of duplicate determinations, and the standard deviation was generally <20%.

results for 3 patients are presented in Figure 2. Leptin increased IL1β and IL6 levels in a dose-dependent manner for all patients, and a plateau (overlapping intervals when comparing mean + standard deviation (SD)) was often reached at ≥1 μg/mL. Even for those patients in whom a plateau was not reached, an enhancement corresponding to >50% of the control levels was observed when testing 1 μg/mL. Similar effects were observed for the other leptin preparation (R&D Systems; *data not shown*). Unless otherwise stated, the following experiments were performed using the Peprotech preparation at 2 μg/mL.

AML blasts derived from 37 consecutive patients were cultured for 48 hours with and without leptin 2 μg/mL before cytokine levels were determined. A majority of patients showed detectable levels of IL1β (20 out of 37), IL6 (29/37), TNFα (13/37), G-CSF (11 out of 17 available patients) or GM-CSF (15/17), and the results for these patients are presented in Figure 3. Leptin significantly increased the levels of IL1β (Wilcoxon's test for paired samples, $p<0.0005$), IL6 ($p<0.005$), TNFα ($p=0.001$), and GM-CSF ($p=0.001$). G-CSF levels were relatively low, and increased levels in the presence of leptin were observed only for a subset

Table 2. Serum and plasma levels of leptin; studies of acute leukemia patients receiving intensive chemotherapy and patients with malignant disorders undergoing PBSC mobilization and harvesting.

Patient group (treatment received)	Samples	Clinical status at the time of examination	Number of individuals	Leptin levels (pg/mL)	
				Median	Variation range
Acute leukemia patients (intensive chemotherapy)	Serum	Before consolidation chemotherapy	n=12	44,500	4,700-160,000
		During neutropenia, no infection	n=16	45,000	5,900-190,000
		During neutropenia, bacterial infections	n=16	52,000	3,000-210,000
		During neutropenia, antibiotic therapy	n=16	38,000	3,100-205,000
		Healthy controls	n=12	36,000	8,400-145,000
Patients with malignant diseases (stem cell mobilization and harvesting)	Citrate plasma	Before apheresis	n=15	3,200	1,090-10,600
		Following apheresis	n=15	3,550	1,200-9,20
		Levels in the stem cell product	n=13	2,800	1,200-8,300
		Healthy controls	n=15	3,050	750-30,000

Leptin levels were determined by ELISA analysis. For each of the two groups of patients separate groups of healthy controls matched for age and sex were examined.

of the patients (5 of 11 patients with detectable release). The effect of leptin did not differ when comparing CD34^{+} (>20% of blasts judged to be positive by flow cytometric analysis, n=21) and CD34^{–} AML blasts populations.

Enriched AML blasts (CD2^{+}, CD19^{+} and adherent cell depleted; >99% blast cells) derived from patients 1-12 (Table 1) were cultured with and without leptin 2 μg/mL. The presence of leptin increased IL6 levels in the supernatants (n=10, *p*=0.008). AML blasts derived from patients 1-10 were in addition cultured in serum-free media (X-vivo 10®, X-vivo 15®, StemSpan™) for 48 hours before IL1β and IL6 levels were determined. Relatively high cytokine levels were then observed for blasts cultured in StemSpan™, and the levels were not significantly altered by leptin (2, 0.2, 0.02 and 0.002 μg/mL). These results were confirmed in repeated experiments (only with leptin 2 μg/mL) that also included 6 additional patients. In contrast, leptin increased IL6 levels significantly for AML blasts cultured in X-vivo 10® (n=8, *p*=0.006) and X-vivo 15® (n=10, *p*=0.02).

AML blasts derived from 5 patients (Table 1, patients 1-4, 7) were cultured with and without leptin 2 μg/mL for two days before the levels of IL1β- and IL6-specific RNA were determined. These patients were selected because they showed a typical leptin-induced enhancement of the release of several cytokines (*see above*) in repeated experiments. IL1β-specific (range 15.0-39.0 amol/μg

total RNA) and IL6-specific RNA (range 32.0-86 amol/mg RNA) reached detectable levels for all patients. Although all these patients showed increased cytokine protein levels in the supernatants, leptin had divergent effects on the cytokine-specific RNA levels (*data not shown*).

