HIF-1α of bone marrow endothelial cells implies relapse and drug resistance in patients with multiple myeloma and may act as a therapeutic target

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Author contributions

RR and IC designed research, performed experiments and wrote the manuscript; SB performed experiments and wrote the manuscript; ADL, AC, CP, VR, MAF, DR, BN, TA, SR performed experiments; AG, CM, EA, DD, PD, contributed material; MM and FD commented on the manuscript; and AV supervised the experiments, provided financing and wrote the manuscript.

List of abbreviations

2-DE, two-dimensional gel electrophoresis; ACN, acetonitrile; AKT, protein kinase B, ANXA1, annexin A1; ANXA4, annexin A4; BM, Bone marrow; CAM, chorioallantoic membrane; CM, conditioned medium; c-MET, mesenchymal-epithelial transition; Ct, threshold cycle; DAPI, 4',6-diamidino-2-phenylindole; DFO, deferoxamine; ECs, endothelial cells; FCS, fetal calf serum; FGF-2, fibroblast growth factor-2; FGFR-2, fibroblast growth factor receptor 2; GRP, gastrin-releasing peptide; GSTP1, glutathione S-transferase P1; H$_2$DCF-DA, 2,7-dichlorodihydrofluorescein-diacetate; HDACi, histone deacetylase inhibitor; HGF, hepatocyte growth factor; HIF-1α, hypoxia-inducible factor-1α; HRE, hypoxia-response element; HSPB1, heat shock protein β-1; HUVECs, human umbilical vein ECs; IEF, isoelectricfocusing; IPG, immobilized pH gradient; LASP1, LIM and SH3 domain protein 1; MGECs, ECs of MGUS patients; MGUS, monoclonal gammapaties of undetermined significance; MM, multiple myeloma; MMECs, MM endothelial cells; MS, mass spectrometry; mTOR, mammalian target of the rapamycin; NCBI, National Center for Biotechnology Information; OD, optical density; OS, overall survival; P4HA2, prolyl 4-
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STATEMENT OF TRANSLATIONAL RELEVANCE

Bone marrow (BM) angiogenesis is an attractive target for treatment of multiple myeloma (MM). Here we demonstrate that constitutive and normoxic expression of the hypoxia-inducible factor-1α (HIF-1α) protein in BM endothelial cells (ECs) of patients with relapsed/refractory MM (MMECs)
is a key inducer of angiogenesis in vitro and in vivo, and mediates resistance to antiangiogenesis exerted by bortezomib and lenalidomide. The expression of HIF-1α protein by MMECs was also associated with shorter overall survival. The HIF-1α inhibition by using siRNA or the histone deacetylase inhibitor (HDACI) panobinostat impaired the angiogenesis-related functions of the HIF-1α protein-expressing MMECs and overcame their resistance to bortezomib and lenalidomide. HIF-1α may thus be envisaged as an attractive target for the antiangiogenic management of relapsed/refractory MM patients, and as a possible prognostic factor.

ABSTRACT

Purpose: To investigate the role of hypoxia-inducible factor-1α (HIF-1α) in angiogenesis and drug resistance of bone marrow (BM) endothelial cells (ECs) of patients with multiple myeloma (MM).

Experimental Design: HIF-1α mRNA and protein were evaluated in ECs of MM patients (MMECs) at diagnosis, at relapse after bortezomib- or lenalidomide-based therapies or on refractory phase to these drugs, at remission; in ECs of patients with monoclonal gammapathies of undetermined significance (MGECs), and of those with benign anemia (controls). The effects of HIF-1α inhibition by siRNA or panobinostat (an indirect HIF-1α inhibitor) on the expression of HIF-1α pro-angiogenic targets, on MMEC angiogenic activities in vitro and in vivo, and on overcoming MMEC resistance to bortezomib and lenalidomide were studied. Patients’ overall survival was also observed.

Results: Compared to the other EC types, only MMECs from 45% of relapsed/refractory patients showed a normoxic HIF-1α protein stabilization and activation that were induced by reactive oxygen species (ROS). The HIF-1α protein correlated with the expression of its pro-angiogenic targets. The HIF-1α inhibition by either siRNA or panobinostat impaired the MMECs angiogenesis-related functions both in vitro and in vivo and restored MMEC sensitivity to bortezomib and lenalidomide. Patients with MMECs expressing the HIF-1α protein had shorter overall survival.
Conclusions: The HIF-1α protein in MMECs may induce angiogenesis and resistance to bortezomib and lenalidomide and be a plausible target for the antiangiogenic management of well-defined relapsed/refractory MM patients. It may also have prognostic significance.

