RESEARCH ARTICLE

Hypoxia increases HIF-1α expression and constitutive cytokine release by primary human acute myeloid leukaemia cells

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ABSTRACT. ***Introduction.*** **Low oxygen tension is able to modulate the expression of several genes involved in physiological and pathological processes. A major regulator of gene expression is the heterodimeric transcription factor hypoxia inducible factor-1 (HIF-1), which also regulates angiogenesis-related genes, including the protein expression of angioregulatory cytokines. Angiogenesis has been shown to play a role in haematological disorders, and low oxygen tension might thereby influence leukaemogenesis and chemosensitivity in human acute myeloid leukaemia (AML).** ***Methods.*** **We examined the effect of a hypoxic environment (1% O_2) on *in vitro*-cultured, primary human AML cells with regard to HIF-1α expression, colony formation and cytokine release.** ***Results.*** **Our study demonstrated that hypoxic culture conditions increased HIF-1α expression in primary AML cells for a majority of the investigated patients when compared to culture at atmospheric (21%) oxygen tension. Hypoxia also increased the release of vascular endothelial growth factor (VEGF), osteopontin, as well as several CCL- (CCL3/4/5/7/8) and CXCL-chemokines (CXCL1 and proangiogenic CXCL8) by AML cells. The constitutive release of antiangiogenic CXCL9-11 was not altered by the low oxygen tension. The wide variation between patients as regards the release of the various cytokines persisted during hypoxia.** ***Conclusion.*** **Culture of primary AML cells under low oxygen tension induces HIF-1α expression and increases the release of several cytokines, including proangiogenic mediators, compared to culture at ambient 21% O_2.**

Keywords: AML, hypoxia, oxygen tension, HIF-1, chemokine, angiogenesis

Acute myeloid leukaemia (AML) is a bone marrow malignancy characterised by a block in the differentiation of immature white blood cells. These abnormal cells grow rapidly, resulting in the accumulation of immature cells in the bone marrow and eventually in the peripheral blood, which suppresses normal bone marrow function. A recent study estimated the mean partial oxygen pressure (pO_2) of normal bone marrow to be 54.9 mmHg (while atmospheric pO_2 corresponds to 140-150 mmHg), thus the physiological oxygen tension within the bone marrow is low and traditionally referred to as physiological hypoxia [1]. Low oxygen tension is also found in AML bone marrow; a recent study reported that the average value for pO_2 in AML patients is 46 mmHg (range 35-63 mmHg) or 6.1 ± 1.7% [2].

The average bone marrow oxygenation corresponded to 6.1% when measured directly in AML bone marrow aspirates, but the authors of this study found it reasonable to assume that the bone marrow oxygen tension varies from below 1% in hypoxic niches to 6% in the sinusoidal cavities [2]. Other estimations of pO_2 of normal bone marrow have also suggested that there is a wide range of oxygen levels in the bone marrow microenvironment [3, 4]. Thus, the oxygen tension in the extracellular space of the bone marrow microenvironment shows a wide variation, which seems to vary according to the localisation in the marrow, cell types occupying the marrow, distance from microvessels and blood flow [2, 3, 5, 6].

An important regulator of hypoxia-inducible genes is the transcription factor hypoxia inducible factor 1 (HIF-1) that has so far been shown to be a direct regulator of more than 70 target genes, though it is likely that hundreds of genes are directly or indirectly regulated by HIF-1 [7]. HIF-1 is a heterodimer composed of the subunits HIF-1β and HIF-1α. The cytoplasmic subunit HIF-1α is regulated by the oxygen level; in the presence of oxygen, the von Hippel-Lindau (VHL) protein targets HIF-1α for proteasomal degradation. In contrast, degradation is inhibited during low oxygen levels (< 5-6% O_2) and HIF-1α dimerises with HIF-1β and leads to activation of transcription [8]. Transcription mediated by HIF-1 is involved in tumour angiogenesis and metastasis, as well

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as several, normal, physiological processes such as vessel remodelling, cell invasion, apoptosis, inflammatory responses and energy metabolism. The role of hypoxia in the development of human malignancies has been mainly studied in solid tumours [9]. Adaption of malignant cells to hypoxia involves changes in gene expression, induction of anti-apoptotic signalling and anaerobic metabolism [10, 11].

The generation of new blood vessels from pre-existing vessels, termed angiogenesis, is suggested to play an important role in AML [12-15]. Angiogenesis is orchestrated by a variety of activators and inhibitors, including vascular endothelial growth factor (VEGF), as well as pro- and antiangiogenic chemokines [13] of which some are regulated by HIF-1. Several studies suggest a role for the HIF pathway in the progression of haematological malignancies. A recent study of AML patients with a normal karyotype found that overexpression of HIF-1α and HIF-2α isoforms were independent prognostic factors in these patients [16].

The main focus of our study was to investigate the effects of low oxygen levels on the expression of HIF-1α protein, and the release of angioregulatory cytokines and growth factors by primary AML cells. Physiological oxygen concentrations are $\leq 6\%$ in the leukaemic bone marrow, though *in vitro* culture of cells is generally performed at ambient, non-physiological 21% O_2; previous studies have shown that the cytokine secretion pattern of haematopoietic cells, as well as cellular response to cytokines, may be determined by the O_2 concentration [2, 17, 18]. We therefore investigated the release of various mediators by primary human AML cells cultured *in vitro*, at a concentration of 1% O_2, which corresponds to an oxygen tension within the expected range for oxygenation in the extravascular space of the bone marrow compartment.