Effects of leptin on AML blast proliferation in suspension cultures

Native AML blasts derived from 9 patients (Table 1, patients 1-8, 10) were cultured with and without leptin (10, 2, 1, 0.2, 0.1, 0.02 and 0.002 μg/mL). Leptin altered spontaneous proliferation only for 3 patients (enhancement for 2 and inhibition for 1 patient), and for all three patients leptin had a dose-dependent effect with a plateau at concentrations ≥ 1 μg/mL (*data not shown*). The effect of leptin 2 μg/mL was then examined for 48 consecutive patients. Undetectable proliferation in cultures both with and without leptin was observed for 25 patients. When comparing the overall results for the other 23 patients leptin caused a significant increase in spontaneous AML blast proliferation (Figure 4, *p*=0.012). This enhancement was observed both for CD34^{+} and CD34^{–} AML blast populations. For patients 1-12 these effects were confirmed in repeated experiments (*data not shown*).

AML blasts derived from 10 patients (Table 1, patients 1-10) were cultured with SCF+IL3+GM-CSF, and leptin (10-0.002 μg/mL) then had a dose-dependent effect on cytokine-dependent proliferation. When leptin 10 μg/mL affected blast prolif-
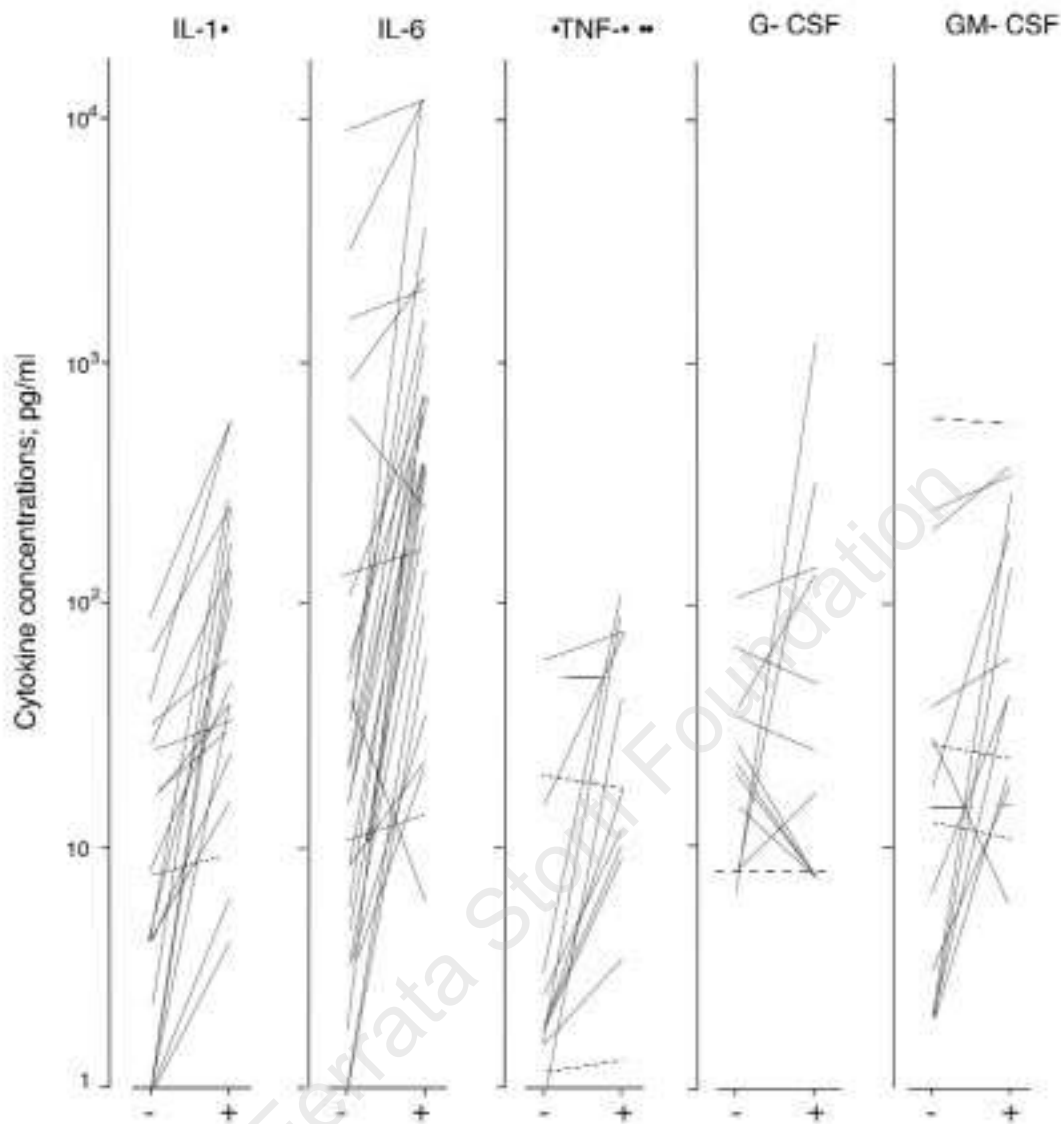
Figure 3. The effect of leptin 2 μg/mL on cytokine levels in supernatants derived from cultures containing native AML blasts. The blasts were cultured at 1 × 10⁶ cells/mL for 48 hours before supernatants were harvested and cytokine levels (IL1β, IL6, TNFα, G-CSF, GM-CSF; see top of the figure) determined by ELISA analysis. The figure compares the cytokine levels for cultures without (-) and with (+) leptin, and includes only the results for patients showing detectable cytokine levels: IL1β: 37 patients examined, 20 patients showing detectable levels; IL6: 37 patients examined, 29 showing detectable levels; TNFα: 37 patients examined, 13 patients showing detectable levels; G-CSF: 17 patients examined, 11 patients showing detectable levels; GM-CSF: 17 patients examined, 15 patients showing detectable levels. All results are presented as the cytokine concentrations (pg/mL) in culture supernatants. The minimal detectable G-CSF level is indicated in the figure (–––).