INTRODUCTION

Angiogenesis plays a critical role in the pathophysiology and progression of multiple myeloma (MM) since it supports the growth and survival of plasma cells (1). Hypoxia is a major angiogenic stimulus (2), and hypoxia-inducible factor-1 (HIF-1) is the master regulator of the cellular response to hypoxia (3). HIF-1 is a heterodimeric transcription factor composed of a constitutively expressed subunit β (HIF-1β) and an oxygen-regulated subunit α (HIF-1α) (3). Under normoxia, HIF-1α is unstable and rapidly degraded via the Von Hippel-Lindau (VHL)-mediated ubiquitin-proteasome pathway. Under hypoxia it escapes the VHL binding and proteasomal degradation, translocates to the nucleus, heterodimerizes with HIF-1β, and induces transcription of numerous target genes, whose products are involved in cell migration, vascular remodeling and angiogenesis (3). Under normoxia, HIF-1α may also be activated in response to growth factors, cytokines and peptide mediators whose binding to their receptor tyrosine kinases activates the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of the rapamycin (PI3K/AKT/mTOR) pathway, which stimulates the HIF-1α expression (4). Also under normoxia, reactive oxygen species (ROS) can activate HIF-1α thus stimulating its transcriptional activity (5). HIF-1α overexpression has been detected in several human tumors (6) as positively related to growth, angiogenesis (7), chemoresistance (8) and poor prognosis (9).

In MM, the expression of HIF-1α has been found in both cell lines and primary plasma cells in normoxic conditions (10, 11) where it induces the expression of vascular endothelial growth factor (VEGF) (12) and other angiogenic cytokines (13). Because of the central role of HIF-1α in tumor aggressiveness and progression, several strategies have been adopted to inhibit its expression and transcriptional activity (4). Histone deacetylase inhibitors (HDACIs) are attractive anti-MM agents.
which suppress the growth and survival of plasma cells in vitro (15), and are currently being used in clinical trials (16). HDACIs induce degradation of HIF-1α independently of the VHL function (17), and repress the transactivation potential of hypoxia-inducible factors (18).

Although HIF-1α has already been studied in MM plasma cells as an angiogenic factor (12, 13), no studies on its expression and function in bone marrow (BM) MM endothelial cells (MMECs) of patients at different disease phases and in ECs of monoclonal gammapathies of undetermined significance (MGUS) patients (MGECs) have been carried out. Here we show that HIF-1α protein is constitutively expressed and activated in MMECs from a well defined proportion of relapsed/refractory patients, and that it confers resistance to antiangiogenesis mediated by bortezomib or lenalidomide. Inhibition of HIF-1α has antiangiogenic power and overcomes the drug resistance. HIF-1α may thus be envisaged as a new target for antiangiogenic management of a fraction of patients with relapsed/refractory MM. Since MMECs expressing the HIF-1α protein implied shorter overall survival, these cells may serve as a prognostic marker.

MATERIALS AND METHODS

Patients and ECs

Patients fulfilling the International Myeloma Working Group diagnostic criteria (19) for MM (n=76) and MGUS (n=35) were studied. The MM patients (52 M and 24 F), aged 45-82 (median 63.5) years, were at diagnosis (n=18), at complete remission (n=16), at relapse after bortezomib- or lenalidomide-based chemotherapies (n=20), or on refractory phase to these drugs (n=22). The M component was IgG (n=46), IgA (n=20), and k or λ (n=10). The MGUS patients (23 M and 12 F), aged 42-79 (median 60.5) years, were IgG (n=22), IgA (n=8), and k or λ (n=5). Normal (control) ECs were derived from 12 subjects with anemia due to iron or vitamin B12 deficiency (20). The study was approved by the Ethics Committee of the University of Bari Medical School, and all patients provided their informed consent in accordance with the Declaration of Helsinki. BM primary MMECs, MGECs, and normal ECs were obtained and cultured as described (21). BM
derived primary macrophages from MGUS and MM patients at different disease phases were obtained as previously described (22).

Reverse transcriptase (RT) PCR, real-time RT-PCR and Western blot

RT-PCR and real-time RT-PCR were performed with the primers shown in Supplementary Table S1 (Invitrogen, Paisley, UK) and the Applied Biosystems methodology (23). The PCR products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. The mRNA level was measured with the comparative threshold cycle (Ct) method using β-actin as the reference and the 2^(-ΔΔCt) formula (24). Total protein lysates (50 μg) from MMECs, MGECs and normal ECs were immunoblotted with anti-HIF-1α (Becton Dickinson-BD Transduction Laboratories, San Jose, CA, USA), anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA), anti-VEGF receptor 2 (VEGFR-2, Cell Signaling Technology, Danvers, MA, USA), anti-fibroblast growth factor-2 (FGF-2) receptor 2 (FGFR-2, Sigma-Aldrich), anti-mesenchymal-epithelial transition (c-MET) and anti-VHL (both from Abcam, Cambridge, UK), anti-AKT and anti-phospho(p)AKT (both from Cell Signaling Technology), as described (25). Immunoreactive bands were detected with enhanced chemiluminescence (LiteAblot®, EuroClone, Milan, Italy), and Gel-Logic1500 system (Eastman Kodak Co., Rochester, NY, USA), and quantified as optical density (OD) units by the Kodak imaging software.