DONORS AND METHODS

Preparation and in vitro *culture of primary human AML cells*

Patients

The study was approved by the local Ethics Committee (Health Region III, Norway), and patient blood samples collected after informed consent. The 30 AML patients used to study cytokine levels and HIF-1α expression had a median age of 61 years (range 29-82 years), and the clinical and biological characteristics are summarised in *table 1*. Leukaemic cells were also isolated from blood

Table 1
Characteristics of AML patients

Patient	Age	Sex	FAB classification	(%) CD34 expression	Karyotype	FLT3 mutations	NPM1 mutations
1	72	M	M5	1	Normal	wt	mut
2	82	M	M5	1	-Y	wt	wt
3	43	M	M5	95	inv(16)	D835	wt
4	32	M	M3	41	t(15;17)	ITD	wt
5	49	F	M2	85	Multiple	nt	nt
6	56	M	M4	14	Normal	ITD	mut
7	40	M	M6	24	Normal	ITD	wt
8	45	F	M1	6	Normal	ITD, D835	mut
9	41	F	M2	31	Normal	ITD	wt
10	61	M	M4	1	Normal	ITD	mut
11	78	F	M0	19	Multiple	wt	wt
12	59	F	M2	99	-7	wt	wt
13	60	M	M4	68	Normal	ITD	wt
14	79	M	M2	100	Normal	ITD	wt
15	74	M	M5	98	Normal	ITD	wt
16	80	F	M2	96	Multiple	wt	wt
17	74	M	M0	99	nt	wt	wt
18	75	F	M1	23	nt	ITD	wt
19	65	M	M2	99	Multiple	wt	wt
20	45	F	M4	1	Normal	wt	mut
21	64	F	M1	95	Multiple	ITD	wt
22	44	F	M1	89	del(7)	ITD	wt
23	48	M	M4	78	inv(16)	wt	wt
24	70	F	M1	81	nt	ITD	wt
25	69	M	M1	97	nt	wt	wt
26	29	M	M4	55	Normal	ITD	mut
27	72	M	M1	84	+8	wt	wt
28	81	F	M2	1	nt	ITD	nt
29	61	F	M5	31	Normal	wt	nt
30	33	M	M1	79	4	ITD	nt

FAB: French-American-British classification system; FLT3: FMS-like tyrosine kinase 3; NPM1:, nucleophosmin1; ITD: internal tandem duplications; D835: Asp 835 mutation; mut: mutated; wt: wild type; nt: not tested.

samples derived from a second group of patients treated with all-*trans* retinoic acid (ATRA), theophylline and valproic acid as described previously [19].

All patients in both groups had high peripheral blood leukocyte counts corresponding to > 20 x 10^9/L, and at least 85% of the blood leukocytes were AML cells. Density gradient centrifugation (Lymphoprep™, Axis-Shield, Oslo, Norway; specific density 1.077 g/mL) of peripheral blood samples could therefore be used for all patients to prepare a mononuclear cell population containing, at least, 95% AML cells as judged from light microscopy of May-Grünwald-Giemsa stained smears [20, 21]. The major contaminating non-leukaemic cell population was small lymphocytes. These gradient-separated, enriched cell populations are therefore referred to as primary human AML cells. After gradient separation, cells were stored in liquid nitrogen until used in studies.

In vitro *culture*

AML cells were cultured in RPMI-1640 (Sigma-Aldrich, St.Louis, MO, USA) supplemented with Streptomycin-Penicillin (50 μg/mL), and 10% heat-inactivated fetal calf serum (BioWhittaker, Walkersville, MD, USA) unless otherwise stated. Initially, primary human AML cells (5 x 10^6 cells in 5 mL) were incubated in 25 cm^2 flasks (Nunc, Nunclon™, Denmark) for 30 min in a humidified incubator with 5% CO_2 and 95% air at 37 °C. Then, cultures were transferred to a sealed humidified chamber, in which the gas mixture contained 5% CO_2, 94% N_2 and 1% O_2, to determine the effect of hypoxia on AML cells. Cells derived from the same patient *(table 1)* were cultured in parallel in 21% O_2 and 1% O_2 for 24-72 h. Cell-free supernatants were collected from culture flasks and stored at - 80°C until further used.

AML cells (1 x 10^6 cells in 1 mL) were cultured for 48 h with or without the HIF-1α transcriptional antagonist chetomin 150 nM [22] (Alexis Biochemicals, NY, USA) in StemSpan SFEM medium (StemCell Technologies; Vancouver, BC, Canada), in the presence of 1% O_2. Cell-free supernatants were collected and the effect of HIF-1-mediated transcription on the release of VEGF and CXCL8 was investigated.

AML cells were also tested in a colony-formation assay as described previously [23]. Briefly, primary human AML cells were cultured in methylcellulose medium containing stem cell factor, erythropoietin, granulocyte-macrophage colony-stimulating factor and IL3 (MethoCult® GF H4434, StemCell Technologies, Vancouver, Canada). Cultures were prepared in 24-well culture plates (Nunc) with 0.5 ml of medium per well (50,000 primary AML cells per well). Then, cells were incubated in an atmosphere of either 21% O_2 or 1% O_2 for 12 days before the number of colonies with >16 cells (corresponding to > 4 cell doublings) was determined by light microscopy. The results are presented as the mean number (duplicate cultures) of colonies per 50,000 seeded cells.

AML cell lines

The two human myeloid leukaemia cell lines were used in the current studies. HL60 (FAB M2) and KG-1a (FAB M0) were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany). Cobalt (II) chloride hexahydrate ($CoCl_2$) (Sigma Aldrich, St Louis, MO, USA) was dissolved in water to make a 50 mM stock solution. Cell lines were cultured *(i)* at a concentration of 4 x 10^5 cells/mL with 50 μM or 100 μM $CoCl_2$ for 48 h before analysis of HIF-1α levels, or *(ii)* in the presence of 50 μM $CoCl_2$ (2 x 10^5 cells/mL) for four days (HL60) and five days (KG1a) before analysis of membrane molecule expression.

Normal haematopoietic cells

Peripheral blood-mobilised normal haematopoietic cells were derived from five patients with multiple myeloma. All patients responded to initial combination chemotherapy with doxorubicin, vincristine and dexamethasone, and normal stem cells were mobilised four to six weeks after the end of this treatment, by cyclophosphamide followed by treatment with granulocyte colony-stimulating factor [24]. Cells were harvested by apheresis and stored in liquid nitrogen until used. The cells were tested in a colony-formation assay, which has been described in a previous publication [25] and is similar to the colony-formation assay used for the primary human AML cells. Briefly, cells were thawed and cultured for 12 days in methylcellulose-medium containing recombinant cytokines (MethoCult® GF H4434) in either 21% O_2 or a hypoxic (1% O_2) atmosphere. The number of erythroid (*i.e.* burst-forming unit erythroid, BFU-E), mixed and colony-forming unit granulocyte-macrophage (CFU-GM) colonies were determined by light microscopy. All cultures were prepared in duplicate, and the results presented as the mean number of colonies per well.