eration, a significant effect (*see above*) was always observed at 2 μg/mL and for certain patients even at lower concentrations (*data not shown*).

Native AML blasts derived from 45 consecutive patients were then cultured in medium containing single cytokines (SCF 20 ng/mL, GM-CSF 100 ng/mL or Flt3-L 20 ng/mLl). Leptin 2 μg/mL altered the cytokine-dependent proliferation only for a subset of patients, and divergent effects were then observed in the presence of all three cytokines (Figure 4). The divergent effects were observed both for CD34+ and CD34– AML cell populations, they were
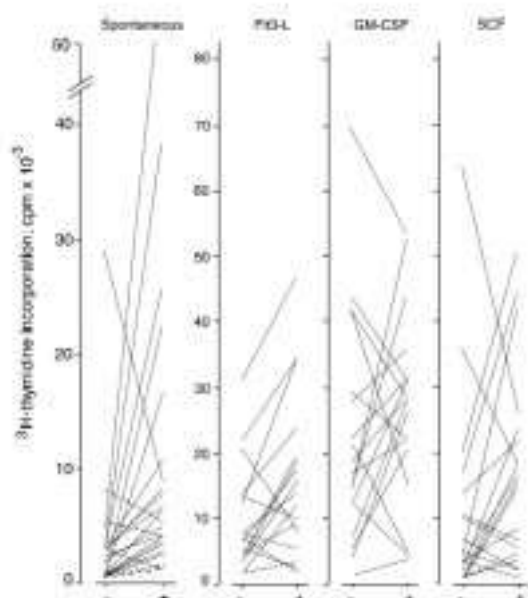
Figure 4. The effect of leptin on spontaneous and cytokine-dependent AML blast proliferation. AML blasts derived from 45 patients (48 for spontaneous proliferation) were cultured in FCS-containing medium for 7 days before ^{3}H-thymidine incorporation was determined. The AML cells were cultured in medium alone and in the presence of Flt3L 20 ng/mL, GM-CSF 100 ng/mL or SCF 20 ng/mL. The results for cultures without (-) and with (+) leptin 2 μg/mL were compared. Leptin increased spontaneous blast proliferation significantly, and the results for all patients showing detectable proliferation (^{3}H-thymidine incorporation corresponding to >1000 cpm) are therefore presented. Leptin had divergent effects in the presence of exogenous cytokines, and for these experiments the figure includes only the results for those patients showing significant alterations in the presence of leptin (^{3}H-thymidine altered by at least 20% and 2,000 cpm compared with the leptin-free control). Spontaneous; the results for 23 patients are presented. Flt3-L: the results for 19 patients are presented, 14 other patients showed undetectable proliferation and 12 patients showed unaltered proliferation. GM-CSF: the results for 18 patients are presented; 12 other patients showed undetectable proliferation and 15 patients showed unaltered proliferation. SCF: the results for 19 patients are presented, 13 patients showed undetectable proliferation and 13 patients showed unaltered proliferation. All the results are presented as the mean cpm of triplicate determinations.

reproduced in repeated experiments for 9 of the patients, and the divergence was observed when testing enriched AML blasts derived from patients 1-12 (*data not shown*). The overall results for spontaneous and cytokine-dependent (Flt3-L, GM-CSF, SCF) AML blast proliferation were also compared for the 45 patients. When the definitions for significant alterations given in the Design and Methods were used, leptin did not have a significant

effect either on spontaneous nor cytokine-dependent AML blast proliferation only for 18 patients.

Effects of leptin on AML colony formation

AML blasts derived from patients 1-18 (Table 1) were pre-incubated in serum-free StemSpan™ with and without leptin 2 μg/mL for 7 days before cells were washed and the frequencies of clonogenic cells estimated in the erythropoietin+conditioned medium assay. Leptin had divergent effects on the formation of both erythroid and non-erythroid colonies (Figure 5), and leptin also had divergent effects on the ratio of erythroid to non-erythroid colonies (*data not shown*). Neither the effect of leptin nor the ability to form erythroid colonies showed any correlation with CD34 expression by the leukemia blasts. This divergence was detected in repeated experiments, and for 5 of these patients (Table 1, patients 1, 2, 6, 8, 9) a similar leptin effect was detected both when AML blasts were preincubated in FCS-containing and serum-free media (StemSpan™, X-vivo-10®, X -vivo-15®) (*data not shown*).

We investigated the effect of leptin 2 μg/mL when it was present during colony formation, and the AML blasts were then cultured in methylcellulose medium containing only GM-CSF (Table 1, patients 7-13) and erythropoietin+conditioned medium (Table 1, patients 1-10). Both erythroid and non-erythroid colonies were detected in the presence of erythropoietin + conditioned medium for a subset of patients. However, leptin had divergent effects and altered colony formation only for a minority of the patients both in the presence of GM-CSF (3/7) and erythropoietin + conditioned medium (2/10) (*data not shown*).

Effects of leptin on AML cell differentiation and apoptosis

Native AML blasts derived from patients 1-10 (Table 1) were incubated with leptin 2 μg/mL for 2 and 7 days before May-Grünwald-Giemsa stained cytospin smears were examined by light microscopy. Incubation with leptin for 2 days did not alter cell differentiation, but after 7 days leptin increased the frequency of promyelocytes (neutrophil granulation without chromatin condensation) with more than 10 % (range 12-31%) for 4 patients (*data not shown*).