Conditioned media and enzyme-linked immunosorbent assay (ELISA)

MMECs and MGECs (1×10^6 cells/mL) were cultured in serum-free DMEM for 24 h, then supernatants centrifuged (380×g for 10 min), and stored at -80°C as CM. VEGF, FGF-2, and hepatocyte growth factor (HGF) were quantified by an ELISA (SearchLight human angiogenesis array 2, Tema Ricerca Srl, Bologna, Italy).

Immunofluorescence and dual immunofluorescence-confocal laser-scanning microscopy

For immunofluorescence, 5×10^3 MMECs, MGECs and normal ECs (these treated or not with deferoxamine [DFO] 380 μM for 6 h as positive and negative control, respectively) were cultured on fibronectin-coated chamber slides (LabTek, Nalge Nunc International, Naperville, IL, USA),
fixed with paraformaldehyde, permeabilized with Triton X-100, and incubated with an anti-HIF-1α antibody (BD Transduction Laboratories) then with a secondary rabbit anti-mouse IgG-TRITC and phalloidin-fluorescein isothiocyanate (both from Sigma-Aldrich); nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI in Vectashield® Hard Set™ mounting medium, Vector, Burlingame, CA, USA).

**HIF-1α DNA binding assay**

HIF-1α activation was measured in MMECs, MGECs, and normal ECs with the TransAM™ HIF-1α assay (Active Motif, Carlsbad, CA, USA). Activated HIF-1α contained in nuclear extracts (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermo Scientific, Rockford, IL, USA) specifically binds to an erythropoietin 3’ hypoxia-response element (HRE)-derived oligonucleotide probe immobilized on a 96-well plate, and it is identified by an anti-HIF-1α antibody. Wild type consensus oligonucleotide was used as a competitor for HIF-1α binding to monitor the assay specificity. A mutated consensus oligonucleotide served as an additional negative control; nuclear extracts of CoCl2-stimulated HeLa cells as the positive control.

**Treatment of MMECs with small interfering RNA (siRNA) and anti-MM drugs**

The HIF-1α protein positive MMECs (5×10⁵) of relapsed or refractory patients were transiently transfected with HIF-1α·siRNA 50 nM for 3-5 days, with control siRNAs (SMART-pool; Dharmacon RNA Technologies, Lafayette, CO, USA) or with the transfection reagent alone (Lipofectamine, RNAiMAX siRNA transfection reagent, Invitrogen) (20). In separate experiments, MMECs (5×10⁵) were treated with bortezomib (Velcade®, Millennium Pharmaceuticals Inc., Cambridge, MA, USA) 10 nM for 24 h (26), lenalidomide (Revlimid®, Celgene Co., Summit, NJ, USA) 1.75 μM for 72 h (25), or panobinostat (LBH589, Novartis Oncology, Varese, Italy) 100 nM for 72 h (27). The HIF-1α·siRNA transfected MMECs were then incubated with/without bortezomib or lenalidomide. All MMECs groups were evaluated in functional studies. Isobologram analysis was performed with the CalcuSyn software (Biosoft, Ferguson, MO, USA; Cambridge, UK), and a combination index (CI) <1.0 indicated synergism.
Functional studies

Viability and apoptosis: Viability was assessed by trypan blue staining, and apoptotic rate by phycoerythrin–annexin V and 7-amino-actinomycin D (Apoptosis detection kit, BD, San Jose, CA, USA) followed by cytofluorimetry on FACScantoII (BD).

Adhesion and spreading: MMECs (1×10⁴) were plated in DMEM (EuroClone) on fibronectin-coated 96-well plates in triplicate for 30 min (adhesion) or 90 min (spreading), fixed with 4% paraformaldehyde, and quantified by the crystal violet assay at 595 nm in a Microplate Reader (20) (Molecular Devices Corp., Sunnyvale, CA, USA).

Chemotaxis: MMECs (5×10⁴) were tested in a Boyden microchamber assay towards DMEM with 1.5% FCS alone (negative control) or added with VEGF (10 ng/mL, Sigma Chemical Co.) and FGF-2 (10 ng/mL, Peprotech Inc., Rocky Hill, NJ, USA) as chemoattractants (20). After 8 h at 37°C, the migrated cells were fixed, stained and counted by the EVOS inverted microscope (EuroClone) at ×400.

Angiogenesis on Matrigel: MMECs were plated on 48-well plates coated with Matrigel (BD) in serum-free medium (SFM): after 12 h, the skeletonization of the mesh was followed by measurement of mesh areas and vessel length in three randomly-chosen fields with the EVOS microscope at ×200.

Chorioallantoic membrane (CAM) assay

Fertilized white Leghorn chicken eggs were incubated at 37°C at constant humidity (25). On day 3, the shell was opened and 2-to-3 mL of albumen removed to detach the CAM. On day 8, the CAMs were implanted with 1 mm³ sterilized gelatin sponges (Gelfoam Upjohn Co., Kalamazoo, MI, USA) loaded with SFM alone (negative control) or with CM from untreated (positive control) or HIF-1α·siRNA- or panobinostat-treated MMECs. On day 12, the angiogenic response was evaluated as the number of vessels converging toward the sponge at ×50 and photographed in ovo by a stereomicroscope (Olympus Italia Srl, Segrate, Milan, Italy).

