Disease-stabilizing treatment with all-trans retinoic acid and valproic acid in acute myeloid leukemia: serum hsp70 and hsp90 levels and serum cytokine profiles are determined by the disease, patient age, and anti-leukemic treatment

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Heat shock protein (HSP) 70 and HSP90 are released by primary human acute myeloid leukemia (AML) cells during stress-induced spontaneous in vitro apoptosis. The AML cells also show constitutive release of several cytokines and the systemic serum levels of several soluble mediators are altered in patients with untreated AML. In the present study, we have investigated serum levels of HSP70/HSP90 and the serum cytokine profiles of patients with untreated AML and patients receiving AML-stabilizing palliative treatment based on all-trans retinoic acid (ATRA) plus valproic acid. Patients with untreated AML showed increased HSP90 levels and a distinct serum cytokine profile when compared with healthy controls, and low pre-therapy HSP90 levels were associated with a prolonged survival during treatment with ATRA + valproic acid + theophyllin. Hierarchical cluster analysis showed a close association between HSP70, HSP90, IL-1 receptor antagonist (IL-1ra), and hepatocyte growth factor (HGF) levels. Furthermore, disease-stabilizing therapy altered the serum-cytokine profile, but the correlations between HSP70/HSP90/IL-1ra/HGF were maintained only when ATRA + valproic acid were combined with theophyllin but not when combined with cytarabine. We conclude that both HSP levels and serum cytokine profiles are altered and may represent possible therapeutic targets or prognostic markers in human AML. Am. J. Hematol. 87:368–376, 2012. © 2012 Wiley Periodicals, Inc.

Introduction

The heat shock response was discovered as chromosome puffs in the salivary gland of Drosophila flies [1]. This led to the discovery of the heat shock proteins (HSPs). The HSPs maintain cellular homeostasis and function as molecular chaperones assisting protein folding and translocation, and their intracellular levels are upregulated in response to many physiological and environmental insults [2]. Intracellular HSPs are cytoprotective effectors and allow the cells to survive otherwise lethal conditions, acting at multiple steps in the apoptotic pathways to ensure that stress-induced damage does not inappropriately trigger cell death [2]. On the other hand, HSPs are also released by cells to the extracellular space. Released HSPs together with their client proteins can then become internalized by antigen-presenting cells and this leads to a more efficient cross-presentation of antigenic epitopes of the client proteins to specific T cells [3,4]. Extracellular HSPs may also initiate release of pro-inflammatory cytokines, stimulate NK cells and facilitate DC maturation [2,5,6].

HSPs are overexpressed in many human malignancies, including both solid tumors and hematological malignancies, and high intracellular levels are usually associated with adverse prognosis [7,8]. High acute myeloid leukemia (AML) cell expression of HSPs seems to be associated with an adverse prognosis both with regard to complete remission rate and survival [9–12]. A correlation between high serum/plasma levels and poor prognosis is also seen for many cytokines [13–16], and these heterogeneous mediators interact through local cytokine networks that regulate growth and differentiation of normal as well as malignant hematopoietic cells [17,18].

HSP70 and HSP90 seem to be the HSPs most commonly expressed at high levels by primary human AML cells [7,11,19]. Previous studies suggest that serum HSP levels may have a prognostic impact in younger AML patients receiving intensive chemotherapy [9,12]; a possible explanation for this could be modulation of anti-leukemic T cell reactivity [20–22]. However, one would expect this effect of AML-cell derived HSPs to be further modulated by the cytokine network. In this study, we have therefore investigated (i) serum levels of HSP70 and HSP90 in a large group of unselected patients (also including elderly patients unfit for intensive chemotherapy), (ii) the possible prognostic impact of serum HSP levels for elderly patients receiving AML-stabilizing treatment, and (iii) the association between potentially immunostimulatory HSP serum levels and the systemic (serum) cytokine profile in AML patients with untreated disease and patients receiving disease-stabilizing therapy base on all-*trans* retinoic acid (ATRA) and valproic acid.

Materials and Methods

Patients. All studies were approved by the local Ethics Committee (Region III, University of Bergen, Norway) and samples collected after informed consent. A large group of consecutive AML patients was examined, and a subset of these patients was treated with disease-stabilizing therapy according to two phase 1/2 clinical protocols:

- The first group included 20 patients (12 males, eight females, median age 70.5 years with variation range 50–86 years) treated with oral ATRA 22.5 mg/m^2 twice daily days 1–14, and valproic acid together with theophyllin from day 3 until disease progression. For both

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TABLE I. Clinical and Biological Characteristics of 82 AML Patients Included in the Study

Age; median (range)	67 years (27–90)
Gender (female/male)	42/40
Percentage of patients with:	
Secondary AML	17%
AML relapse	20%
De novo AML	63%
FAB classification (percentage of patients)	
M0/M1	48%
M2	20%
M4/M5	32%
Expression of CD34 (percentage of patients)[a]	71%
Cytogenetic abnormalities (percentage)[a]	
Normal	50%
Intermediate	13%
Good	7%
Adverse	30%
Flt3-internal tandem duplication (percentage)	24%
NPM-1 mutation (percentage)	20%

[a] CD34 positivity was defined as at least 20% of the cells staining positive for the CD34 stem cell marker. Cytogenetic abnormalities were classified according to the MRC guidelines. Cytogenetic analyses were available for 67 patients. FAB classification was available for 66 patients, Flt3 analysis for 55 patients and NPM-1 analysis for 40 patients.

the drugs, this treatment with valproic acid and theophyllin started with an initial intravenous loading dose and thereafter 48 hr of intravenous infusion therapy guided by serum level estimations before the treatment was continued as oral administration. Serum samples were then collected before treatment (day 1), after 2 days of treatment with ATRA alone (day 3), and after 5 additional days of treatment with the triple combination (day 8) (Study registration: ClinicalTrials.gov no. NCT00175812 and EudraCT no.2004-00166 3-22) [23].

- The second group included 22 patients (eight males and 14 females, median age 77 years with variation range 58–90 years) treated with valproic acid from day 1 and until disease progression, oral ATRA 21.5 mg/m^2 twice daily days 8–22, and subcutaneous cytarabine 10 mg/m^2 administered once daily days 15–24. Treatment with valproic acid started with an intravenous loading dose and thereafter, a continuous intravenous infusion for 24 hr before the treatment was continued as oral administration guided by the serum valproic acid level. Samples were collected before treatment (day 1), after 7 days of treatment with valproic acid alone (day 8), after treatment of ATRA plus valproic acid (day 15), and after completed treatment with ATRA, valproic acid and cytarabine (day 25–27) (Study registration: ClinicalTrials.gov no. NCT00995332 and EudraCT no. 2007-2007-001995-36).

Forty additional patients were included in our studies of patients with untreated AML, and together all these patients represent a group of 82 unselected patients. Their clinical and biological characteristics are summarized in Table I, the cytogenetic abnormalities then being classified according to the MRC criteria [24]. Primary human AML cells derived from 28 patients were also included in our study. We included serum samples derived from 20 healthy controls with a similar age and gender distribution as the untreated AML patients. For all the blood samples, the serum was separated within 2 hr after sampling and was stored at −80°C until analyzed.

Analysis of heat shock protein and cytokine levels in serum. EIA/ELISA kits (Enzo life sciences, DE-79539 Lörrach, Germany) were used to determine HSP70 (HSP70 high sensitivity EIA kit EKS-715) and HSP90 serum levels (Hsp90α ELISA kit EKS-895). For analysis of serum cytokine levels, we used the Fluorokine MAP Multiplex Kits, human cytokine panel A and B (R&D Systems; Abingdon, UK). All analyses were performed strictly according to the manufacturers' instruction. The cytokine studies included analyses of (i) immunomodulatory cytokines (CD40-Ligand, TNFα, IFNγ), (ii) interleukins (IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17), (iii) chemokines (CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/Rantes, CCL11/Eotaxin, CXCL5/ENA-78, CXCL8/IL-8, CXCL10/IP10, CXCL11/I-TAC), and (iv) the growth factors granulocyte- and granulocyte-macrophage colony-stimulating factor (G-CSF and GM-CSF), epithelial growth factor (EGF), hepatocyte growth factor (HGF), basic fibroblast growth factor, thrombopoietin (Tpo), vascular endothelial growth factor (VEGF), and leptin.