The frequency of apoptotic AML blasts after *in vitro* culture was also examined for 10 patients. For 9 of these patients the frequencies of apoptotic cells were <30%, and the difference between cultures with and without leptin 2 μg/mL was <5% except for one patient in whom the frequency was

reduced from 54 % to 38% by addition of leptin (this effect was reproduced in a repeated experiment). Caspase 3 activity in cultured AML blasts was also determined for three patients showing a typical leptin-induced enhancement of cytokine release, and leptin 2 µg/mL then had divergent effects on the caspase activity without any induction of caspase-inhibitory activity.

Discussion

Leptin is released by adipocytes and is involved in the regulation of fat metabolism.[5,6] An additional role of leptin in the regulation of leukemic hematopoiesis is suggested by (i) the expression of leptin by bone marrow stromal cells,[8,19,28-31] and (ii) the detection of leptin receptors on normal and leukemic hematopoietic cells.[8-12,18] However, our present results suggest that leptin usually affects AML functions only at concentrations exceeding the serum levels.

Total body fat mass can be used as a predictor of systemic leptin levels, but due to the influence of other factors there is great heterogeneity in leptin levels at any given index of body fat.[10,32,33] Leptin levels are affected by energy imbalance, and within 24 hours of fasting leptin levels decline to approximately 30%.[32,33] The levels are also influenced by acute infections and inflammation as well as systemic levels of several cytokines and hormones.[10,32] Konopleva *et al.*[12] reported normal serum levels when they compared the results for 56 patients (AML or myelodysplasia) with the levels in 5 healthy individuals. In contrast, our results demonstrate that systemic leptin levels are decreased in AML patients, although the patients' levels showed a wide variation and a large overlap with the levels in the controls. The reduced levels were not secondary to a reduced body mass index, the cause rather seems to be multifactorial and related to the severe illness, altered energy balance and disease complications. Furthermore, systemic leptin levels showed a similar wide variation before chemotherapy and during chemotherapy-induced cytopenia, febrile neutropenia and antibiotic therapy. Acute infections can increase leptin levels,[10] but this effect can be counteracted by general illness with reduced food intake.[32,33] This is probably the explanation why acute infections did not cause any significant alterations in our patients.

We prepared native AML blasts by density gradient separation from the peripheral blood of consecutive patients with high blast counts. This simple technique was chosen because more extensive *ex vivo* manipulation can induce functional alter-
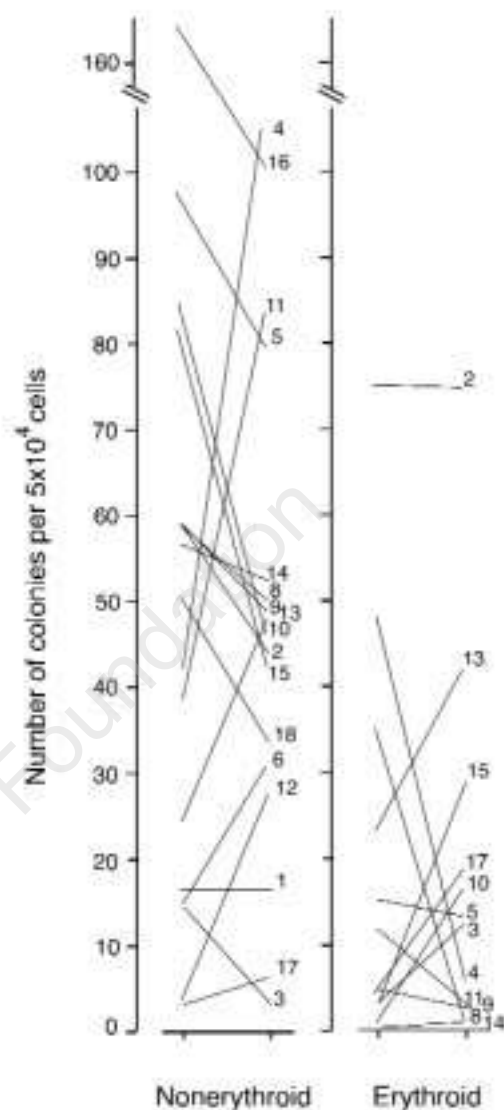