Western blot analysis

Primary AML cells (5 x 10^6) were washed with ice-cold phosphate-buffered saline (PBS) and pelleted by centrifugation at 4°C. Subsequently, cells were lysed at 4°C in lysis buffer. Samples were kept cold, homogenised and centrifuged (14,000 g, 15 min), before the protein content was measured using the Bradford protein assay (BioRad Lab., Hercules, CA, USA). Samples containing 30 μg of protein were added to 3 x sodium dodecyl sulphate (SDS) loading buffer, and boiled for ten min before separation in SDS-polyacrylamide gel electrophoresis (PAGE) minigels with 10% polyacrylamide.

After electrophoresis, gels were electroblotted to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences), and then blocked for 1 h in blocking buffer [21] at room temperature. Membranes were incubated with mouse IgG1 anti-HIF-1α antibody (Becton-Dickenson, BD Tranduction Laboratories, San José CA, USA; diluted 1:500) for 1 h at room temperature, before being washed in Tris-buffered-saline (TBS)-buffer and incubated for 1 h with a secondary donkey anti-mouse antibody conjugated to horse-radish peroxidase (HRP) (Jackson Immunoresearch Lab., Suffolk, UK). The membrane was washed, and protein bands were visualised using the Supersignal West Femto Chemiluminescent Substrat (Pierce, Rockford, IL, USA). The immunoblots were imaged using a Kodak Image Station 2000R (Eastman Kodak Company, NY, USA). Detection of beta-actin (Abcam, Cambridge, UK) was used as a gel loading control.

Analysis of cytokine concentrations

Enzyme-linked immunosorbent assays (ELISA) were used to measure cytokine concentrations in AML supernatants. Assays were performed strictly according to the manufacturer`s instructions (Quantikine colorimetric sandwich ELISA kits, R&D Systems, Minneapolis, MN, USA). The mean minimum detectable levels were < 5 pg/mL for VEGF; 0.1 pg/mL for osteopontin (OPN), 8.3 pg/mL for Angiopoietin-2 (Ang-2), 3.5 pg/mL for Ang-1 and 6.4 pg/mL for CXCL8. Alternatively, chemokine levels were measured using the Luminex 100™ instrument with an XY platform and the human Chemokine ten-plex kit from Biosource™ (Nivelles, Belgium). The sensitivities of these assays were < 5 pg/mL CCL2/MCP-1, < 15 pg/mL CCL3/MIP-1α, < 10 pg/mL CCL4/MIP-1β, < 25 pg/mL CCL5/rantes, < 10 pg/mL CCL7/MCP-3, < 5 pg/mL CCL8/MCP-2, < 5 pg/mL CCL11/eotaxin, < 10 pg/mL CXCL1/GRO-α, < 50 pg/mL CXCL9/MIG and < 5 pg/mL CXCL10/IP-10 respectively. The concentrations were calculated from the standard curves using a five-parameter regression formula, and data analysis performed with StarStation software program (Applied Cytometry Systems, Sheffield, UK).

Flow cytometry

Leukaemic cells were washed with phosphate-buffered saline (PBS), centrifuged and resuspended in blocking solution [250 μg/mL human immunoglobulin G (IgG), Octagam®, Octapharma, Lachen, Switzerland] for 10 min. Cells were then incubated with fluorochrome-conjugated antibodies (aCD11b-PE, aCD13-PE, CD14-PerCP, aCD15-APC, aCD33-FITC, aCD34-APC, aCD38-PE, aCD61-PerCP, aCD64-FITC, aCD71-FITC, CD117-APC, CXCR4-APC; all from Becton-Dickenson and aTie2-PE from R&D Systems). Unstained cells were used as negative controls. Subsequently, cells were washed with PBS and analysed for the expression of differentiation markers.

Double staining of AML cells with FITC-conjugated Annexin V and propidium iodide (PI) (Apoptest™ FITC kit; NeXins Research, Kattendijke, Netherlands) was used to discriminate viable from apoptotic and dead cells.

For detection of intracellular HIF-1α protein, cells were washed with ice-cold PBS, pelleted and resuspended in 4% paraformaldehyde (PFA) for 15 min. Then cells were washed with PBS and resuspended in ice-cold 100% methanol for 15 min. Subsequently, cells were washed twice with PBS, blocked with 250 μg/mL Octagam® for 10 min and incubated with primary mouse anti-HIF-1α antibody (5 μg per mL in 2% BSA-solution) overnight at 4°C. Cells were washed three times with PBS and stained with goat anti-mouse secondary antibody (Alexa488 or Alexa647, Molecular Probes; diluted 1:500 in 2% BSA-solution), for 30 min, at room temperature. Cells were then washed with 0.01% Tween-20 in PBS before being resuspended in PBS before analysis. Dead (nonviable) cells were identified by their change in light scatter properties, and were excluded by gating before analysis. Unstained cells and cells incubated only with the secondary antibody were used as negative controls. All samples were analysed using a dual laser FACSCalibur™ flow cytometer (Becton Dickinson, 488 nm and 635 nm lasers). At least 10,000 events were collected and data analyzed using Flow Jo (Tree Star Inc., Ashland, OR).

Statistics

Wilcoxon's test for paired samples was used for comparing levels of angiogenic factors. Spearman's rho test was used for correlation studies, and the Mann-Whitney test for comparison studies. The level of significance was set at ≤ 0.05, except for the cytokine release studies where the level of significance was set at $p < 0.02$ due to the large number of comparisons, and p-values corresponding to 0.02-0.05 are then referred to as borderline in these studies. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc, Chicago, USA).