Measurement of ROS and treatment of MMECs with antioxidants
MMECs and MGECs (2×10^5) treated or not with 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) 0.5 mM for 24 h were trypsinized and incubated with 2,7-dichlorodihydrofluorescein-diacetate (H$_2$DCF-DA, both from Sigma-Aldrich) 2 µM at 37°C for 15 min, washed and resuspended in PBS. Each sample was analyzed for 20,000 events on the FACSCanto II cytofluorimeter.

RESULTS

Normoxic activation of HIF-1α in MMECs correlates with relapse and drug resistance

HIF-1α mRNA and protein were measured in normoxic conditions in MMECs from patients at different disease phases as well as in MGECs and normal ECs. While mRNA levels overlapped between all the ECs types (Fig. 1A), the protein was only expressed in MMECs of 19 out of 42 (45%) patients at relapse after bortezomib- or lenalidomide-based therapies or on refractory phase to these drugs (Fig. 1B). HIF-1α was active as assessed by a DNA-binding assay (Fig. 1C), and found in the nucleus (Fig. 1D, top panels). In contrast, it was always absent (or irrelevant) in patients at diagnosis (Fig. 1D, middle left panel) or in remission (data not shown), or in those with MGUS (Fig. 1D, middle right panel), as well as in control subjects’ ECs (Fig. 1D, bottom right panel). The normoxic expression of HIF-1α in MMECs from relapsed/refractory patients was not confirmed in another BM hematopoietic cell population, such as macrophages (Supplementary Figure S1).

HIF-1α induces overexpression of its pro-angiogenic targets

We wondered whether HIF-1α protein expression may result in the upregulation of its pro-angiogenic targets: VEGF, FGF-2, and c-MET. These were significantly overexpressed in conjunction with the HIF-1α protein, as both mRNA (Fig. 2A) and protein (Fig. 2B). A similar correlation was also found with VEGFR-2, FGFR-2 and HGF (the c-MET ligand) (Supplementary Fig. S2). To determine whether VEGF, FGF-2 and c-MET overexpression was induced by the HIF-1α activation, we measured each mRNA following HIF-1α knockdown by siRNA. Interestingly,
this produced a 90% reduction (as average) of HIF-1α mRNA at 72 h (Fig. 2C, left graph) while the protein was reduced by 70% (as average) only at day 5 (Fig. 2C, right panel) suggesting that it was stabilized substantially in the MMECs. The HIF-1α silencing sizeably suppressed the VEGF (-60% as average), FGF-2 (-54%) and c-MET (-50%) mRNAs (Fig. 2D, left graph). No effect on cell viability was observed upon siRNA after 5 days (Fig. 2D, right graph).

**HIF-1α knockdown affects key MMEC angiogenesis-related functions**

To investigate whether HIF-1α plays a role in MM angiogenesis the effects of HIF-1α-siRNA on the functions of MMECs expressing the HIF-1α protein were studied. The HIF-1α silencing impacted chemotaxis (-60% as average) cell adhesion (-50%), and spreading (-47%) but not cell viability (Fig. 3A) nor apoptosis (data not shown). The HIF-1α-siRNA MMECs seeded onto the Matrigel surface lacked angiogenesis as assessed by substantial reduction of vessel length (-62% as average) and empty areas (-70%) (Fig. 3B). On the *in vivo* CAM assay, when CAMs were implanted with a gelatine sponge soaked with the CM of MMECs expressing the HIF-1α protein, many newly-formed capillaries converging radially toward the sponge in a “spoked-wheel” pattern were seen (vessel count = 31±6, positive control; Fig. 3C, middle). In contrast, the CM of the HIF-1α-siRNA MMECs gave poor angiogenesis (12±5; Fig. 3C, right) which was similar to physiological angiogenesis obtained with SFM (9±3, negative control; Fig. 3C, left).

**HIF-1α knockdown restores MMECs sensitivity to bortezomib and lenalidomide**

Interestingly, the HIF-1α protein mediated drug resistance of MMECs. Indeed, HIF-1α protein expressing MMECs of patients at relapse or on refractory phase to bortezomib- or lenalidomide-based therapies were resistant to the previously shown (26, 25) antiangiogenic effect of these drugs: neither bortezomib nor lenalidomide impaired MMECs chemotaxis, adhesion, spreading (Fig. 4A) and the whole angiogenesis (Fig. 4B). In contrast, HIF-1α protein negative MMECs of the same patients’ series were sensitive to the drugs’ antiangiogenic effect (Supplementary Fig. S3). When HIF-1α was knocked down by siRNA, resistance to bortezomib or lenalidomide was overcame. Specifically, the combination of HIF-1α-siRNA+bortezomib showed a synergistic inhibition of
chemotaxis (-60% as average), adhesion (-38%), spreading (-45%; Fig. 4A) and whole angiogenesis (-80% vessel length; -78% empty areas; Fig. 4B) compared to HIF-1α·siRNA alone (combination index [CI]<1; isobologram analysis). The combination of HIF-1α·siRNA+lenalidomide also showed a synergistic inhibition of chemotaxis (-54%) (Fig. 4A) and whole angiogenesis (-63% vessel length; -60% empty areas; Fig. 4B). No changes in cell viability (Fig. 4A) nor apoptosis (data not shown) were seen in HIF-1α protein expressing MMECs treated with bortezomib or lenalidomide singularly or in association with HIF-1α·siRNA.