Figure 5. The effect of preincubation with leptin on AML blast colony formation. Native AML blasts were cultured for 7 days without (-) and with (+) leptin 2 µg/mL, and colony-formation was thereafter assayed in methylcellulose medium supplemented with erythropoietin+conditioned medium. The figure shows the numbers of non-erythroid (17 patients) and erythroid colonies (only detected for 12 patients) for each patient (Table 1, patients 1-18). The patient's identity is given in the figure.

ations in the blasts.[34-38] However, high blast counts seem to be an adverse prognostic factor that may reflect intrinsic properties of the AML cells,[36] and our results may thus be representative only for the selected subset of patients.

Previous studies have demonstrated that AML cells derived from peripheral blood and bone mar-

row of the same patients can have different phenotypes.[39] Flow cytometric studies of our patients also demonstrated minor quantitative differences between blood and marrow-derived cells for a subset of patients, but no qualitative differences were observed. Furthermore, circulating AML cell populations seem to have a similar hierarchical organization as the bone marrow cells, including long-term proliferating primitive progenitors.[40] Thus, by using peripheral blood-derived AML cells we probably get a representative cell population that can be prepared by a simple separation procedure.

The expression of leptin receptors by native AML blasts has been characterized in detail previously,[12,18] but functional effects have mainly been characterized for AML cell lines.[12] Konopleva *et al.*[12] investigated the effects on proliferation of native AML blasts only for a small group of 14 patients, whereas we investigated effects on proliferation (colony formation, spontaneous and cytokine dependent proliferation in suspension cultures), cytokine secretion, differentiation and regulation of apoptosis in a large group of consecutive patients.

Leptin had a dose-dependent enhancing effect on the release of IL1β, IL6, TNFα and GM-CSF by AML blasts, whereas the usually low or undetectable G-CSF levels were not significantly altered. Two different leptin preparations had similar effects that usually were observed at relatively high concentrations, and similarly high serum levels were detected only for a minority of patients. Although leptin can increase cytokine release by monocytes,[41] the high levels were observed for various AML-subtypes and not only for the myelomonocyte variant. The leptin effects did not differ between CD34+ and CD34– AML cell populations either. The increase was observed both when using FCS-containing and X-vivo® serum-free media, but not when the serum-free StemSpan™ medium was used. This last medium is associated with increased levels of AML blast cytokine release[22] and may thus represent optimal *in vitro* conditions even in the absence of leptin.

We examined the levels of IL1β- and IL6-specific RNA for five AML populations that showed increased cytokine protein levels in the presence of leptin. However, leptin had divergent effects on these RNA levels, and these results suggest that increased cytokine release is not dependent on an increase in cytokine-specific RNA levels. Our results also demonstrated that leptin's effect on cytokine levels was not dependent on reduced cytokine consumption due to reduced blast proliferation, differentiation induction, or reduced caspase 3 activ-

ity that often inhibits apoptosis. Furthermore, leptin increased the cytokine release by AML blasts cultured under serum-free conditions and when testing enriched AML blast populations, and these results demonstrate that leptin has a direct effect on the blasts that is not mediated via contaminating cell populations or an unidentified serum factor.

Leptin increased spontaneous AML blast proliferation in suspension cultures, and increased release of autocrine growth factors may then contribute to this enhancement. However, the leptin-induced alterations of AML colony-formation were also detected after preincubation in StemSpan™ medium when leptin does not enhance cytokine secretion. This observation suggests that leptin also modulates blast proliferation by additional direct effects and not only indirectly via altered growth factor release. In contrast, the leptin effects on cytokine-dependent proliferation were divergent, and the reproducibility of this divergence strongly suggests that it represents a true biological variation.