RESULTS

Hypoxia increased HIF-1α protein in primary human AML cells

Western blot analysis was performed to compare the expression of HIF-1α protein in AML cell lysates after culture for 24 h under normoxic (21% O_2) *versus* hypoxic (1% O_2) conditions. Primary AML cells derived from 17 unselected patients were examined. A minority of patient samples showed a low expression of HIF-1α when cells were cultured under 21% O_2 *(figure 1)*. In contrast, hypoxia induced AML-cell expression of HIF-1α in 11 out of 17 patients examined *(figure 1)*. Undetectable HIF-1α levels were shown for five patients, even after hypoxic culture, while one patient had unaltered levels when comparing protein levels at 1% O_2 and 21% O_2. Similar results were observed for patient samples cul-

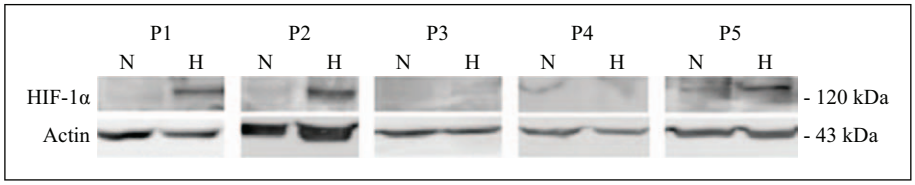

Figure 1

Expression of HIF-1α in primary AML patient samples in response to hypoxia. The figure shows Western blots of HIF-1α expression in primary human AML cell samples derived from five patients. AML cells were incubated at atmospheric oxygen levels (normoxia, N) or in the presence of 1% oxygen (hypoxia, H) for 24 h. Increased HIF-1α protein levels were observed for certain patients after incubation under hypoxic conditions. Expression of actin served as a loading control.

tured for 48 h (data not shown). Thus, a variation in HIF-1α expression is seen between cultured patient samples, which can be upregulated under hypoxic conditions.

We examined intracellular HIF-1α levels by flow cytometry in primary AML samples derived from four patients, before and after treatment with all-*trans* retinoic acid, theophylline and valproic acid. Low, basal levels of intracellular HIF-1α protein were found in all patient samples, and the expression was not influenced by treatment (data not shown).

HIF-1α protein could not be detected under hypoxic culture for five patients. Leukaemic cells were available for additional studies for four of these patients, and we investigated the effect of the HIF-1α inhibitor chetomin during hypoxia for these four patients and for six additional, unselected patients. Chetomin disrupts the binding of transcriptional coactivator p300 to HIF-1α, which thereby inhibits HIF-1-mediated transcription [22]. Decreased viability of AML cells was observed for all 10 patients in the presence of chetomin compared with control cultures prepared in medium alone. According to the Annexin V assay, the patients had a mean of 35% viable cells (range 17%-60%) in the control cultures, while a mean of 4% (range 0-23%) was found in the presence of chetomin. The four patients with undetectable HIF-1α levels had a mean of 43% viable cells (range 27%-60%) in the control cultures and a mean of 2% (range 0%-9%) in the presence of chetomin. We furthermore examined levels of CXCL8 and VEGF after hypoxic culture in the presence or absence of chetomin. Detectable CXCL8 levels were observed for nine of the patients, including all four patients with undetectable HIF-1α levels (median CXCL8 level 239 ng/mL; variation range 1.8-877 ng/mL). The patients with no detectable HIF-1α showed a median CXCL8 level of 378 ng/mL (variation range 1.8-877 ng/mL) in the controls, and a median level of 80 ng/mL (range 3.6-244 ng/mL) in the presence of chetomin; the inhibitor caused a minor increase in CXCL8 levels for one of these patients. Detectable VEGF release was observed for only four patients, and HIF-1α inhibition decreased these levels for all patients. Taken together, our results suggest that although Western blot analysis showed undetectable HIF-1α levels, this does not represent absence, but rather the presence of low HIF-1α levels as HIF-1α seems to be important both for regulation of AML cell viability and/or cytokine release during hypoxic *in vitro* culture for all 10 patients investigated. Even though the negative effect of HIF-1α inhibition on cell viability may contribute to reduced cytokine levels, the specific HIF-1α-mediated effect on cytokine expression must be more important because a general increase in cytokine levels was not observed; only certain mediators seem to be regulated by low oxygen tension (see below).

Low oxygen levels increased the release of angiogenic mediators from primary AML cells

Primary AML cells usually show constitutive release of several angioregulatory chemokines. The release of angioregulatory chemokines then occurs in clusters where proangiogenic CXCL8 is released together with CCL2-4/CXCL1 (referred to as Cluster I) and antiangiogenic CXCL9-11 together with CCL5 (Cluster II) [15]. We investigated the constitutive release of these chemokines in addition to the proangiogenic growth factors OPN and VEGF, when cells were incubated in 21% O_2 or 1% O_2 for 24 h. Culture supernatants were then collected and cytokine levels quantified. The majority of AML patients showed detectable CCL2-4, CXCL8 and OPN release at normal oxygen tension, while only a minority released CCL5, CCL7-8, CXCL1, CXCL9-10 and VEGF *(table 2)*.

We compared the cytokine concentrations after culture of the primary AML cells in 21% O_2 for 24h with those found after culture under hypoxia (overall results summarised in *table 2*). Firstly, the protein levels of VEGF and OPN increased significantly during hypoxic culture conditions (*figure 2A*, Wilcoxon's test for paired samples, $p < 0.0005$ and $p = 0.006$ respectively). Secondly, the levels of proangiogenic CXCL8 were significantly increased by hypoxia (*figure 2A*, $p < 0.0005$). Increased levels were also detected for the other cluster I chemokines CCL3 (*figure 2B*, $p < 0.0005$) and CCL4 (*figure 2B*, $p = 0.006$); detectable CXCL1 levels were observed for only six patients, and reached borderline significance ($p = 0.043$, data not shown), whereas CCL2 levels were not altered (data not shown). Thirdly, the levels of the antiangiogenic CXCL9-11 were decreased for most patients although these differences did not reach statistical significance, and the levels of the last cluster II chemokine CCL5 were not significantly altered either (data not shown). Finally, we also investigated the effect of hypoxia on CCL7, CCL8 and CCL11 levels *(table 2*, *figure 2B)*. Increased levels were then observed for CCL7 ($p = 0.006$) and CCL8 ($p = 0.043$), whereas CCL11 levels did not differ; the CCL7/CCL8 results demonstrate that the hypoxia-induced enhancement of chemokine release is not specific for cluster I chemokines, but is also seen for certain other chemokines.