Analysis of HSP cellular levels and HSP release by in vitro cultured primary human AML cells. Total AML cell levels (28 patients, see above) of HSP70 and HSP90 were determined as described in detail earlier [25]. Primary human AML cells ($n = 14$) were cultured in vitro and HSP levels determined in the supernatants as described in our earlier study [19].

Statistics and bioinformatical approaches. For statistical comparisons between different groups, we used the Mann–Whitney *U*-test. For statistical analyses of paired observations, the Wilcoxon's signed rank test was used and for correlation analyses, we used the Spearman's correlation test. Differences were generally regarded as statistically significant when *P*-values were <0.05. For the characterization of the cytokine profile in patients and healthy controls, we investigated 31 different cytokines; due to this large number of comparisons, we only regarded *P*-values < 0.02 as statistically significant ($0.02 < P < 0.05$ is then referred to as borderline significance) in this particular part of the study to reduce the risk of finding, a significant *P*-value < 0.05 by chance due to the large number of cytokines/comparisons analyzed. Bioinformatical analyses were performed using the J-Express (MolMine AS, Bergen, Norway) [26]. For hierarchical clustering, all values were median variance standardized and log(2) transformed. The complete linkage was used as linkage method and for distance measure, the Pearson correlation test was used.

Results

Untreated AML patients have increased HSP90 serum levels compared with healthy controls

Serum levels of HSP70 and HSP90 were determined for 82 unselected patients with untreated AML. The patients showed significantly higher HSP90 levels (Fig. 1A; mean level 295 ng/ml, Mann–Whitney *U*-test, $P < 0.0001$) than the healthy controls (mean level 12.1 ng/ml). The serum levels of HSP70 were significantly lower than the HSP90 levels both for the patients (mean level 2.0 versus 295 ng/ml, Wilcoxon's signed rank test, $P < 0.0001$) and for the healthy controls (Fig. 1; mean level 0.5 versus 12.1 ng/ml,

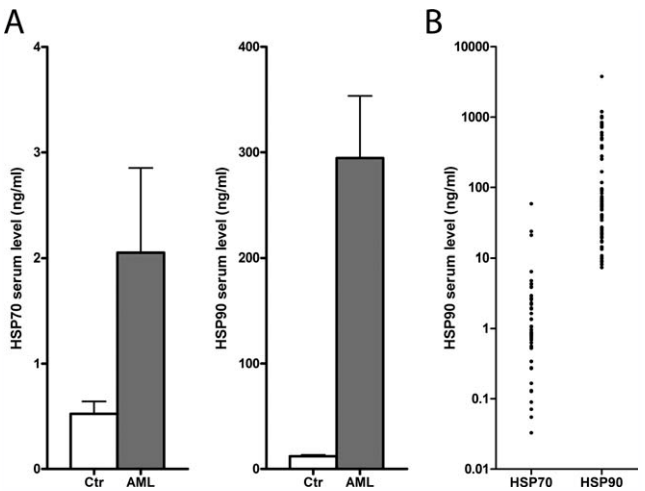

Figure 1. Serum HSP levels in patients with untreated AML and healthy controls. Serum levels of HSP70 and HSP90 in 82 patients with untreated AML and 20 healthy controls (A) The HSP90 and HSP70 serum levels were determined for patients with untreated AML and for healthy controls. (B) HSP70 levels were generally lower than the HSP90 serum levels in untreated AML patients as can be seen from the figure, but despite this the HSP70 and HSP90 levels showed a statistically significant correlation (Spearman $R = 0.425$, $P < 0.01$).

$P < 0.0001$), and the HSP70 serum levels did not differ significantly between patients and controls. Finally, the HSP70 serum levels were generally lower than the corresponding HSP90 levels (Fig. 1B), but despite this the HSP70 and HSP90 serum levels were significantly correlated for the untreated-AML patients (Fig. 1B; Spearman, $P < 0.01$, $R = 0.425$). A similar correlation was not observed for the healthy controls (data not shown).

Primary AML cells derived from 14 unselected patients were cultured in vitro and the HSP levels in culture supernatants determined. There was no significant correlation between HSP70 or HSP90 levels in the supernatants and the corresponding serum samples (data not shown).

HSP serum levels in AML patients depend on age, AML cell differentiation, and the leukemia cell burden

Patients age showed a significant inverse correlation to HSP90 ($P = 0.271$, $P < 0.05$), and elderly patients above 70 years of age showed significantly lower HSP90 levels than patients below 50 years of age ($P = 0.033$). Then we investigated whether there was any association between HSP serum levels and AML cell characteristics. First, morphological signs of AML cell differentiation were evaluated by the French–American–British (FAB)-classification. Patients with minimal signs of differentiation showed significantly lower HSP90 levels (FAB 0/1; median level 35.7 ng/ml, range 7.4–3,785 ng/ml) than patients with neutrophil differentiation (FAB-2; median 94.3 ng/ml, range 17.3–1,198 ng/ml, Mann–Whitney *U*-test $P = 0.0238$) and patients with monocytic differentiation (FAB-4/5, median 386 ng/ml, range 25.3–1,198 ng/ml, Mann–Whitney *U*-test $P = 0.0006$). The HSP90 serum levels were also significantly lower for patients with $CD34^+$ leukemic cells (>20% $CD34^+$ cells; median level 39 ng/ml, range 7–3,785 ng/ml, Mann–Whitney *U*-test $P = 0{,}0009$) than for patients with $CD34^-$ AML cells (median level 389 ng/ml, range 21–1,198 ng/ml). Second, there were no significant differences in serum HSP90 levels between patients with intermediate, normal, and adverse cytogenetic abnormalities; we had very few patients with low-risk abnormalities and for this reason these patients were not included in these analyses. HSP90 levels did not show any associations with FLT3-ITD or NPM1 mutations either. Finally, HSP70 serum levels showed no association with age, morphological differentiation, CD34-expression, cytogenetic, or molecular genetic abnormalities.

We investigated consecutive patients admitted to our hospital, including both patients with relapse and newly diagnosed AML, but the HSP70/HSP90 serum levels did not differ between relapse and newly diagnosed leukemia. No differences were seen between de novo and secondary AML either.

There is a wide variation between patients with regard to the total HSP protein levels in the AML cells [25]. We therefore investigated whether there were any correlations between HSP90 serum levels and (i) total HSP90 levels in the leukemia cells or (ii) the leukemia cell burden. First, the total cellular HSP70 and HSP90 protein levels were determined for 28 unselected patients. The HSP90 levels showed a wide variation with regard to both total cellular protein level (median 12.9 ng/ml, range 2.0–35.8) and serum levels (48.7 ng/ml, range 7.4–3,785 ng/ml), but without any significant correlation. Similarly, HSP70 cellular levels (median 43.3, range 2.8–454 ng/ml) and serum levels (median 0.13 ng/ml, range not detectable–4.32 ng/ml) also showed wide variations without significant correlation. Thus, the total HSP protein levels in the AML cells seem to have only a minor influence on the serum levels. Second, we also investigated whether there was any association between the AML cell burden and HSP70/HSP90 levels. Detailed information was available only for patients included in the two clinical protocols, and we evaluated four different parameters that we regard, at least partly, to reflect the leukemia cell burden:

- *Percentage of bone marrow blasts.* Only HSP70 serum levels (Spearman's correlation test; $P < 0.05$, $R = 0{,}343$) showed a significant correlation with the blast percentage (median 55%, range 28–99%).
- *Serum lactate dehydrogenase (LDH) levels.* Both HSP70 ($P < 0.05$, $R = 0{,}314$) and HSP90 ($P < 0.01$, $R = 0{,}707$) showed significant correlations with serum LDH levels (median 265 IU/L, range 133–1,677 IU/L). There was also a correlation of borderline significance between bone marrow blast percentage and serum LDH levels ($P < 0.05$, $R = 0{,}338$).
- *The peripheral blood blast count.* We observed a significant correlation between the level of circulating blasts and both HSP90 ($P < 0.01$, $R = 0.617$) and HSP70 serum levels ($P < 0.01$, $R = 0.360$). For most patients, the blood blast counts were relatively low corresponding to $<5 \times 10^9$/l, and there was no significant correlation between percentage of bone marrow blasts and levels of circulating blasts.
- *Remaining normal bone marrow function.* No significant correlations were detected between HSP70 or HSP90 levels and peripheral blood platelet counts or the hemoglobin levels.