The effect of leptin on AML blast differentiation was investigated under the same experimental conditions as those for cytokine release and ³H-thymidine incorporation. Differentiation was analyzed by light microscopy because we regard morphologic alterations as a robust sign of irreversible differentiation. An increased fraction of promyelocytes was thus observed after 7 days of culture only for a few patients. Furthermore, leptin had divergent effects on erythroid and non-erythroid AML colony formation, and leptin alone did not induce erythroid differentiation. Taken together these results suggest that leptin itself has only minimal effects on AML blast differentiation, but the divergent effects on colony formation are maintained after induction of erythroid differentiation.

Although induction of differentiation and apoptosis often occur simultaneously in AML blasts, they should be regarded as separate events with different regulatory mechanisms.[2,3] The effect of leptin on AML cell apoptosis has previously been studied only in cell lines.[12] Our present results demonstrate that leptin had minimal effects on spontaneous *in vitro* apoptosis in native AML blasts, and the enhancing effect of leptin on AML blast cytokine release cannot be explained by altered caspase 3 activity with inhibition of spontaneous *in vitro* apoptosis in the cell population.

Autologous stem cell transplantation is investigated as a possible post-remission therapeutic approach in AML.[4,21] We regard autotransplantation as an experimental approach in AML,[4] and for this

reason we had to examine leptin levels in autografts derived from patients with other malignant disorders. However, our results are probably representative also for other patients with hematologic malignancies (including AML in remission) when similar mobilization and harvesting procedures are used. These procedures did not affect systemic leptin levels, but in the absence of additional local release these levels alone will probably not affect contaminating AML blasts in autografts.

Our present results for a large group of consecutive patients demonstrate that leptin enhances spontaneous proliferation and constitutive cytokine release by native AML blasts, but the systemic levels were generally lower than the concentrations required to affect these *in vitro* functions. Several studies have demonstrated that leptin is released by various bone marrow stromal cells,[8,19,28-31] and local release together with the systemic levels may thus affect leukemic hematopoiesis. This local bone marrow release is difficult to quantify. However, a recent study described a correlation between serum leptin and the degree of bone marrow infiltration for patients with lymphoproliferative disorders.[31] One possible explanation for this observation could be that bone marrow release is sufficiently high to affect the systemic levels, and increased binding by leptin receptor-expressing malignant cells in the bone marrow may then cause decreased systemic levels. A similar mechanism may explain our present results with decreased levels in our patients, and this would also support the hypothesis that bone marrow-released leptin is functionally important in AML. Systemic leptin levels in combination with local release may thus represent a functional interaction between leukemic hematopoiesis and metabolic regulation.

Contribution and Acknowledgments

All authors took part in the design of these studies, interpretation of the overall data and preparation of the manuscript. The authors especially contributed to the following parts: ØB: collection of cell samples and serum samples, investigation of AML blast proliferation and cytokine secretion, colony formation and differentiation. T-SH: analysis of cytokine-specific RNA levels; BTG: analysis of apoptosis and regulation of apoptosis; NG: collection of serum samples, ELISA analysis, interpretation of the data for serum levels; BF: collection, preparation and analysis of plasma samples from autografted patients. All authors approved the final version to be published.

Disclosures

Conflict of interest: none.
Redundant publications: no substantial overlapping with previous papers.

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PEER REVIEW OUTCOMES

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Elihu Estey, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Estey and the Editors. Manuscript received January 10, 2002; accepted April 11, 2002.

What is already known on this topic

Leptin receptors are present on AML blasts. This study explored the possible role of serum leptin in pathogenesis of AML.

What this study adds

Serum leptin levels were decreased in AML patients (n=20) compared to age- and sex-matched healthy controls. Leptin's effects on leukemic blasts were complex and seemed to occur at *in vitro* concentrations above those found in the sereum in most patients

Potential implications for clinical practice

This study points out the complex biological regulation of leukemic proliferation and thus implies that approaches to specifically target this process will likely need to be quite sophisticated.

Elihu Estey, Associate Editor