Levels of Ang-2 were not significantly altered, though Ang-1 levels were significantly increased after hypoxic culture ($p = 0.01$). However, Ang-1 release did not vary considerably after culture at different oxygen concentrations for the large majority of patients with a median level of 372 pg/mL at 21% O_2, and 412 pg/mL at 1% O_2.

The cytokine release was also measured during 48 and 72 hours of *in vitro* culture at different oxygen concentrations for primary AML cells derived from 11 randomly selected patients, and similar results were also observed for these cell cultures (data not shown). Finally, increased HIF-1α expression was not associated with increased cytokine levels for all patients, and increased HIF-1α was seen for several patients without detectable cytokine release, even after culture in a hypoxic atmosphere.

Hypoxia decreases the proliferation of normal haematopoietic progenitors, but has divergent effects on AML clonogenic cells

We investigated the effect of hypoxia on colony-forming cells from normal haematopoietic progenitors and cells derived from 10 unselected AML patients. The

Table 2
Cytokine levels in cultured AML cells in response to hypoxia

	Oxygen concentration	Range (pg/mL)	Median (pg/mL)	Detectable release by patients	p-value (21% O_2 *versus* 1% O_2)
VEGF	21%	0-210	3.0	15 of 28	< 0.0005*
	1%	0-760	6.6	17 of 28	
OPN	21%	0-204,000	400	22 of 28	0.006*
	1%	0-300,000	800	23 of 28	
CCL2	21%	0-11269	37	17 of 29	> 0.05
	1%	0-7735	100	21 of 29	
CCL3	21%	0-11300	113	18 of 29	< 0.0005*
	1%	0-12967	216	19 of 29	
CCL4	21%	0-10966	163	20 of 29	0.006*
	1%	0-13430	250	22 of 29	
CCL5	21%	0-715	0	9 of 29	0.050
	1%	0-1700	0	12 of 29	
CCL7	21%	0-2458	0	12 of 29	0.006*
	1%	0-2250	19	17 of 29	
CCL8	21%	0-482	0	5 of 29	0.043
	1%	0-166	0	5 of 29	
CCL11	21%	0-25	0	6 of 29	> 0.05
	1%	0-12	0	6 of 29	
CXCL1	21%	0-158	0	6 of 29	0.043
	1%	0-383	0	6 of 29	
CXCL8	21%	0-180,800	2993	26 of 27	< 0.0005*
	1%	4.2-221,080	6715	27 of 27	
CXCL9	21%	0-102	0	7 of 29	> 0.05
	1%	0-218	0	9 of 29	
CXCL10	21%	0-1896	0	14 of 29	> 0.05
	1%	0-2050	0	11 of 29	

Wilcoxon's test for paired samples (culture at 21% O_2 *versus* 1% O_2) was used for all statistical tests. A p-value < 0.02 was considered statistically significant (*). Only patients with at least one detectable value were included in the statistical analyses. Chemokine levels of CCL2, CCL4 and CXCL8 exceeded the maximum detection level of the assay for three, two and one patient(s), respectively. These patients were excluded from the statistical analysis.

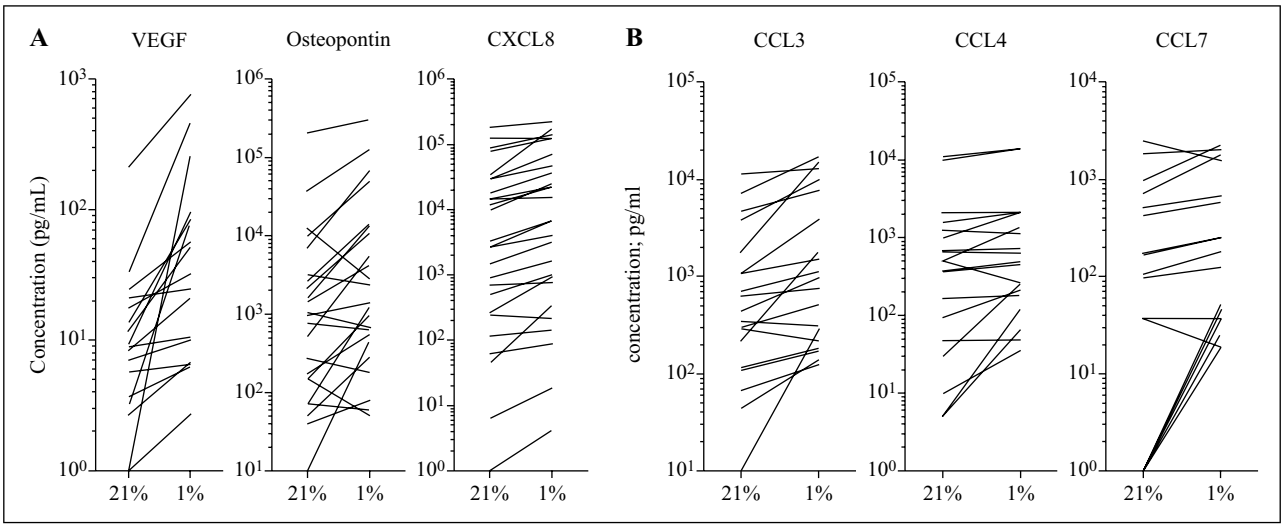

Figure 2
Effect of hypoxia on cytokine release by cultured AML cells. Primary human AML cells were cultured under atmospheric (21% O_2) or hypoxic (1% O_2) conditions for 24 h before supernatants were harvested and the chemokine levels determined in the culture supernatants. The figure shows only the levels for those patients with detectable cytokine levels (see *table 2*). **A**) Levels of VEGF, OPN and CXCL8 released by AML cells. For one patient, CXCL8 levels are not presented as the levels exceeded the maximum detection level for the assay. **B**) Levels of CCL3, CCL4 and CCL7 released by AML cells. Levels of CCL4 exceeded the maximum detection level of the assay for two patients, and are therefore not presented in the figure for these patients.

number of colonies decreased for all five donors of peripheral blood-mobilised stem cells, when cells were cultured at 1% O_2 compared to 21% O_2, and this decrease was observed for erythroid, mixed and CFU-GM except for one patient with an increased amount of erythroid colonies *(figure 3)*. We also compared the number of colony-forming cells for primary human AML cells when leukaemic cells were cultured

under different oxygen concentrations; hypoxia then had divergent effects and increased colony-formation was observed for only a minority of patients *(figure 3)*. Thus, hypoxia has a general effect on the local cytokine network, with increased levels of several cytokines, whereas the effect of hypoxia on AML cell proliferation is divergent and increased colony formation is seen only for a minority of patients.