Panobinostat inhibits MMEC angiogenesis by downregulating the HIF-1α transcriptional activity

We investigated whether panobinostat may exert an antiangiogenic effect on the HIF-1α protein expressing MMECs, since it is a HDACi, i.e., an indirect HIF-1α inhibitor (18). Similarly to what was seen in HIF-1α·siRNA MMECs, panobinostat impacted chemotaxis (-61% as average), cell adhesion (-50%) and whole angiogenesis (-64% vessel length; -64% empty areas; Fig. 5A-B). The combination of panobinostat+bortezomib induced a synergistic inhibition of chemotaxis, cell adhesion, and spreading (-64%, -50%, -33% respectively) (Fig. 5A), and of whole angiogenesis (-77% vessel length and -47% empty areas; Fig. 5B, combination index [CI]<1) compared to panobinostat alone. Also, panobinostat+lenalidomide showed a synergistic inhibition of chemotaxis (-64%; Fig. 5A) and of whole angiogenesis (-80% vessel length; -54% empty areas; Fig. 5B). No changes in cell viability (Fig. 5A) were seen in HIF-1α protein expressing MMECs treated with panobinostat singularly or in association with bortezomib or lenalidomide. Interestingly we also demonstrated that panobinostat inhibits MMEC angiogenesis either directly by acting on MMECs and indirectly by acting on total BM cells. We observed that the CM of the total BM cells (BMCM) increased MMEC angiogenesis because of the pro-angiogenic factors and cytokines released by BM cells (Supplementary Fig. S4A, right panel). Indeed, the direct inhibitory effect of panobinostat, on the isolated MMEC angiogenesis (Supplementary Fig. S4B, left panel), was partially subverted by the BMCM (Supplementary Fig. S4B, middle panel). However when the total BM cells were
pretreated with panobinostat, their BMCM inhibited MMEC angiogenesis (Supplementary Fig. S4B, right panel). In the CAM assay, different from the CM of untreated MMECs (vessel count = 37±7; Figure 5C, middle panel) the CM of panobinostat-treated MMECs gave irrelevant angiogenesis (10±5, \(P<0.01\); Fig. 5C, right), re-equilibrating the vessel counts to physiological angiogenesis (8±3; Fig. 5C, left). Next, we wondered whether treatments with panobinostat impacted on the HIF-1\(\alpha\) protein expression and/or activation: the drug did not reduce the protein levels (Fig. 5D, left panel), while it reduced its activation when given singularly (-50%) and in combination with bortezomib (-79%) or lenalidomide (-53%) (Fig. 5D, middle graph). These treatments respectively inhibited VEGF (-50%; -80%; -68%), FGF-2 (-40%; -70%; -50%) and c-MET (-45%; -70%; -50%) transcription (Fig. 5D, right graph).

HIF-1\(\alpha\)-siRNA and panobinostat induce similar changes in the MMEC proteome

To further examine the inhibitory effects of HIF-1\(\alpha\)-siRNA and panobinostat at the molecular level, we compared the proteome of HIF-1\(\alpha\) protein expressing MMECs following HIF-1\(\alpha\)-siRNA or panobinostat treatment. At least three 2-DE-gels were run per sample followed by computer-assisted spot matching to enable the identification of spots variations. Nine proteins were identified as differentially expressed by peptide sequencing and tandem mass spectrometry (MS-MS) followed by database searching (Supplementary Fig. S4C,D). Eight proteins were downregulated by both HIF-1\(\alpha\)-siRNA and panobinostat (2-fold changes vs control): glutathione S-transferase P1 (GSTP1), heat shock protein \(\beta\)-1 (HSPB1), annexin A4 (ANXA4), protein disulfide-isomerase A3 (PDIA3), prolyl 4-hydroxylase subunit \(\alpha\)-2 (P4HA2), gastrin-releasing peptide (GRP), LIM and SH3 domain protein 1 (LASP1) and annexin A1 (ANXA1); the polymerase I and transcript release factor (PTRF) was instead upregulated. These proteins govern cell shape, cell metabolism, chemotaxis and angiogenesis (Supplementary Table S2).