Thus, HSP70 serum levels showed statistically significant correlations with three out of the four parameters, whereas HSP90 showed significant correlations only for two parameters.

ATRA alone or in combination with valproic acid plus theophyllin (protocol 1) does not alter serum HSP70/HSP90 but pretherapy HSP90 levels show an inverse correlation with survival

We investigated HSP serum levels for 20 patients included in the first protocol; they initially received ATRA alone for 2 days before valproic acid and theophyllin were added. HSP70 and HSP90 serum levels were determined before (day 1) and after two days of ATRA therapy (day 3) and after five additional days with the triple combination (day 8). The median HSP70 level was 0.9 ng/ml (range not detectable (nd)–58.7 ng/ml) before treatment, not detectable (range nd–65.6 ng/ml) on day 3 and 1.2 ng/ml (range nd–43.2 ng/ml) on day 8. The median HSP90 levels before treatment and after 3 and 8 days were 48.5 ng/ml (range 8.2–1,023 ng/ml), 19.7 ng/ml (range 9.7–1,170 ng/ml), and 63.1 ng/ml (range 8.8–2,968 mg/ml), respectively. None of these differences reached statistical significance, but there was a similar correlation between HSP70 and HSP90 both on day 3 ($P < 0.05$, R = 0.447) and day 8 ($P < 0.01$, $R = 0.664$) as described above for untreated-AML patients.

For this clinical study, we had follow-up data for all included patients [23], and the pre-therapy HSP90 serum levels showed a significant inverse correlation with survival ($P = 0.004$, $R = -0.617$; Spearman's correlation test). The HSP90 levels for patients with survival shorter (13 patients, median level 118 ng/ml, range 8–1,023 ng/ml) and longer than 100 days (7 patients, median 19 ng/ml (range 10–90 ng/ml, Mann–Whitney *U*-test $P = 0.04$) differed significantly. In contrast, there was no significant correlation between HSP70 levels and survival.

As a comparison, 10 of our patients with newly diagnosed AML received only supportive treatment, and for this group the HSP90 levels showed no correlation with the survival (data not shown).

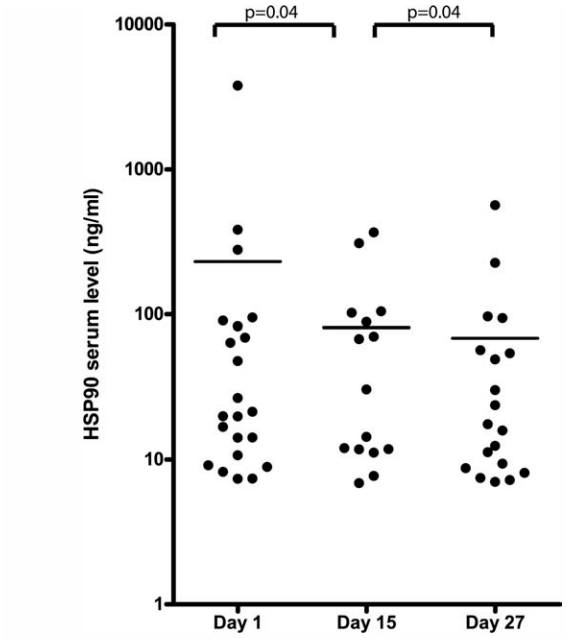

Figure 2. HSP90 serum levels in patients treated with valproic acid, ATRA, and low dose cytarabine. HSP90 serum levels (ng/ml) were determined for 22 patients before and after treatment with valproic acid (from day 1), oral ATRA (21.5 mg/m^2 twice daily, days 8–22), and low-dose subcutaneous cytarabine (corresponding to a total dose 100 mg/m^2 for a 10-day period, days 15–24). The figure presents the serum HSP90 levels before treatment (day 1, 22 samples), treatment with valproic acid plus ATRA (day 15, 15 samples) and after treatment with the triple combination of valproic acid, ATRA, and low-dose cytarabine (day 27, 19 samples). The mean levels are indicated in the figure and the *P*-values are given at the top of the figure (Wilcoxon's signed rank test).

HSP90 but not HSP70 levels are significantly altered during treatment with ATRA, valproic acid, and low-dose cytarabine

The 22 patients included in the second protocol received valproic acid alone for the first 7 days, this drug was thereafter combined with ATRA for days 8–22 and low-dose cytarabine for days 15–25. Finally, valproic acid was continued as the only drug from day 26. We compared HSP serum levels for samples collected on day 1 (without any treatment), day 8 (after valproic acid alone), day 15 (valproic acid plus ATRA), and day 25–27 (after valproic acid, ATRA, and low-dose cytarabine). The median HSP70 levels at all four time points were undetectable with maximal levels 23.8, 30.3, 23.1, and 18.1 ng/ml, respectively. Thus, HSP70 levels did not differ during treatment.

The HSP90 levels were also determined on day 1 before treatment and on days 8, 15, and 25–27 during treatment. The median level before treatment was 20.7 ng/ml (range 7.4–3,785 ng/ml) and after 7 days with valproic acid alone 14.0 ng/ml (range 6.6–380 ng/ml), but this difference did not reach statistical significance ($P = 0.11$). However, HSP90 levels were significantly reduced on day 15 (median level 17.3 ng/ml, range 6.9–368 ng/ml; $P = 0.04$) compared with pretherapy levels and further significantly reduced between days 15 and 25–27 (median level 16.7 ng/ml, range 7.0–567 ng/ml, $P = 0.04$, Fig 2). Finally, a significant correlation between HSP70 and HSP90 levels was observed on day 1 ($P < 0.05$, $R = 0.493$), day 8 ($P < 0.05$, $R = 0.570$), and day 15 ($P < 0.01$, $R = 0.666$) but not on day 27.

Patients with untreated AML show an altered serum cytokine profile

We compared the serum-cytokine profile for the 82 untreated-AML patients and the healthy controls. The overall results are summarized in Fig. 3, and the results for cytokines showing statistically significant differences are presented in detail in Table II. The cytokines were classified as (i) immunoregulatory mediators; (ii) chemokines, (iii) interleukins, and (iv) growth factors. Due to the large number of comparisons, differences were regarded as significant when $P < 0.02$ (see Table II). The serum levels differed between patients and healthy controls for several cytokines, including immunomodulatory cytokines (TNFα), interleukins (IL-1α, IL-4, IL-5, IL-6, IL-10), CXCL chemokines (CXCL5, CXCL8/IL-8, CXCL10), and growth factors (G-CSF, GM-CSF, HGF). Only two additional cytokines

