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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Abstract

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

**Experimental Design:** HIF-1α mRNA and protein were evaluated in patients with multiple myeloma endothelial cells (MMEC) at diagnosis, at relapse after bortezomib- or lenalidomide-based therapies or on refractory phase to these drugs, at remission; in endothelial cells of patients with monoclonal gammapathies of undetermined significance (MGUS; 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 proangiogenic targets, on MMEC angiogenic activities *in vitro* and *in vivo* and on overcoming MMEC resistance to bortezomib and lenalidomide were studied. The overall survival of the patients was also observed.

**Results:** Compared with the other endothelial cell 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 proangiogenic 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 may be a plausible target for the antiangiogenic management of patients with well-defined relapsed/refractory multiple myeloma. It may also have prognostic significance. *Clin Cancer Res; 1–12. ©2013 AACR.*

Introduction

Angiogenesis plays a critical role in the pathophysiology and progression of multiple myeloma because 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 α (HIF-1α) and an oxygen-regulated subunit α (HIF-1α; ref. 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 proteosomal 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...
Bone marrow angiogenesis is an attractive target for the treatment of multiple myeloma. 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 the 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 patients with relapsed/refractory multiple myeloma, and as a possible prognostic factor.

**Translational Relevance**

Bone marrow angiogenesis is an attractive target for the treatment of multiple myeloma. 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 the 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 patients with relapsed/refractory multiple myeloma, and as a possible prognostic factor.

Materials and Methods

**Patients and endothelial cells**

Patients fulfilling the International Myeloma Working Group diagnostic criteria (19) for multiple myeloma (n = 76) and MGUS (n = 35) were studied. The patients with multiple myeloma (52 male and 24 female), ages 44 to 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 male and 12 female), ages 42 to 79 (median 60.5) years, were IgG (n = 22), IgA (n = 8), and k or λ (n = 5). Normal (control) endothelial cells 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 (Bari, Italy), and all patients provided their informed consent in accordance with the Declaration of Helsinki. Bone marrow primary MMECs, MGECs, and normal endothelial cells were obtained and cultured as described previously (21). Bone marrow derived primary macrophages from MGUS and multiple myeloma patients at different disease phases were obtained as previously described (22).

**Reverse transcriptase PCR, real-time RT-PCR, and Western blot analysis**

Reverse transcriptase PCR (RT-PCR) and real-time RT-PCR were performed with the primers shown in Supplementary Table S1 (Invitrogen) 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 endothelial cells were immunoblotted with anti-HIF-1α (BD Biosciences), anti-β-actin (Sigma-Aldrich), anti-VEGF receptor 2 (VEGFR-2; Cell Signaling Technology), anti-fibroblast growth factor receptor-2 (FGFR-2), anti-mesenchymal-epithelial transition (c-MET), and anti-VHL (both from Abcam), anti-AKT and anti-phospho(p)AKT (both from Cell Signaling Technology), as described previously (25). Immunoreactive bands were detected with enhanced chemiluminescence (LiteAblot; EuroClone), and Gel-Logic1500 system (Eastman Kodak Co.), and quantified as optical density units by the Kodak imaging software.

**Conditioned media and ELISA**

MMECs and MGECs (1 × 10⁶ cells/mL) were cultured in serum-free Dulbecco’s Modified Eagle Medium (DMEM) for 24 hours, then supernatants centrifuged (380 × g for 10 minutes), and stored at −80°C as conditioned media. VEGF, FGF-2, and hepatocyte growth factor (HGF) were
quantified by ELISA (SearchLight human angiogenesis array 2; TEMA RICERCA SRL).

**Immunofluorescence and dual immunofluorescence–confocal laser-scanning microscopy**

For immunofluorescence, 5 × 10⁴ MMECs, MGECs, and normal endothelial cells (these treated or not with deferoxamine 380 μmol/L for 6 hours as positive and negative control, respectively) were cultured on fibronectin-coated chamber slides (LabTek; Nalge Nunc International), fixed with paraformaldehyde, permeabilized with Triton X-100, and incubated with an anti-HIF-1α antibody (BD Transduction Laboratories) then with a secondary rabbit antimouse IgG-TRITC and phalloidin–fluorescein isothiocyanate (both from Sigma-Aldrich); nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Vectashield Hard_Set mounting medium; Vector).