The HIF-1\(\alpha\) protein is stabilized in MMECs by reactive oxygen species (ROS) and has a prognostic value
To search the mechanism that stabilizes the HIF-1α protein in MMECs, we studied the pathways governing its expression in normoxia. The expression was not associated with neither the loss of pVHL nor the activation of the AKT pathway (Supplementary Fig. S5). Interestingly, the ROS production was highly correlated with the HIF-1α protein expression (Fig. 6A), as assessed in HIF-1α protein positive vs negative MMECs of relapsed/refractory patients and of the other patients’ groups, or vs MGECs. Accordingly, the antioxidant Trolox reduced both the ROS (Fig. 6B left) - without affecting cell viability (Fig. 6B right) - and the HIF-1α protein levels (Fig. 6C), suggesting a key role of ROS in mediating the HIF-1α protein stabilization in normoxia.

Worth of note is that a 12-month follow-up of patients with HIF-1α protein positive and negative MMECs showed a significantly shorter overall survival in the former (Fig. 6D).

DISCUSSION

Among hematological malignancies, chronic lymphocytic leukemia (28), diffuse large B cell and follicular non-Hodgkin lymphomas (29), Hodgkin lymphoma (30), and MM (10,11) express HIF-1α in tumor cells. HIF-1α has been found in plasma cells cultured in normoxia of 28% of MM patients (10), and it was enhanced by plasma cell growth factors such as insulin-like growth factor-1 and IL-6 (11). Here the role of HIF-1α in MM angiogenesis and drug resistance was investigated in MMECs harvested from patients at different disease phases and cultured in normoxia. HIF-1α mRNA overlapped between MMECs, MGECs and normal ECs whereas the protein was expressed, stabilized and activated only in MMECs of 45% of patients who were relapsed after bortezomib- or lenalidomide-based therapies or were refractory to these drugs. Since these MMECs had the protein in normoxic conditions one can hypothesize that it was regulated at post-translational level. Accordingly, hypoxia-independent mechanisms may govern HIF-1α in MMECs from patients relapsed/ refractory to bortezomib or lenalidomide as already found in plasma cells (10). HIF-1α inducers in normoxic cells include reduced expression of the tumor suppressor protein VHL (31), activation of PI3K/AKT/mTOR pathway (32) and ROS (5). In MMECs, the HIF-1α protein did not
correlate neither with expression of VHL nor with activation of the AKT pathway, but it did with increased ROS production; and the ROS inhibition by Trolox reduced the HIF-1α protein stabilization. Data support a key role of ROS in mediating the HIF-1α protein stabilization in MMECs similarly to what observed in human prostate (33) and gastric carcinoma cells (5). HIF-1α was closely involved in the MMECs overangiogenic phenotype. BM angiogenesis is a constant hallmark of MM progression (21), and enhanced by an autocrine VEGF loop of MMECs (34). The HIF-1α stabilization may plausibly lead, in turn, to enhanced angiogenesis because it increases the expression of the angiogenic factors VEGF, FGF-2 and c-MET. In plasma cells too the HIF-1α activation leads to the production of angiogenic factors (13).

To further elucidate the role of HIF-1α in MM angiogenesis we evaluated the effects of its knockdown in the MMECs. HIF-1α mRNA was reduced by 90% at 72 h after transfection while the protein was reduced by 70% five days after transfection, which implied its strong stabilization. HIF-1α knockdown affected MMECs adhesion, spreading and migration, as others have found in cells of malignant glioma (35) and renal carcinoma (36). It also inhibited MMEC angiogenesis in vitro (Matrigel) and in vivo (CAM) assays. Recently, Calvani et al. reported that human umbilical vein ECs (HUVECs) cultured in growth factors-enriched medium form tube-like structures under both normoxia and hypoxia, while they form vessels only under hypoxia when cultured in a growth factors-reduced medium (37). Here the enhanced angiogenesis shown by MMECs in normoxic conditions could be explained with constitutive HIF-1α protein stabilization and consequent production of its angiogenic targets.

The stabilization of HIF-1α protein in MMECs of a well-defined percentage of relapsed/refractory patients suggests its involvement in MM drug resistance. In fact MMECs expressing the HIF-1α protein showed resistance to bortezomib and lenalidomide compared to MMECs negative for expression as shown previously (26, 25). Interestingly, the HIF-1α knockdown restored the sensitivity to bortezomib or lenalidomide showing a synergistic effect in combination with these
drugs. All these evidences suggest that HIF-1α may be an antiangiogenic target in relapsed/refractory patients having MMECs with the HIF-1α protein.