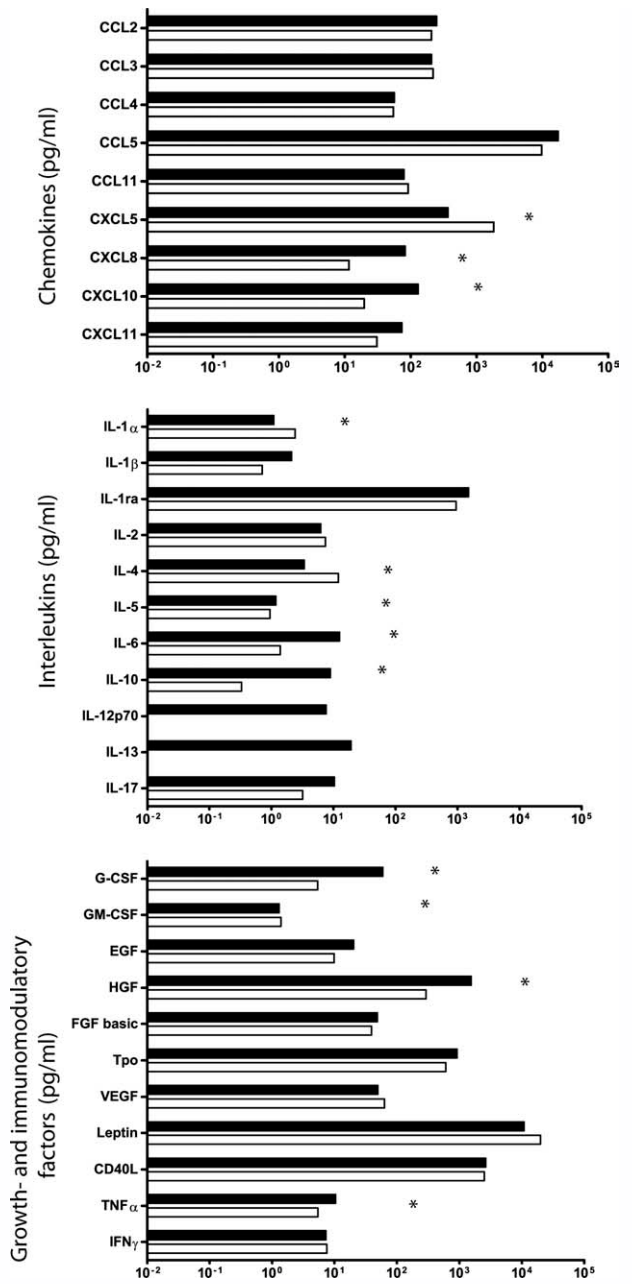

Figure 3. The serum cytokine profile for untreated AML patients and healthy controls. Serum cytokine levels were determined for 82 AML patients and 20 healthy controls with a similar age and gender distribution. The cytokines were classified as (i) chemokines, (ii) interleukins, and (iii) growth- and immunomodulatory factors (see left part of the figure). A total of 31 cytokines were analyzed, and the figure presents the mean for each cytokine in patients (dark columns) and healthy controls (white columns). Cytokines showing statistically significant differences between patients and healthy controls are marked with an asterisk to the right in the figure.

TABLE II. Serum Cytokine Levels in AML Patients and Healthy Controls: A Detailed Presentation of the Results for Cytokines Showing Statistically Significant Differences Between Patients and Controls

Cytokine		Patients	Healthy controls	*P*-value
Immunomodulatory cytokines				
TNF-α	↑	8.4 (<1.5–68.5)	4.5 (2.3–13.4)	0.016
Interleukins				
IL-1α	↓	0.5 (<0.4–7.0)	2.0 (2.0–2.9)	<0.0001
IL-2[a]	↓	2.5 (<2.23–61.5)	7.2 (<2.23–13.8)	0.0227
IL-4	↓	<4.5 (<4.5–53.9)	11.8 (5–17.5)	<0.0001
IL-5	↑	<0.7 (<0.7–39.1)	0.9 (<0.7–0.9)	0.0001
IL-6	↑	5.8 (<1.1–204.6)	<1.1 (<1.1–7.7)	<0.0001
IL-10	↑	1.3 (<0.3–238.0)	0.3 (<0.3–0.8)	0.0005
Chemokines				
CXCL5	↓	128.4 (<4.1–3252.1)	1283.5 (341–5307)	<0.0001
CXCL8/IL-8	↑	30.5 (<2.0–1103.2)	10.7 (2.2–22.8)	0.0003
CXCL10	↑	56.7 (0.5–1958.7)	15.25 (8.5–58.4)	<0.0001
Growth factors				
EGF[a]	↑	5.2 (<1.58–292.6)	<1.58 (<1.58–135)	0.0375
HGF	↑	797.3 (1–9703.0)	262.4 (117.3–729.3)	<0.0001
G-CSF	↑	32.2 (<1.5–1029.5)	<1.5 (<1.5–63.2)	<0.0001
GM-CSF	↑	0.5 (<2.0–17.2)	0.9 (0.9–3.6)	0.0096

The table shows the serum levels for 82 AML patients and a group of 20 healthy controls. The results are presented as the cytokine analyzed, the alteration in the patients (↑ increased, ↓ decreased), the patients levels (median and range), levels in healthy controls (median and range). Due to the large number of comparisons a significant p-value was defined as $P < 0.02$ (Mann–Whitney *U*-test); the table in addition presents the results for IL2 and EGF that showed a borderline significance with $0.02 < P < 0.05$. All cytokine concentrations are presented as pg/ml, for cytokine levels not detected the values are set to < the detection limit.

[a] Cytokines showing a borderline significance value with $0.02 < P < 0.05$.

showed a difference corresponding to a borderline significance with $0.02 < P < 0.05$, namely IL2 ($P = 0.0227$) and EGF ($P = 0.0375$). Despite these differences, the overall cytokine profiles showed several similarities in the two groups: immunomodulatory cytokines showed detectable levels, the interleukins generally showed low/undetectable levels, whereas the chemokines usually showed relatively high levels, and the growth factors showed detectable levels but with relatively low G-CSF and GM-CSF levels. Finally, platelets can also release several cytokines [27,28] and we detected significant correlations between peripheral blood platelet counts and the platelet-released mediators VEGF ($R = 0.233$, $P < 0.05$), CD40-Ligand ($R = 0.391$, $P < 0.01$), and CCL5 ($R = 0.270$, $P < 0.05$). However, these mediators did not cluster together (Fig. 4), an observation indicating that their levels are influenced also by other factors. For this reason, they were included in the cluster analysis.

Patient age and AML cell differentiation influence the serum cytokine profile in patients with untreated AML

We investigated whether the serum-cytokine profiles were different for patients above 70 years of age when compared with younger patients. Due to the large number of comparisons, we only regarded *P*-values < 0.02 as statistically significant. Patients above 70 years of age showed increased levels of CCL5 (even though platelet counts did not differ, $P = 0.31$) and EGF whereas the levels of IFNγ and CD40-Ligand were reduced (Table III). IL4 showed an increase and FGF basic a decrease of borderline significance ($0.02 < P < 0.05$) in the elderly patients.

We also examined whether there were any associations between serum cytokine levels and the expression of the CD14 monocyte marker or the CD34 stem cell marker by the patients' AML cells. Flowcytometric detection of at least 20% positive cells was then used as the cut-off between positive and negative staining. CD14 positivity was associated with increased serum levels of IL-1ra and reduced levels of CCL2, whereas CD34 positivity was associated with decreased levels of IL-1ra and IL5 (Table IV, $P < 0.02$). Finally, CCL11 showed an association of borderline significance ($0.02 < P < 0.05$) to CD14 expression, whereas IL-1β and Tpo showed associations of borderline significance to CD34 expression (Table IV).

Hierarchical cluster analysis of serum cytokine levels in patients with untreated AML: the HSPs cluster together with potentially immunosuppressive cytokines

We did an unsupervised hierarchical cluster analysis for all 82 patients and all cytokines together with HSP70 and HSP90. The soluble mediators could then be divided into four main clusters (Fig. 4, lower part). The cluster A (left) is referred to as the chemokine cluster and consisted of 12 soluble mediators, among them 7 chemokines. The smaller cluster B (middle left), consisted of three interleukins and three growth factors. Cluster C, referred to as the interleukin cluster, consisted of eight cytokines, among them five interleukins, and cluster D (right, referred to as the HSP cluster) consisted of seven soluble mediators including HSP70 and HSP90 that clustered close to HGF, TNFα, and IL-1ra. There was a significant correlation (Spearman, $P < 0.01$) between the mediators that clustered close to each other in cluster D, i.e., HSP90, HSP70, HGF, TNFα, and IL-1ra (Table V). The two remaining cytokines in this cluster (i.e., IL-10 and CXCL10) showed significant correlations only with HSP90 and IL-1ra, respectively (Spearman, $P < 0.01$).