Increased cytokine release during hypoxia is not associated with signs of AML cell differentiation

Primary human AML cells derived from 10 patients were cultured for 48 hours at atmospheric oxygen levels

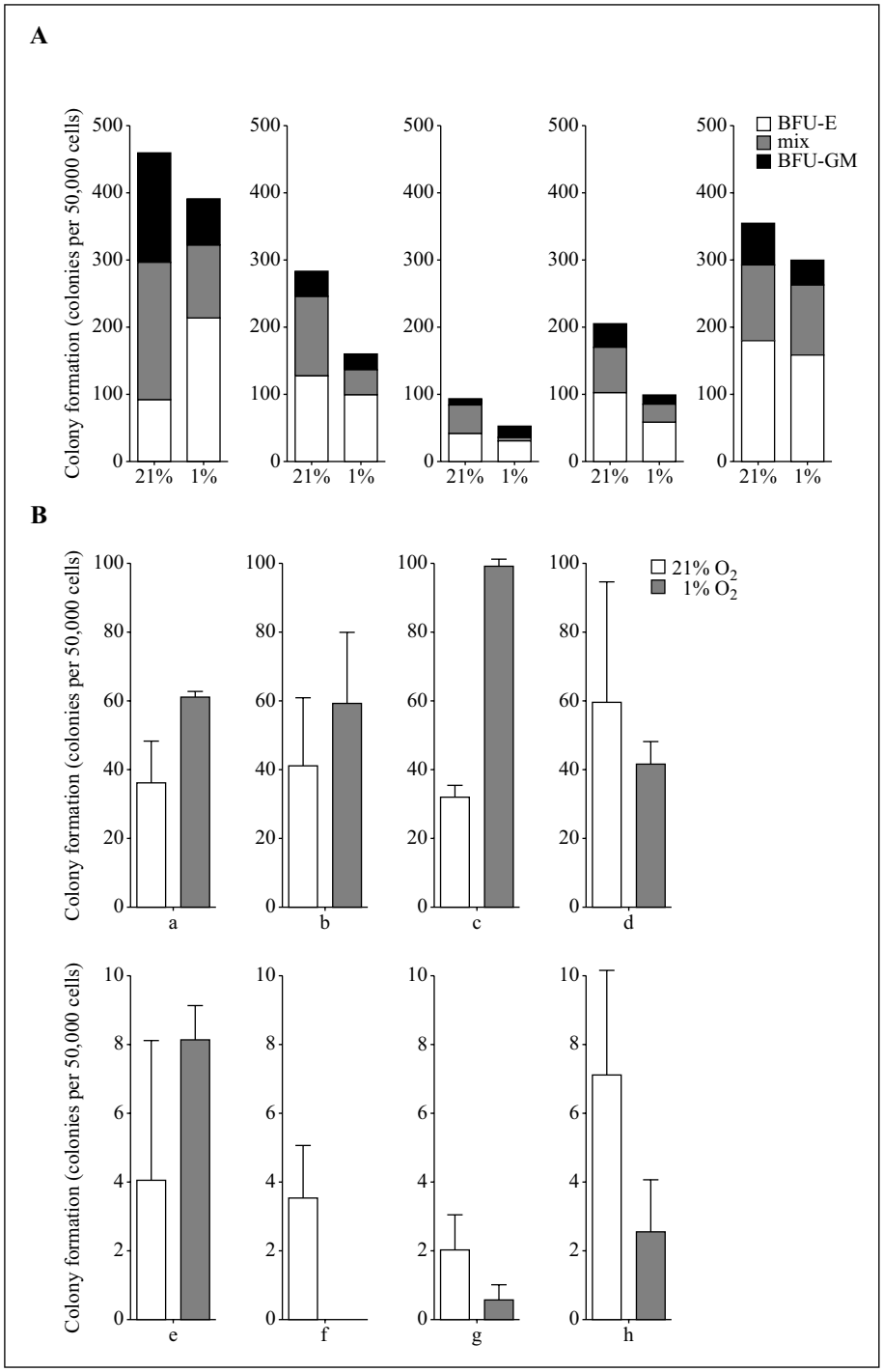

Figure 3

The effect of hypoxia on the growth of normal and leukaemic clonogenic cells. **A**) Peripheral blood-mobilised normal progenitors were derived from five myeloma patients. The cells were mobilised by chemotherapy plus G-CSF after initial disease stabilisation by chemotherapy. The figure shows the number of colonies for each of the patients when cells were cultured under atmospheric (21%) oxygen concentrations and hypoxia (1% O_2). The results are presented as the total number of erythroid, mixed and nonerythroid colonies (mean of duplicate cultures). The standard error of the mean (SEM) for each type of colony was less than 20% of mean values (not shown). **B**) The number of AML colonies was determined for 10 unselected patients; two patients did not form colonies in either culture condition and the results are therefore presented for the eight patients with detectable colonies. The results are presented as the number of colonies per 50,000 seeded cells (mean + SEM of duplicate cultures) when cells were cultured under 21% O_2 and 1% O_2 conditions.

(21% O_2) or hypoxia (1% O_2) before the cellular expression of stem cell markers (CD34, CXCR4, CD117) and lineage-associated markers (CD11b, CD15) was compared. We only observed relatively small variations in single markers without any major alterations in the differentiation marker profile during hypoxia; expression of stem cell markers were generally maintained and only minor variations in single lineage-associated markers were observed for certain patients (data not shown).