**HIF-1α DNA–binding assay**

HIF-1α activation was measured in MMECs, MGECs, and normal endothelial cells with the TransAM HIF-1α assay (Active Motif). Activated HIF-1α contained in nuclear extracts (NE-PER Nuclear and Cytoplasmic Extraction Reagents; Thermo Scientific) specifically binds to an erythropoietin 3′ hypoxia–response element–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 siRNA and anti-multiple myeloma drugs**

The HIF-1α protein–positive MMECs (5 × 10⁴) of relapsed or refractory patients were transiently transfected with HIF-1α siRNA 50 nmol/L for 3 to 5 days, with control siRNAs (SMART-pool; Dharmacon RNA Technologies) or with the transfection reagent alone (Lipofectamine, RNAiMax siRNA transfection reagent; Invitrogen; ref. 20). In separate experiments, MMECs (5 × 10⁴) were treated with bortezomib (Velcade; Millennium Pharmaceuticals Inc.) 10 nmol/L for 24 hours (26), lenalidomide (Revlimid; Celgene Co.) 1.75 × 10⁻⁵ mol/L for 24 hours (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), and a combination index (CI) < 1.0 indicated synergism.

**Functional studies**

**Viability and apoptosis.** Viability was assessed by trypsin blue staining, and apoptotic rate by phycoerythrin–Annexin V and 7-amino-actinomycin D (Apoptosis detection kit; BD Biosciences) followed by cytofluorimetry on FACScantoII (BD Biosciences).

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

**Chemosensitivity.** MMECs (5 × 10⁴) were tested in a Boyden microchamber assay toward DMEM with 1.5% fetal calf serum alone (negative control) or added with VEGF (10 ng/mL; Sigma Chemical Co.), and FGF-2 (10 ng/mL; Peprotech Inc.) as chemoattractants (20). After 8 hours 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 Biosciences) in serum-free medium (SFM): after 12 hours, 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 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 chorioallantoic membrane (CAM). On day 8, the CAMs were implanted with 1 mm³ sterilized gelatin sponges (Gelfoam Upjohn Co.) loaded with SFM alone (negative control) or with conditioned media 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 vivo by a stereomicroscope (Olympus Italia S.R.L.).

**Measurement of ROS and treatment of MMECs with antioxidants**

MMECs and MGECs (2 × 10⁵) treated or not with 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) 0.5 mmol/L for 24 hours were trypsinized and incubated with 2,7-dichlorodihydrofluorescein-diacetate (H₂DCF-DA; both from Sigma-Aldrich) 2 μmol/L at 37°C for 15 minutes, washed, and resuspended in PBS. Each sample was examined 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 endothelial cells. Although mRNA levels overlapped between all the endothelial cells types (Fig. 1A), the protein was only expressed in MMECs of 19 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). In contrast, it was always absent (or irrelevant) in patients at diagnosis (Fig. 1D, middle left) or in remission (data not shown), or in those with MGUS (Fig.
The normoxic expression of HIF-1α in MMECs from relapsed/refractory patients was not confirmed in another bone marrow hematopoietic cell population, such as macrophages (Supplementary Fig. S1).

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 endothelial cells were arbitrarily set as 1. B, HIF-1α protein was examined by Western blot analysis (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 the Wilcoxon signed-rank test. D, immunofluorescence for HIF-1α (red signal), actin (green signal), and nuclei (blue signal) in endothelial cells from representative patients with multiple myeloma and MGUS, and control subjects. Merge (pink signal) shows colocalization of HIF-1α, nuclei, and actin. Top, merge for HIF-1α and nuclei in MMECs from relapsed/refractory patients. Middle, merge for HIF-1α and nuclei in MMECs at diagnosis and MGECs. Bottom, merge for HIF-1α and nuclei in normal endothelial cells 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.
HIF-1α induces overexpression of its proangiogenic targets

We wondered whether HIF-1α protein expression may result in the upregulation of its proangiogenic 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 hours (Fig. 2C, left graph), whereas the protein was reduced by 70% (as average) only at day 5 (Fig. 2C, right) suggesting that it was stabilized substantially in the MMECs. The HIF-1α silencing sizably 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 multiple myeloma 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 conditioned media 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 conditioned media of the HIF-1α siRNA MMECs gave poor angiogenesis (12 ± 5; Fig. 3C, right), which was similar to physiologic 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 antiangiogenic effect of the drugs (Supplementary Fig. S3). When HIF-1α was knocked down by siRNA, resistance to bortezomib or lenalidomide was overcome. 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 with HIF-1α siRNA alone (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 and −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 because 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 and 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, CI < 1) compared with 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 bone marrow cells. We observed that the conditioned media of the total bone marrow cells (BMCM) increased MMEC angiogenesis because of the proangiogenic factors and cytokines released by bone marrow cells (Supplementary Fig. S4A, right). Indeed, the direct inhibitory effect of panobinostat, on the isolated MMEC angiogenesis (Supplementary Fig. S4B, left), was partially subverted by the BMCM (Supplementary Fig. S4B, middle). However, when the total bone marrow cells were pretreated with panobinostat, their BMCM inhibited MMEC angiogenesis (Supplementary Fig. S4B, right). In the CAM assay, different from the conditioned media of untreated MMECs (vessel count = 37 ± 7; Fig. 5C, middle) the conditioned media of panobinostat-treated MMECs gave irrelevant angiogenesis (10 ± 5, P < 0.01; Fig. 5C, right), reequilibrating the vessel counts to physiologic angiogenesis (8 ± 3; Fig. 5C, left). Next, we wondered whether treatments with panobinostat impacted on the HIF-1α protein
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. (Continued on the following page.)
expression and/or activation: the drug did not reduce the protein levels (Fig. 5D, left), whereas 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%; and −68%), FGF-2 (−40%–70%; and −50%), and c-MET (−45%–70%; and −50%) transcription (Fig. 5D, right graph).