The clustering analysis identified two major patient subsets I and II. There was a significant difference between these for both HSP70 (Mann–Whitney *U*-test, $P < 0.0001$) and HSP90 (Mann–Whitney *U*-test, $P = 0.0038$), where the lower cluster II showed the highest levels. In addition to the increased HSP levels, the lower cluster II also showed significantly higher levels of CXCL10 ($P < 0.0001$) and IL1-ra ($P = 0.009$). In contrast, the cluster I patients showed

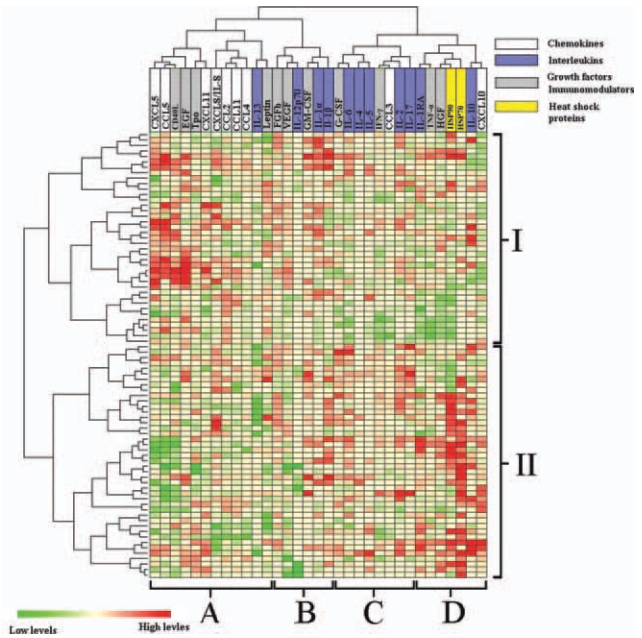

Figure 4. Unsupervised hierarchical cluster analysis of soluble mediators serum profile in 82 untreated-AML patients. Serum levels for nine chemokines (indicated by white color at the top of the figure), 11 interleukins (blue), 11 growth- and immunomodulatory factors (gray), and 2 heat shock proteins (yellow) were determinate from 82 consecutive AML patients. The concentrations were median normalized and log(2) transformed before unsupervised hierarchical clustering with Pearson's correlation as distance measure and complete linkage was performed, resulting in a heatmap for visualization and interpretation. The mediator expression profiles identified two main patient subsets that are referred to as clusters I and II (see the right margin). The soluble mediators formed four major clusters as indicated at the bottom of the figure (clusters A–D).

TABLE III. The Serum Cytokine Levels in Patients with Untreated AML Above and Below 70 Years of Age. The Table Only Shows the Results for Those Cytokines with *P*-values < 0.05

Altered cytokine	Patients above >70 years of age	Patients below 70 years of age	*P*-value
Increased levels in elderly patients			
CCL5	1782 (<1.9–369,139)	4235 (556.4–177,579)	0.0071
EGF	10.1 (<1.6–174.8)	3.8 (<1.6–292.6)	0.0063
IL-4[a]	<4.5 (<4.5–53.9)	<4.5 (<4.5–25.6)	0.0407
Decreased levels in elderly patients			
IFNγ	4.5 (<1.3–56.2)	8.6 (<1.3–20.8)	0.0067
FGF basic[a]	17.3 (<4.9–103.9)	76.6 (<4.5–163.1)	0.0334
CD40 ligand	811.1 (51.4–15,508)	1340 (243.3–34,712)	0.0193

The results are presented as the median level, the variation range is given in parenthesis. The Mann–Whitney *U*-test was used for the statistical comparison.
[a] Cytokines showing a borderline significance value with $0.02 < P < 0.05$.

higher levels of CCL5 ($P < 0.0001$), CXCL5 ($P < 0.0001$), VEGF ($P = 0.0049$), CD40-Ligand ($P < 0.0001$), and leptin ($P = 0.0162$). The difference between cluster I and II did not reach statistical significance for HGF ($P = 0.05$), IL10 ($P = 0.05$), and TNFα ($P = 0.33$) even though they all clustered close to the HSPs. Finally, there was no significant difference between the two patient clusters with regard to age, gender, cytogenetic abnormalities or frequencies of FLT3, or NPM1 mutations (data not shown).

The cytokine clustering is altered after ATRA + valproic acid-based disease-stabilizing treatment

We compared single cytokine levels before (day 1) and during treatment for patients included in protocol 1 (ATRA, valproic acid, and theophyllin; day 8 samples) and protocol 2 (ATRA, valproic acid, and cytarabine; day 25–27 samples). IL-6 was the only cytokine that showed a significant increase in both protocols (*P*-values 0.011 and 0.010, respectively), and in protocol 2, we, in addition, observed a significant decrease in IL-4 levels ($P = 0.016$). The other cytokines showed no significant differences (data not shown).

We also did an unsupervised clustering of the post-treatment cytokine levels for protocol 1 and 2 patients (day 8 and day 25–27 samples, respectively, Fig. 5). Even though the effects of the treatment on the cytokine network was limited when investigating single cytokines (see above), the different cytokine profiles when compared with the untreated patients (see Fig. 4) show that there are effects on the cytokine network even though the effects on individual cytokines do not reach statistical significance. Furthermore, the two protocols differed with regard to these cytokine profiles after treatment. The close correlations between HSP levels and the levels of IL-1ra, HGF, and TNFα were maintained in protocol 2. In contrast, for patients included in protocol 1, these mediators clustered further apart. Despite these differences in clustering, the significant correlations between HSP70/HSP90/IL1-ra/HGF levels were generally maintained after disease stabilizing therapy (Fig. 5, Table IV). Thus, the two protocols differ with regard to their effects on the serum-cytokine profile.

DISCUSSION

The aims of the present study were to examine the serum levels of potentially immunostimulatory HSP70/HSP90 in a large group of unselected AML patients (also including elderly patients not fit for intensive chemotherapy), to investigate the potential prognostic impact of the HSPs in elderly patients receiving disease-stabilizing therapy, and to examine whether altered HSP serum levels are associated with specific alterations in the immunoregulatory cytokine networks. We then compared the HSP70 and HSP90 levels for patients with untreated AML and a group of healthy controls. The HSP90 levels were significantly higher than the HSP70 levels both for patients and controls, and the patients showed significantly higher HSP90 levels than the controls. We have recently described that HSP90 is constitutively released at relatively high levels by primary human AML cells whereas HSP70 is released at lower levels [19]. Even though these increased HSP90 levels may at least partly be determined by the release from the leukemic cells and then initiate leukemia-directed immune responses, this effect may be counteracted by increased levels of the immunosuppressive HGF and IL-1ra cytokine levels.

HSP90 serum levels were dependent on several factors, including patient age, morphology/FAB classification,

TABLE IV. The Association Between Differentiation Status of Primary Human AML Cells and Serum Cytokine Levels. The Table Shows the Results for Those Cytokines that Showed Significant Alteration ($P < 0.05$) When Comparing Patients with More/less than 20% Positive Cells Based on Flow Cytometric Analyses

Membrane molecule	Positive staining	Negative staining	*P*-value
CD14 expression			
IL-1ra	2,646 (1,299–10,705)	529.4 (<10.9–12,105)	0.0007
CCL2	72.1 (40.5–86.9)	163.9 (17.9–1,414)	0.0039
CCL11[a]	42.4 (<8.2–51.7)	66.9 (<8.2–381)	0.0272
CD34 expression			
IL-1β[a]	<0.57 (<0.57–24.3)	1.8 (<0.57–8.1)	0.0439
IL-1ra	454.6 (<10.9–12,105)	1,404 (121–10,705)	0.0009
IL-5	0.5 (<0.7–4.4)	0.5 (0.5–39.1)	0.0035
Tpo[a]	511 (<9.9–2,892)	947 (234–2,933)	0.0285

The results are presented as the median serum level, the variation range is given in parenthesis. The Mann–Whitney *U*-test was used for the statistical comparison. All concentrations are given as picogram per milliliter.
[a] Cytokines showing a borderline significance value with $0.02 < P < 0.05$.