The expression of the CD34 stem cell marker by primary human AML cells showed a wide variation between patients *(table 1)*. We therefore compared the effect of hypoxia on the cytokine release of VEGF/OPN/CXCL8/CCL3-4/CCL7 for patients with high (> 80%) and low (< 20%) CD34 expression. Hypoxia increased the release of angioregulatory cytokines independent of the CD34 expression level for most patients, though a low CD34 expression correlated with a higher fold-increase of VEGF and OPN levels after hypoxic culture (Mann Whitney test, $p < 0.02$ for both). Furthermore, expression of CD34 was significantly lower in the AML M4/M5 subtypes that show monocytic differentiation compared to the minimally differentiated AML M0/M1/M2 subtypes (Mann Whitney test, $p = 0.007$), and the M4/M5 subtypes were also associated with a higher fold-increase of VEGF and OPN levels after hypoxic culture (Mann Whitney test, $p < 0.02$ for both).

Expression of differentiation markers by human AML cell lines were only slightly altered after treatment with a hypoxia-mimicking agent

We investigated whether induction of HIF-1α in human AML cells was also associated with induction of differentiation. For these studies, we used a highly standardised model of two well-characterised AML cell lines. Several previous studies have documented monocytic and granulocytic differentiation of these cells in response to different types of stimuli [26, 27]. Increased HIF-1α expression in leukaemic cell lines after exposure to the chemical hypoxia-mimicking agent $CoCl_2$ was verified by flow cytometry. Differentiation was investigated by flow cytometric analysis of stem cell-associated (CD34 and CD117) and lineage-associated (CD11b, CD13, CD14, CD15, CD33, CD38, CD61, CD64, CD71 and Tie2) membrane molecules. Even though HIF-1α expression was induced by $CoCl_2$, we did not observe a general reduction in stem cell-associated markers or any increase in lineage-associated markers; thus, only a minor increase in single membrane molecules (CD11b, CD15 and CD33) were observed (data not shown). The transferrin receptor, CD71, increased in both cell lines after culture in the presence of $CoCl_2$ (data not shown); though this receptor is known to be induced by $CoCl_2$ [28]. Thus, we conclude that the hypoxia-mimicking agent $CoCl_2$ only slightly alters the expression of single lineage-associated membrane molecules, and these results are similar to our observations for primary human AML cells cultured under hypoxic conditions.

HIF-1α expression and constitutive chemokine release by circulating AML cells are not altered after in vivo *exposure to ATRA*

In a recent article, we described that *in vivo* exposure to the differentiation agent ATRA could alter the levels of several intracellular mediators [29]. *In vitro* studies have also shown that ATRA can increase the intracellular levels of HIF-1α in AML cells [30]. In this context, we investigated the intracellular levels of HIF-1α in primary AML samples derived from four patients before and after two days of ATRA therapy. Low basal levels of intracellular HIF-1α protein were seen for all patients. We did not observe an ATRA-induced increase in intracellular HIF-1α levels for any patient during treatment, and the expression was not influenced by five additional days of treatment with ATRA in combination with valproic acid and theophylline (data not shown). Finally, we also examined the constitutive chemokine release by primary human AML cells derived from a total of 10 patients before ATRA, after two days of ATRA and after five additional days with the combination therapy. This *in vivo* treatment did not significantly alter the constitutive chemokine release of CCL2-5, CXCL8 or CXCL10, not even for those three patients showing a clinical response to the treatment (data not shown). Thus, altered chemokine release is probably not involved in the AML-stabilizing effect of ATRA plus valproic acid.

DISCUSSION

AML is characterised as a bone marrow disease, where the leukaemic cells rapidly accumulate in the bone marrow compartment and interfere with normal haematopoiesis. AML bone marrow is considered physiologically hypoxic, with oxygen levels approximately three times lower than that usually applied during *in vitro* cell culture [1, 2]. In the bone marrow, the most primitive haematopoietic cells reside in locations with very low oxygen tensions [5]. As all cells require oxygen for survival, low oxygen levels are generally considered toxic to cells, though rapid cell division and blood vessel formation can also create a hypoxic microenvironment that promotes and selects for a more aggressive cell phenotype. Various oxygen levels have been reported to affect gene expression profiles, and also result in phenotypic changes in cells [31, 32]. Low oxygen tension may modulate the functional phenotype of primary human AML cells and alter membrane receptor expression and trafficking [2], but it is not known how other functions are altered. Hypoxia is important in regulation of angiogenesis, and in the present study we therefore investigated the effect of low oxygen tension on HIF-1α expression and constitutive AML release of angioregulatory cytokines by primary human AML cells.

In our present study, we included only patients with a high level of circulating AML blasts among peripheral blood leukocytes, but otherwise the patients were unselected. This methodological strategy has also been used in previous studies and does not induce any bias with regard to important biological characteristics of the

AML cells and major prognostic parameters, as discussed in detail in [21]. By combining this patient inclusion strategy and a simple gradient-separation procedure, we were able to prepare highly enriched AML cell populations with a minimal risk of separation-induced functional modifications. More extensive separation procedures have been shown to induce functional alterations in primary human AML (reviewed in [20, 33]). Nevertheless, our results should be interpreted with great care as we cannot exclude that they may be representative for only this particular subset of patients.

The average bone marrow oxygenation corresponded to 6.1% when measured directly in AML bone marrow aspirates [2], but there will always be a risk of peripheral blood contamination with aspiration of such relatively large bone marrow volumes (the level is close to the level in mixed venous blood) and these observations definitely do no exclude the possibility that lower levels can be present in the extravascular space of the bone marrow microenvironment. Actually, a study using mathematical modelling estimated the pO_2 distribution in the bone marrow compartment; the results suggested that there will be up to 80-90% reduction in oxygen tension at a distance of 150 μm from a microvessel, and there will also be a similar reduction from the periphery to the centre of an haematopoietic island with a radius of five immature myeloid cells [3]. We would also expect similar gradients to occur in AML marrow that contains an excess of morphologically immature myeloid cells. In fact, a study of AML progression in the Brown Norwegian rat model showed that increased cellular hypoxia occurs as the disease progresses, and leukaemic proliferation during hypoxia may contribute to further reduce the oxygen tension of bone marrow [34]. In our opinion, the use of 1% O_2 during *in vitro* culture should be within the range for extravascular oxygen tension in the bone marrow microenvironment.