**HIF-1α siRNA and panobinostat induce similar changes in the MMEC proteome**

To further examine the inhibitory effects of HIF-1α siRNA and panobinostat at the molecular level, we compared the proteome of HIF-1α protein expressing MMECs following HIF-1α siRNA or panobinostat treatment. At least three two-dimensional electrophoresis (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 followed by database searching (Supplementary Fig. S4C and S4D). Eight proteins were downregulated by both HIF-1α siRNA and panobinostat (2-fold changes vs. control): glutathione S-transferase P1 (GSTP1), HSPB1, Annexin A4 (ANXA4), protein disulfide-isomerase A3, prolyl 4-hydroxylase subunit α-2, gastrin-releasing peptide (GRP), LIM and SH3 domain protein 1 (LASP1), and ANXA1; the polymerase I and transcript release factor was instead upregulated. These proteins govern cell shape, cell metabolism, chemotaxis, and angiogenesis (Supplementary Table S2).

The HIF-1α protein is stabilized in MMECs by 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 versus -negative MMECs of relapsed/refractory patients and of the other groups of the patients, or versus 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 OS in the former (Fig. 6D).

**Discussion**

Among hematologic malignancies, chronic lymphocytic leukemia (28), diffuse large B cell and follicular non-Hodgkin lymphomas (29), Hodgkin lymphoma (30), and multiple myeloma (10, 11) express HIF-1α in tumor cells. HIF-1α has been found in plasma cells cultured in normoxia of 28% of patients with multiple myeloma (10), and it was enhanced by plasma cell growth factors such as insulin-like growth factor-1 and interleukin-6 (11). Here, the role of HIF-1α in multiple myeloma 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 endothelial cells, 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. Because these MMECs had the protein in normoxic conditions one can hypothesize that it was regulated at posttranslational 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 either with expression of VHL or 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. Bone marrow angiogenesis is a constant hallmark of multiple myeloma 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 multiple myeloma angiogenesis, we evaluated the effects of its knockdown in the MMECs. HIF-1α mRNA was reduced by 90% at 72 hours after transfection, whereas the protein was reduced by 70% 5 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 and colleagues reported that human umbilical vein endothelial cells cultured in...
growth factors enriched medium form tube-like structures under both normoxia and hypoxia, whereas 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 multiple myeloma drug resistance. In fact MMECs expressing the HIF-1α protein showed resistance to bortezomib and lenalidomide compared with 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, an HDACI (i.e., an indirect HIF-1α inhibitor; ref. 18), which has already been shown to deliver potent in vitro (38, 39) and in vivo (38) anti-multiple myeloma activity. The deacetylase activity is needed for the transactivation potential of HIF-1α (18). We show, in fact, that

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 analysis (β-actin as loading control) and shown as 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 the Wilcoxon signed-rank test. D, Kaplan–Meier functions of 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 the log-rank test.
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 with 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 antiangiogenic activity of the drugs in a synergistic way. Others have shown that panobinostat was able to potentiate cytotoxic activity of bortezomib, dexamethasone, and melphalan in multiple myeloma 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 multidrug resistances (40). This provides tumor cells with a selective survival advantage over normal cells by enhancing drug efflux, and consequently decreasing the therapeutic efficacy of the drugs. In multiple myeloma 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 multiple myeloma plasma cells (43) and in resistant multiple myeloma 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 actin-binding cytoskeletal protein localized at focal adhesions along stress fibres, which regulates cell migration (45); and GRP that elicits endothelial cells migration and cord formation in vitro, and enhances angiogenesis in vivo (46).

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

In conclusion, HIF-1α 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

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Authors’ Contributions

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