TABLE V. Serum Levels of HSP70, HSP90, HGF, IL1-ra, and TNFα: A Presentation of a Correlation Map for These Five Mediators that Usually Cluster Together in the Bioinformatical Analyses

	HSP90	HGF	IL1-ra	TNFα
Untreated AML ($n = 82$)				
HSP70	<0.01	<0.01	<0.01	<0.01
HSP90		<0.01	<0.01	<0.01
HGF			<0.01	<0.01
IL1-ra				<0.01
ATRA + valproic acid + theophyllin (protocol 1): levels AFTER treatment				
HSP70	<0.01	<0.01	<0.01	<0.01
HSP90		<0.01	<0.05	<0.05
HGF			<0.05	<0.01
IL1-ra				<0.01
ATRA + valproic acid + low-dose cytarabine (protocol 2): levels AFTER treatment				
HSP70	ns	ns	ns	<0.01
HSP90		<0.01	ns	ns
HGF			<0.05	ns
IL1-ra				<0.05

The Spearman test was used for the correlation analyses, *P*-values as stated.

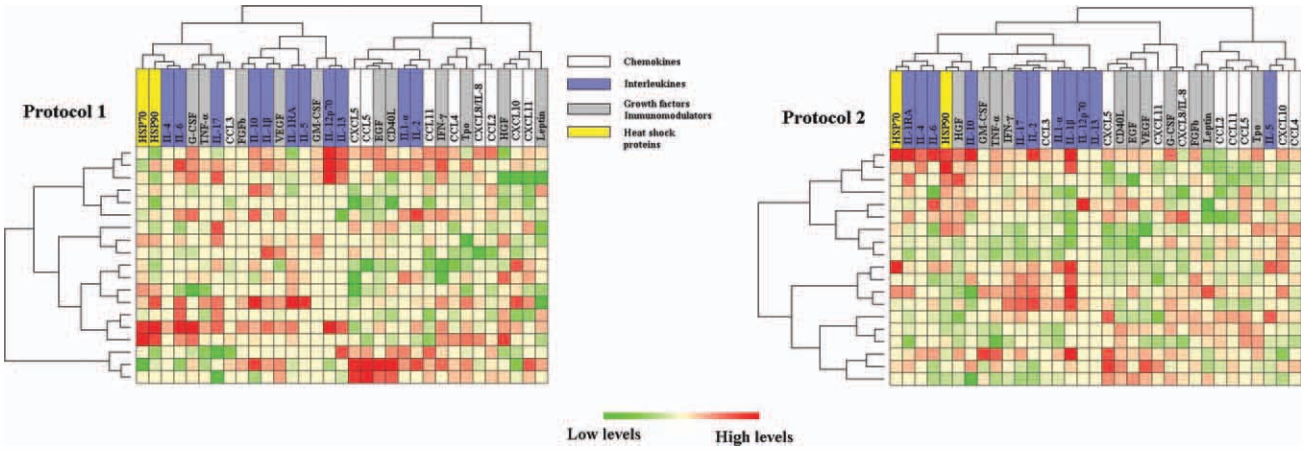

Figure 5. Unsupervised hierarchical cluster analysis of the serum cytokine profile during AML-stabilizing therapy. The upper part shows the results for patients treated in the ATRA/valproic acid/theophyllin protocol (protocol 1) for 7 days, and the lower part shows the results for patients treated in the ATRA/valproic acid/cytarabine protocol (protocol 2) for 25–27 days. The concentrations were median normalized and log(2) transformed before unsupervised hierarchical clustering with Pearson's correlation as distance measure and complete linkage was performed, resulting in a heatmap for visualization and interpretation.

peripheral blood blast counts, and serum LDH levels; but the strongest correlation was to express the CD34 stem cell marker. This observation demonstrates that the biological characteristics of AML cells are major determinants for the serum HSP90 level. A remaining question is then whether the quantity and not only the quality of AML cells has any additional influence on the HSP serum levels. The leukemia cell burden cannot be estimated accurately in AML patients, but the percentage of bone marrow blasts as well as the degree of peripheral blood leukemization, serum LDH, and bone marrow failure would be expected to at least partly reflect the total AML cell burden. First, the bone marrow percentage was only correlated with HSP70 but not HSP90 levels. Second, there was no correlation between bone marrow blast percentage and leukemization, an observation suggesting that other factors than the total AML cell burden have a major impact on the level of circulating blasts. This is also consistent with previous studies demonstrating that leukemization is associated with the surface membrane molecule profiles in AML cells [29]. In our opinion, the association between serum HSP levels and blood blast counts probably reflects confounding biological characteristics rather than a causal relationship. Third, serum LDH levels showed significant correlations with HSP70/90 levels. Finally, the degree of bone marrow failure is not only dependent on the degree of bone marrow infiltration but also probably on the degree of bone marrow suppression due to the AML cells' constitutive cytokine release profile that differs between patients [30,31]. However, for HSP90, the strongest correlation is still between serum HSP90 levels and AML cell expression of the CD34 stem cell marker. Taken together, our observations suggest that at least for HSP90 the most important determinants for the variation in serum levels are qualitative (i.e., the biological characteristics of the AML cells) rather than quantitative differences in AML cell burden. These biological characteristics are not dependent on the HSP level per AML cell because there was no correlation between total cellular levels and serum levels; it seems more likely that the variation depends on the ability to release HSPs.

Our patients were heterogeneous with regard to genetic abnormalities, we included patients both with secondary and de novo disease, and with newly diagnosed and relapsed AML. However, HSP70/HSP90 levels did not differ when comparing patients with high-risk disease (high-risk cytogenetics, FLT3-ITD, secondary AML) with the corresponding group with lower risk (intermediate and normal cytogenetics/FLT3-wt, de novo AML). Thus, the HSP serum levels show no association with any of these conventional high-risk criteria.

We have previously characterized the HSP release by in vitro cultured primary human AML cells [19]. These in vitro studies demonstrated that (i) there is a wide variation between patients in AML cell release of HSP70 and HSP90; (ii) this difference is not dependent on drug-induced or spontaneous stress-induced in vitro apoptosis; (iii) there is a significant correlation between HSP70 and HSP90 in vitro release; and (iv) the release does not differ for cells collected before and during ATRA + valproic acid based anti-leukemic treatment. These observations further support our conclusion given above: the serum levels are mainly dependent on biological AML cell differences between patients in the control of HSP release. This is further supported by the comparison between HSP70 and HSP90; HSP70 shows relatively high cellular but low serum levels, whereas HSP90 shows low cellular and high serum levels. This observation also supports the hypothesis that nonspecific AML cell release together with other intracellular molecules (e.g., the LDH) or release due to cell death is not a major determinant of the serum levels. However, for HSP70 shedding of surface-expressed molecules may also contribute to the serum levels [10], and this may explain the closer association by HSP70 to parameters reflecting the total AML cell burden.