We investigated the pharmacologic inhibition of HIF-1α by panobinostat, a histone deacetylase inhibitor (HDACI, i.e., an indirect HIF-1α inhibitor (18)) which has already been shown to deliver potent in vitro (38, 39) and in vivo (38) anti-MM activity. The deacetylase activity is needed for the transactivation potential of HIF-1α (18). We show, in fact, that panobinostat does not induce HIF-1α degradation, but reduces its binding to DNA, hence its transcriptional activity. This was especially seen when panobinostat was associated to bortezomib, as demonstrated by the intense decrease of VEGF, FGF-2 and c-MET transcripts. Moreover, panobinostat impacted MMEC angiogenesis-related functions such as cell adhesion and chemotaxis, as well as the whole angiogenesis in vitro and in vivo. Similarly to HIF-1α·siRNA, the panobinostat treatment was able to overcome MMECs resistance to bortezomib and lenalidomide, thus potentiating the drugs’ antiangiogenic activity in a synergistic way. Others have shown that panobinostat was able to potentiate cytotoxic activity of bortezomib, dexamethasone and melphalan in MM cell lines resistant to these drugs by impairing cell growth and survival (27).

We investigated more deeply into the molecular mechanisms involved in the inhibition of MMEC angiogenesis by HIF-1α·siRNA and panobinostat through proteomic analysis. The differentially expressed MMECs proteins in response to the HIF-1α·siRNA and panobinostat treatment were involved in drug resistance (GSTP1), as well as cell shape, cytoskeletal remodeling, migration and invasiveness (GRP; LASP-1). Specifically, GSTs are enzymes that catalyze the conjugation of xenobiotics with glutathione, thereby facilitating their subsequent efflux through multi-resistance pumps (40). This provides tumor cells with a selective survival advantage over normal cells by enhancing drug efflux, and consequent decreasing the drugs’ therapeutic efficacy. In MM plasma cells, indeed, GSTs entail a well characterized mechanism of drug resistance (41). We suggest that the downregulation of GSTP1 in HIF-1α·siRNA- and panobinostat-treated MMECs may be a way to overcome drug resistance. The restoration of drug sensitivity in the HIF-1α·siRNA- and
panobinostat-treated MMECs could also be explained by the downregulation of HSB1 (or HSP70), a member of the “stress-associated early response gene” family involved in a wide range of cell functions under stress conditions and oncogenesis (42). Of note, HSB1 expression is upregulated in MM plasma cells (43) and in resistant MM cell lines, and its inhibition reduces cell adhesion and reverts drug resistance (44), much in the same way as we show in MMECs. Among the downregulated proteins we found LASP-1, an acting-binding cytoskeletal protein localized at focal adhesions along stress fibres, that regulates cell migration (45); and GRP that elicits ECs migration and cord formation \textit{in vitro}, and enhances angiogenesis \textit{in vivo} (46).

Moreover, here we emphasize that patients with MMECs expressing the HIF-1\(\alpha\) protein had shorter overall survival than those with MMECs negative for expression suggesting that endothelial HIF-1\(\alpha\) may represent a poor prognosis factor. Further confirmatory studies in larger series may be encouraged.

In conclusion, HIF-1\(\alpha\) of MMECs of a well defined percentage of relapsed patients after treatment with bortezomib or lenalidomide-based therapies or refractory to these drugs may be targeted for antiangiogenic management, and be regarded as a new prognostic factor.

REFERENCES


FIGURE LEGENDS

Figure 1
HIF-1α is expressed and activated in MMECs from patients with relapsed/refractory disease cultured in normoxia.

A) HIF-1α mRNA levels were analyzed by RT-PCR and real-time RT-PCR and normalized to endogenous β-actin mRNA. Gene expression fold changes in normal ECs was arbitrarily set as 1.

B) HIF-1α protein was examined by Western blot (β-actin=loading control), and data shown as optical density (OD). C) HIF-1α transcription activity was quantified by spectrophotometry at 450 nm. **P<0.01 by Wilcoxon signed-rank test. D) Immunofluorescence for HIF-1α (red signal), actin (green signal) and nuclei (blue signal) in ECs from representative MM and MGUS patients, and control subjects. Merge (pink signal) shows colocalization of HIF-1α, nuclei and actin. Top panels: merge for HIF-1α and nuclei in MMECs from relapsed/refractory patients. Middle panels: merge for HIF-1α and nuclei in MMECs at diagnosis and MGECs. Bottom panels: merge for HIF-1α and nuclei in normal ECs treated or not with deferoxamine (DFO). DFO mimics hypoxia and served as the positive control. Pictures by an Olympus photomicroscope (Olympus, Milan, Italy) equipped with the DP20-5E digital camera. Magnification ×600; Scale bar: 16 µm.

Figure 2

HIF-1 pathway promotes the transcription of VEGF, FGF-2 and c-MET in MMECs.

A) Real-time RT-PCR (normalized to β-actin) for VEGF, FGF-2, and c-MET. B) Protein levels of VEGF, FGF-2 by ELISA (left graphs) and of HIF-1α and c-MET by western blot measured by optical density (OD, right panel). Data are expressed as mean ± SD of MMECs from patients at diagnosis (n=12), in remission (n=14), relapsed after bortezomib- or lenalidomide-based therapies (n=9) or on refractory phase to these drugs (n=10) and of MGECs (n=20); C) MMECs treated with HIF-1α·siRNA compared to non-targeting siRNA (control) or untreated MMECs showed significant reduction (-90% as average) of HIF-1α mRNA at 72 h after transfection (on real-time RT-PCR normalized to β-actin, left graph), and of HIF-1α protein (-70% as average) at 5 days (on western blot with β-actin as loading control, right panel); D) Downregulation of HIF-1α target
genes: VEGF, FGF-2 and c-MET following the HIF-1α-siRNA (on real-time RT-PCR, left graph) and cell viability upon siRNA after 5 days (right graph).