Primary human AML cells show constitutive or spontaneous cytokine release, *i.e.* these cells usually secrete several cytokines when incubated in serum-free medium alone [35, 36]. Since AML is a bone marrow malignancy characterised by the accumulation of immature myeloid cells, we regard normal $CD34^+$ bone marrow progenitor cells as the normal counterpart to the primary AML cells. In contrast to the primary AML cells, normal bone marrow CD34+ cells do not show constitutive release of cytokines, including CCL2-5, CXCL10 and VEGF [37], which were examined in our present study. CXCL8 is also released at very low levels by normal CD34+ cells [37], whereas high levels are commonly released by primary human AML cells [35]. Thus, the constitutive release of a wide range of cytokines, at relatively high levels and including several chemokines, should be regarded as an AML-associated phenotypic characteristic. Furthermore, our studies of clonogenic AML cells clearly demonstrate that the immature leukaemic progenitors/stem cells constitute a small minority of the enriched AML cell population; the major determinant for the local cytokine levels thus being the large majority of more mature progenitors.

In our study, some patients had detectable, but low, HIF-1α expression after culture under atmospheric oxygen levels (21% O_2). Similar to our results, a previous study had shown constitutive expression of HIF-1α by leukaemic cell lines also under non-hypoxic conditions [38]. It is possible that mechanisms other than those relying on oxygen, such as genetic alterations or extracellular stimuli, can induce expression of HIF-1α [7, 8]. We also demonstrated that low oxygen tension altered the *in vitro* cytokine expression profile of primary leukaemia cells. AML cells released increased levels of several cytokines (CXCL1, CXCL8, CCL34, CCL7, VEGF and OPN) of which several are known to be involved in angiogenesis, whereas the antiangiogenic chemokines CXCL9-11 were not altered by low oxygen tension. However, one should emphasise that a wide variation in constitutive cytokine release between patients persisted, even during low oxygen tension. Several of the mediators that are shown to have angiogenic activity such as VEGF, OPN and the chemokines CXCL1 and CXCL8 that contain the ELR sequence motif characteristic of potent promoters of angiogenesis [39], are demonstrated to have prognostic value in AML [14, 40]. Low oxygen tension in leukaemic bone marrow may therefore affect bone marrow angioregulation.

Although HIF-1α is considered to be the main regulator of hypoxic signalling, hypoxia can also regulate several other transcription factors such as NF-kB, AP-1 and CREB (reviewed in [41]) which play a role in the transcriptional response to hypoxia. Activation of NF-kB, in particular, is known to regulate the expression of several cytokines and there is accumulating evidence of a cross-talk between HIF-1α and NF-kB pathways. In addition, the HIF-1α paralogue HIF-2α, which is also regulated by oxygen levels, can modify gene expression. Lack of correlation between chemokine release and HIF-1α levels can then be explained by the influence of other hypoxia-regulated transcription factors, apart from HIF-1α, on the constitutive chemokine release. Furthermore, even though hypoxia increased constitutive cytokine release, a wide variation between patients was also maintained during hypoxia. This last observation strongly suggests that patient-specific biological characteristics still have a major influence on the constitutive cytokine release also during hypoxia.

We could not detect HIF-1α by Western blot analysis after hypoxic culture for five AML patients. Additional studies showed that HIF-1α inhibition reduced AML cell viability even for these patients, and reduced levels of CXCL8 and VEGF (two of the mediators with increased levels during hypoxia) were also observed. These observations suggest that undetectable HIF-1α levels in patient samples represent low expression levels rather than the absence of the protein. Furthermore, even though inhibition of HIF1-α affects AML cell viability and thus cytokine release in general, additional cytokine-specific mechanisms must be present during low oxygen levels because increased cytokine levels (including increased CXCL8 and VEGF) were observed only for certain mediators after culture at 1% O_2.

We investigated the effect of hypoxia on the proliferation of clonogenic normal and leukaemic progenitors. Hypoxia decreased the proliferation of different normal progenitors when investigating normal peripheral

blood-mobilised haematopoietic progenitors derived from cancer patients. However, hypoxia had divergent effects on AML cells, and even increased proliferation was observed for a minority of AML patients. Previous studies have shown a better maintenance of normal haematopoietic stem cells at low oxygen concentrations as well as slower cell cycling when compared to cultures at 21% O_2 [4, 42]. A possible explanation for the increased proliferation of leukaemic progenitors could be the increased constitutive release of several chemokines during hypoxia; previous studies have demonstrated that several chemokines have a growth-enhancing effect for a minority of AML patients [35].

We investigated effects of hypoxia on AML cell differentiation in well-characterised human cell lines. This experimental approach was chosen because primary AML cells are very heterogeneous with regard to differentiation, whereas several previous studies have demonstrated that these cell lines are able to differentiate in response to various stimuli [26, 27, 43]. In these experiments, we treated cells with the hypoxia-mimicking agent $CoCl_2$ to induce HIF-1α expression. Our results show that HIF-1α induction was only associated with minor alterations in single differentiation markers, whereas we could not detect any general decrease in the expression of stem cell-associated markers. Similar observations were made when investigating primary human AML cells cultured for 48 h under hypoxia, suggesting that the increased release of certain cytokines during hypoxia is not associated with a hypoxia-induced, major alteration of the differentiation status of the leukaemic cells.

ATRA, in combination with the histone deacetylase inhibitor valproic acid and possibly theophyllin, is being considered as a disease-stabilizing treatment in AML. *In vitro* exposure to ATRA increases HIF-1α protein levels in AML cell lines [30]. We therefore investigated HIF-1α levels and constitutive chemokine release for primary human AML cells derived during *in vivo* ATRA therapy, but neither HIF-1α levels nor constitutive chemokine release was significantly altered during this treatment.

To conclude, we have demonstrated the importance of oxygen levels on the release of cytokines by AML cells. Low oxygen levels more closely resemble the bone marrow environment where leukaemic cells are physiologically exposed to much lower oxygen levels than the atmospheric (20-21%) oxygen concentrations typically used during *in vitro* culture. Low oxygen concentrations induce HIF-1α expression and also the release of several proangiogenic cytokines by leukaemia cells. These mechanisms may be operative during leukaemogenesis or possibly be involved in the chemoresistance found in the AML bone marrow.

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