We compared serum HSP70 and HSP90 levels during disease-stabilizing AML treatment containing valproic acid and ATRA. These drugs have also been used by others in different regiments [32–35]. We observed that neither ATRA nor valproic acid alone caused any significant alteration of the serum levels. Furthermore, in our first study, we could not detect any effect on HSP70 and HSP90 levels after 7 days of treatment with ATRA + valproic acid + theophyllin, whereas in the second study, we observed decreased HSP90 levels after treatment with ATRA plus valproic acid plus low-dose cytarabine, and the correlation between HSP70 and HSP90 levels was also lost after this treatment. One possible explanation for the difference between the two protocols could be that for Protocol

1 patients the HSP levels were determined relatively early (within 7 days versus 24–26 days in Protocol 2). However, in our opinion, this is not the most likely explanation because biological effects on the AML cells as well as altered systemic levels of other soluble mediators were observed even after 2–7 days of treatment in Protocol 1 patients [23,36]; differences in biological effects seem more likely.

HSP90 is released by primary human AML cells [9,11]. The increased serum levels in untreated AML and the decreased HSP90 levels during treatment may therefore reflect the AML cell burden and an anti-leukemic effect during treatment with the cytarabine-containing regimen. This is also consistent with the observation that a clinical anti-leukemic effect is usually seen after 2–3 weeks and cannot be detected after only 1 week of treatment [23]. Increased HSP release is a characteristic of chemotherapy-induced immunogenic apoptosis [5,21], and the decreased levels after cytarabine therapy suggest that the anti-leukemic effect of in vivo cytarabine is caused by a reduced AML cell burden without induction of immunogenic apoptosis or nonspecific release by dying AML cells.

AML-stabilizing treatment based on ATRA plus valproic acid has a clinical effect only for a minority of patients, and complete remissions are uncommon [37,38]. The results from our first clinical study suggest that pre-therapy serum HSP90 levels show an inverse correlation with survival. In our previous report from this study, we described a difference in survival between responders and nonresponders to the treatment [23]. Thus, serum HSP90 levels may become useful to identify patients that are more likely to respond to therapy based on ATRA plus valproic acid.

Serum cytokine levels were determined for a large group of unselected AML patients; the levels of single cytokines were compared between the groups and the cytokine profile was in addition analyzed by unsupervised hierarchical cluster analysis. The patients showed altered levels mainly of immunoregulatory interleukins and chemokines, but the growth factors G-CSF and HGF (both showing increased levels) also have immunoregulatory/immunosuppressive effects (for references see [39,40]). Such alterations may thus reflect/contribute to the immunocompromised status of AML patients. Furthermore, four main cytokine clusters were detected when the HSP70/HSP90 levels were included in this analysis, whereas the patients could be classified into two main subsets that differed both with regard to HSP and cytokine levels.

We compared the serum cytokine levels for elderly AML patients with younger patients and observed differences in the levels of several cytokines. The serum levels of CCL17 have previously shown age dependence in human AML [41]. Thus, patient age is one of the factors that influence the serum-cytokine profile. Expression of the stem cell marker CD34 or the monocyte marker CD14 was another factor that was associated with differences in the serum profile. However, one had to emphasize that both age and differentiation status affected the levels only for relatively few cytokines.

Serum-cytokine profiles have also been examined in a previous study by Kornblau et al., and the profiles were then shown to have a prognostic impact in AML patients receiving intensive chemotherapy [15]. The cytokine panel in this previous study was partly overlapping with our present study, and for the 23 overlapping cytokines similar results were seen for 12 cytokines when comparing patients with untreated AML and healthy controls. The most likely explanation for this difference is that we investigated an unselected AML patient population with a higher median age and including patients with AML relapse, whereas Kornblau et al. [15] investigated younger patients receiving intensive chemotherapy for primary AML. Our present results extend the previous observations and demonstrate that the disease-induced alterations in the serum cytokine profile are relevant also during low-intensity AML-stabilizing treatment.

We examined the HSP and cytokine levels during disease-stabilizing therapy based on ATRA plus valproic acid. The cytokine-cluster analysis showed several alterations during treatment, an observation suggesting this treatment alters the cytokine network. Several of the cytokines included in the present analyses have effects on primary human AML cells [30,42–45], and the altered cytokine network may thereby directly affect the AML cells and also have immunomodulatory effects.

HSP90, HSP70, HGF, and IL1-ra levels clustered together or were significantly correlated both in untreated patients and after the cytarabine-including AML-stabilizing therapy. IL-1ra seems to have immunosuppressive effects [46] and based on studies in allogeneic stem cell transplantation models HGF may also mediate immunosuppressive effects [47–49]. Thus, release of soluble HSPs is regarded as an immunostimulatory event [5,21], but the correlation between HSP70/90 and immunosuppressive soluble mediators (and possibly also the maintenance of relatively high levels of potentially immunosuppressive G-CSF, see above) may then counteract or limit the potentially immunostimulatory effects of the soluble HSPs. Even though immunostimulatory TNFα also showed a close clustering, this cytokine was generally detected at relatively low levels. The decreased HSP90 levels during treatment in Protocol 2 may also limit the immunostimulatory effects.

In a previous study [30], we examined the constitutive chemokine release by primary human AML cells, and the chemokines then formed the three clusters CCL2-4/CXCL1/8, CCL5/CXCL9-11 (possibly also CCL23), and CCL13/17/22/24/CXCL5 (possibly also CXCL6). For those cytokines that were included in our present study, we could not detect any similarities in the clustering. Thus, the serum levels of these chemokines do not solely reflect the constitutive AML cell release and thereby the leukemia cell burden, it seems more likely that the serum levels reflect a balance between release (including constitutive AML cell release) and receptor binding/degradation.

To conclude, patients with untreated AML show altered systemic levels of HSP90 and several cytokines and thereby also a very different serum cytokine profile compared with healthy individuals. This profile is altered during disease-stabilizing therapy. There is a strong association between the levels of immunostimulatory soluble HSP90/HSP70 and the immunosuppressive HGF and IL-1ra. Our observations suggest that future studies of immunotherapy alone or in combination with chemotherapy in human AML had to include studies of this balance between potential immunostimulatory soluble HSPs and immunomodulatory mechanisms.

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References

1. Ritossa F. A new puffing pattern induced by temperature shock and DNP in Drosophila. Cell Mol Life Sci 1962;18:571–573.
2. Schmitt E, Gehrmann M, Brunet M, et al. Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. J Leukoc Biol 2007;81:15–27.
3. Radons J, Multhoff G. Immunostimulatory functions of membrane-bound and exported heat shock protein 70. Exerc Immunol Rev 2005;11:17–33.