\*P<0.05; and \**P<0.01 by Wilcoxon signed-rank test.

**Figure 3**

HIF-1α knockdown impairs angiogenesis-related functions of MMECs from relapsed/refractory patients.

HIF-1α-siRNA transfected MMECs were tested for: **A** chemotaxis, adhesion, spreading and viability; **B** angiogenesis on Matrigel (measurement of vessel length and empty areas by the EVOS image software). Matrigel magnification: ×200; Scale bar: 50 µm. **C** CAM assay: poor angiogenesis by CM of HIF-1α-siRNA (right panel) vs untreated MMECs (positive control, middle) from a representative relapsed patient; physiological angiogenesis with serum-free medium (SFM, left). Pictures with a stereomicroscope: original magnification ×50. Data are means ± SD of MMECs from relapsed (n=9) or refractory (n=10) patients. \*P<0.05; and \**P<0.01 by Wilcoxon signed-rank test.

**Figure 4**

HIF-1α knockdown sensitizes MMECs to bortezomib and lenalidomide.

MMECs treated with bortezomib or lenalidomide singularly, or in conjunction with HIF-1α-siRNA were tested for: **A** viability, chemotaxis, adhesion and spreading; **B** angiogenesis on Matrigel, (measurement of vessel length and empty areas by the EVOS image software). Original magnification: ×200; Scale bar: 50 µm. Histograms are means ± SD of MMECs from relapsed (n=9) or refractory (n=10) patients. \*P<0.05 or \**P<0.01 by Wilcoxon signed-rank test. The combination index (CI) <1.0 indicates synergism (isobologram analysis).

**Figure 5**

Angiogenesis inhibition by panobinostat in MMECs of relapsed/refractory patients is mediated by downregulation of the HIF-1α transcriptional activity.
MMECs treated with panobinostat, bortezomib or lenalidomide singularly or in combination were tested for: A) viability, chemotaxis, adhesion and spreading; B) angiogenesis on Matrigel (measurement of vessel length and empty areas by the EVOS image software). Matrigel magnification: ×200; Scale bar: 50 µm. C) CAMs were incubated with gelatine sponges loaded with serum-free medium (physiological angiogenesis, left panel), with the CM of MMECs not treated (positive control, middle panel) or treated with panobinostat (right panel) showing insufficient angiogenesis. Pictures with a stereomicroscope: original magnification ×50. D) Evaluation of: HIF-1α protein (on Western blot with β-actin as loading control; left panel); HIF-1α activation (by a DNA binding assay; middle panel); expression of the HIF-1α target genes VEGF-A, FGF-2, c-MET (by real-time RT-PCR; right panel) of MMECs treated with panobinostat, bortezomib and lenalidomide singularly or in combination. All histograms are means ± SD of MMECs from relapsed (n=9) or refractory (n=10) patients. *P<0.05 or **P<0.01 by Wilcoxon signed-rank test. The combination index (CI) <1.0 indicates synergism (isobologram analysis).

Figure 6
HIF-1α of MMECs from relapsed/refractory patients is stabilized by ROS
A) Intracellular ROS production by flow cytometry analysis in MMECs and MGECs treated with the oxidation sensitive dye H2DCF-DA. Data are expressed as mean fluorescence intensity ± SD of MMECs of patients at diagnosis (n=12), in remission (n=14), or relapsed after bortezomib- or lenalidomide-based therapies (n=15), or on refractory phase to these drugs (n=14), and of MGECs (n=20). B) ROS levels measured by flow cytometry analysis in MMECs treated with the antioxidant Trolox and then incubated with H2DCF-DA (left graph); evaluation of cell viability after Trolox treatment (right graph). C) HIF-1α protein levels examined by Western blot (β-actin as loading control) and shown as optical density (OD) in MMECs treated with Trolox. Data are expressed as mean fluorescence intensity ± SD of HIF-1α positive MMECs of relapsed (n=9) or refractory (n=8) patients. *P<0.05 and **P<0.01 by Wilcoxon signed-rank test. D) Kaplan-Meier functions of overall survival (OS) of patients relapsed after bortezomib- or lenalidomide-based
therapies or refractory to these drugs, and calculated from the day of bone marrow sampling at relapse/refractory phase to the date of final follow-up (12 months). Patients with MMECs expressing the HIF-1α protein were 19 (continuous line); those with MMECs negative for the HIF-1α protein were 23 (sketched line). $P<0.001 = $ significance by Log-Rank test.
Figure 1
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HIF-1α of bone marrow endothelial cells implies relapse and drug resistance in patients with multiple myeloma and may act as a therapeutic target

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