4. Callahan MK, Garg M, Srivastava PK. Heat-shock protein 90 associates with N-terminal extended peptides and is required for direct and indirect antigen presentation. Proc Natl Acad Sci U S A 2008;105:1662–1667.
5. Garg AD, Nowis D, Golab J, Vandenabeele P, Krysko DV, Agostinis P. Immunogenic cell death, DAMPs and anticancer therapeutics: an emerging amalgamation. Biochim Biophys Acta 2009;1805:53–71.
6. Kepp O, Tesniere A, Schlemmer F, et al. Immunogenic cell death modalities and their impact on cancer treatment. Apoptosis 2009;14:364–375.
7. Ciocca DR, Calderwood SK. Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. Cell Stress Chaperones 2005;10: 86–103.
8. Reikvam H, Ersvaer E, Bruserud O. Heat shock protein 90 – a potential target in the treatment of human acute myelogenous leukemia. Curr Cancer Drug Targets 2009;9:761–776.
9. Flandrin P, Guyotat D, Duval A, et al. Significance of heat-shock protein (HSP) 90 expression in acute myeloid leukemia cells. Cell Stress Chaperones 2008;13:357–364.
10. Steiner K, Graf M, Hecht K, et al. High HSP70-membrane expression on leukemic cells from patients with acute myeloid leukemia is associated with a worse prognosis. Leukemia 2006;20:2076–2079.
11. Thomas X, Campos L, Mounier C, et al. Expression of heat-shock proteins is associated with major adverse prognostic factors in acute myeloid leukemia. Leuk Res 2005;29:1049–1058.
12. Yeh CH, Tseng R, Hannah A, et al. Clinical correlation of circulating heat shock protein 70 in acute leukemia. Leuk Res 2010;34:605–609.
13. Aguayo A, Kantarjian HM, Estey EH, et al. Plasma vascular endothelial growth factor levels have prognostic significance in patients with acute myeloid leukemia but not in patients with myelodysplastic syndromes. Cancer 2002;95:1923–1930.
14. Kim JG, Sohn SK, Kim DH, et al. Clinical implications of angiogenic factors in patients with acute or chronic leukemia: hepatocyte growth factor levels have prognostic impact, especially in patients with acute myeloid leukemia. Leuk Lymphoma 2005;46:885–891.
15. Kornblau SM, McCue D, Singh N, et al. Recurrent expression signatures of cytokines and chemokines are present and are independently prognostic in acute myelogenous leukemia and myelodysplasia. Blood 2010;116: 4251–4261.
16. Tsimberidou AM, Estey E, Wen S, et al. The prognostic significance of cytokine levels in newly diagnosed acute myeloid leukemia and high-risk myelodysplastic syndromes. Cancer 2008;113:1605–1613.
17. Lotem J, Sachs L. Cytokine control of developmental programs in normal hematopoiesis and leukemia. Oncogene 2002;21:3284–3294.
18. Kiss C, Benko I, Kovacs P. Leukemic cells and the cytokine patchwork. Pediatr Blood Cancer 2004;42:113–121.
19. Fredly H, Ersvaer E, Gjertsen BT, Bruserud O. Immunogenic apoptosis in human acute myeloid leukemia (AML): primary human AML cells expose calreticulin and release heat shock protein (HSP) 70 and HSP90 during apoptosis. Oncol Rep 2011;25:1549–1556.
20. Jimbo J, Sato K, Hosoki T, Shindo M, Ikuta K, Torimoto Y, Kohgo Y. Induction of leukemia-specific antibodies by immunotherapy with leukemia-cell-derived heat shock protein 70. Cancer Sci 2008;99:1427–1434.
21. Tesniere A, Panaretakis T, Kepp O, et al. Molecular characteristics of immunogenic cancer cell death. Cell Death Differ 2008;15:3–12.
22. Fucikova J, Kralikova P, Fialova A, et al. Human tumor cells killed by anthracyclines induce a tumor-specific immune response. Cancer Res 2011;71: 4821–4833.
23. Ryningen A, Stapnes C, Lassalle P, et al. A subset of patients with high-risk acute myelogenous leukemia shows improved peripheral blood cell counts when treated with the combination of valproic acid, theophylline and all-trans retinoic acid. Leuk Res 2009;33:779–787.
24. Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. Blood Rev 2004;18:115–136.
25. Reikvam H, Hatfield KJ, Ersvìr E, et al. Expression profile of heat shock proteins in AML patients reveals a distinct signature strongly associated with FLT3 mutation status – consequences and potentials for pharmacological intervention. Br J Haematol 2011. doi: 10.1111/j.1365-2141.2011.08960.x./ PMID:22150087.
26. Stavrum A, Petersen K, Jonassen I, Dysvik B. Analysis of gene-expression data using J-Express. Curr Protoc Bioinformatics 2008; chapter 7: unit 7.3.
27. von Hundelshausen P, Petersen F, Brandt E. Platelet-derived chemokines in vascular biology. Thromb Haemost 2007;97:704–713.
28. Smyth SS, McEver RP, Weyrich AS, et al. Platelet functions beyond hemostasis. J Thromb Haemost 2009;7:1759–1766.
29. Reuss-Borst MA, Buhring HJ, Schmidt H, Muller CA. AML: immunophenotypic heterogeneity and prognostic significance of c-kit expression. Leukemia 1994;8:258–263.
30. Bruserud O, Ryningen A, Olsnes AM, et al. Subclassification of patients with acute myelogenous leukemia based on chemokine responsiveness and constitutive chemokine release by their leukemic cells. Haematologica 2007;92:332–341.
31. Bruserud O, Tore Gjertsen B, Brustugun OT, et al. Effects of interleukin 10 on blast cells derived from patients with acute myelogenous leukemia. Leukemia 1995;9:1910–1920.
32. Bug G, Ritter M, Wassmann B, et al. Clinical trial of valproic acid and all-trans retinoic acid in patients with poor-risk acute myeloid leukemia. Cancer 2005;104:2717–2725.
33. Corsetti MT, Salvi F, Perticone S, et al. Hematologic improvement and response in elderly AML/RAEB patients treated with valproic acid and low-dose Ara-C. Leuk Res 2011;35:991–997.
34. Kuendgen A, Knipp S, Fox F, et al. Results of a phase 2 study of valproic acid alone or in combination with all-trans retinoic acid in 75 patients with myelodysplastic syndrome and relapsed or refractory acute myeloid leukemia. Ann Hematol 2005;84:61–66.
35. Raffoux E, Cras A, Recher C, et al. Phase 2 clinical trial of 5-azacitidine, valproic acid, and all-trans retinoic acid in patients with high-risk acute myeloid leukemia or myelodysplastic syndrome. Oncotarget 2010;1:34–42.
36. Ryningen A, Stapnes C, Paulsen K, et al. In vivo biological effects of ATRA in the treatment of AML. Expert Opin Investig Drugs 2008;17:1623–1633.
37. Bruserud O, Stapnes C, Tronstad KJ, et al. Protein lysine acetylation in normal and leukaemic haematopoiesis: HDACs as possible therapeutic targets in adult AML. Expert Opin Ther Targets 2006;10:51–68.
38. Stapnes C, Gjertsen BT, Reikvam H, Bruserud O. Targeted therapy in acute myeloid leukaemia: current status and future directions. Expert Opin Investig Drugs 2009;18:433–455.
39. Reikvam H, Mosevoll KA, Melve GK, et al. The pretransplant serum cytokine profile in allogeneic stem cell recipients differ from healthy individuals and different profiles are associated with different risk of posttransplant complications. Biol Blood Marrow Transplant 2012;18:190–199.
40. Melve GK, Ersvssr E, Kittang AO, Bruserud O. The chemokine system in allogeneic stem-cell transplantation: a possible therapeutic target? Expert Rev Hematol 2011;4:563–576.
41. Olsnes AM, Motorin D, Ryningen A, et al. T lymphocyte chemotactic chemokines in acute myelogenous leukemia (AML): local release by native human AML blasts and systemic levels of CXCL10 (IP-10), CCL5 (RANTES) and CCL17 (TARC). Cancer Immunol Immunother 2006;55:830–840.
42. Bruserud O. Effects of endogenous interleukin 1 on blast cells derived from acute myelogenous leukemia patients. Leuk Res 1996;20:65–73.
43. Bruserud O. IL-4, IL-10 and IL-13 in acute myelogenous leukemia. Cytokines Cell Mol Ther 1998;4:187–198.
44. Bruserud O. Acute myelogenous leukemia blasts as accessory cells during T lymphocyte activation: possible implications for future therapeutic strategies. Leukemia 1999;13:1175–1187.
45. Bruserud O, Aasen I, Akselsen PE, et al. Interleukin 1 receptor antagonist (IL1RA) in acute leukaemia: IL1RA is both secreted spontaneously by myelogenous leukaemia blasts and is a part of the acute phase reaction in patients with chemotherapy-induced leucopenia. Eur J Haematol 1996;57:87–95.
46. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol 2009;27:519–550.
47. Bian L, Guo ZK, Wang HX, et al. In vitro and in vivo immunosuppressive characteristics of hepatocyte growth factor-modified murine mesenchymal stem cells. In Vivo 2009;23:21–27.
48. Imado T, Iwasaki T, Kataoka Y, et al. Hepatocyte growth factor preserves graft-versus-leukemia effect and T-cell reconstitution after marrow transplantation. Blood 2004;104:1542–1549.
49. Kuroiwa T, Kakishita E, Hamano T, et al. Hepatocyte growth factor ameliorates acute graft-versus-host disease and promotes hematopoietic function. J Clin Invest 2001;107:1365–1373.