Published OnlineFirst August 11, 2009; DOI: 10.1158/1535-7163.MCT-09-0150

Inhibition of hypoxia-inducible factor-1 function enhances the sensitivity of multiple myeloma cells to melphalan

Yongzhen Hu, Keita Kirito, Kozue Yoshida, Toru Mitsumori, Kei Nakajima, Yumi Nozaki, Satoshi Hamanaka, Takahiro Nagashima, Masae Kunitama, Kumi Sakoe, and Norio Komatsu

Department of Hematology and Oncology, University of Yamanashi, Yamanashi, Japan

Abstract

Abnormal activation of hypoxia-inducible factor-1 (HIF-1), one of the most important transcription factors for the adaptation of cells to hypoxia, is frequently observed in numerous types of solid tumors. Dysregulation of HIF-1 induces tumor angiogenesis and enhances the expression of anti-apoptotic proteins and glycolysis-associated enzymes in cancer cells, which in turn leads to the promotion of tumor growth. In the present study, we examined the pathophysiologic role of HIF-1 in multiple myeloma. Furthermore, we explored the possibility that HIF-1 may be a molecular target for myeloma therapy. We identified constitutive expression of the hypoxia-inducible factor-1 α (HIF-1α)-subunit in established myeloma cell lines and in primary myeloma cells. Treatment with insulin-like growth factor-1 (IGF-1) significantly increased HIF-1α expression through activation of the AKT and mitogen-activated protein kinase signaling pathways. Inhibition of HIF-1 function either by echinomycin, a specific HIF-1 inhibitor, or a siRNA against HIF-1α resulted in enhanced sensitivity to melphalan in myeloma cells. This inhibition of HIF-1 also reversed the protective effect of IGF-1 on melphalan-induced apoptosis. Inhibition of HIF-1 drastically reduced both basal and IGF-1–induced expression of survivin, one of the most important anti-apoptotic proteins in myeloma cells. We conclude that HIF-1 inhibition may be an attractive therapeutic strategy for multiple myeloma. [Mol Cancer Ther 2009;8(8):2329–38]

Received 2/23/09; revised 5/18/09; accepted 5/19/09; published OnlineFirst 8/11/09.

Grant support: Ministry of Health, Welfare, and Labor of Japan and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Technology of Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Requests for reprints: Keita Kirito, University of Yamanashi, 1110 Shimokato, Chuo-shi, Yamanashi 409-3898, Japan. Phone: 81-55-273-9432; Fax: 81-55-273-1274. E-mail: kirito@yamanashi.ac.jp

doi:10.1158/1535-7163.MCT-09-0150

Introduction

Despite new therapeutic approaches, multiple myeloma remains incurable. Myeloma cell intrinsic factors and microenvironmental stimuli contribute to the unfavorable biology of the disease (1). Cytogenetic abnormalities, including immunoglobulin H translocations, confer a growth advantage to myeloma cells through deregulated expression of cyclin D family proteins. Several cytokines and chemokines also support the growth and survival of myeloma cells, which ultimately leads to chemotherapy resistance. The biological functions of these intrinsic factors and microenvironmental stimuli are mediated by a series of transcription factors, including those of the nuclear factor κB, signal transducers and activators of transcription 3, and Forkhead transcription factor families (1). These transcription factors regulate the expression of anti-apoptotic proteins and enhance cytokine production, which in turn induces pathologic angiogenesis, osteoblast activation, and the growth of myeloma cells in an autocrine fashion.

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that serves as a master regulator of cellular responses to hypoxia (2) and regulates dozens of genes required for adaptation to hypoxia. HIF-1 is composed of two subunits, α-subunit and β-subunit. Although the expression of hypoxia-inducible factor-1 β (HIF-1β)-subunit is constitutive, HIF-1α protein levels are regulated in response to oxygen tension. Under normoxic conditions, HIF-1α is degraded by the proteasome, whereas HIF-1 remains inactive. In hypoxia, HIF-1α is stabilized and forms a complex with HIF-1β that allows HIF-1 to function as a transcription factor. Thus, HIF-1 is activated only during hypoxia under normal physiologic conditions. In contrast, HIF-1 is frequently activated in cancer cells, including under normoxic conditions (3), by oncogene products, impaired activity of tumor suppressor genes (4), or the accumulation of metabolic glucose intermediates (5, 6). Constitutively activated HIF-1 enhances neovascularization, increases glucose metabolism, and induces the expression of anti-apoptotic proteins in cancer tissues. HIF-1 is thought to be one of the most important molecular targets in the treatment of cancer (7), and a variety of chemical inhibitors for HIF-1 have been introduced to date (8).

In contrast to solid tumors, only a limited number of studies on the role of HIF-1 in hematologic malignancies have been published to date. HIF-1 is activated by cytokine stimuli in leukemic cells under normoxic conditions (9). HIF-1 regulates vascular endothelial growth factor production in leukemic cells, which supports cell survival in an intracellular autocrine fashion (9). HIF-1 has important functions in multiple myeloma. First, numerous important genes for the progression of multiple myeloma [i.e., vascular endothelial growth factor, stromal cell-derived factor-1, and myeloid cell

Published OnlineFirst August 11, 2009; DOI: 10.1158/1535-7163.MCT-09-0150

leukemia sequence-1 (MCL-1)] are putative targets of HIF-1 (3). Second, several transcription factors that play important roles in the pathophysiology of multiple myeloma regulate HIF-1 expression and function. signal transducers and activators of transcription 3 protects against HIF-1 degradation (10, 11). Several studies have shown that nuclear factor κB regulates HIF-1α transcription (12–14). Proteins from the Forkhead transcription factor family, which are often inactivated in myeloma cells, negatively regulate HIF-1 function (15). In addition to these basic findings, recent studies have proposed that HIF-1 may be a molecular target of certain new generation antimyeloma agents, including bortezomib (16) and heat shock protein 90 inhibitors (17). However, the biological significance of HIF-1 in multiple myeloma is under investigation, and to date, no study has investigated a new HIF-1–based strategy for multiple myeloma treatment.

We investigated the molecular function of HIF-1 in multiple myeloma cells using established myeloma cell lines and primary $CD138^+$ myeloma cells derived from patients. HIF-1 is constitutively activated in these cells, even under normoxic conditions, and insulin-like growth factor-1 (IGF-1), one of the most crucial growth factors for myeloma cells, was clearly shown to enhance HIF-1 levels. In addition, blocking HIF-1 function was found to heighten the sensitivity of myeloma cells to melphalan. Our observations suggest that inhibiting HIF-1 function may be a new and effective therapeutic approach for multiple myeloma.

Materials and Methods

Cell Culture

Human MM cell lines KMM-1, U266, and RPMI8226 were obtained from Japanese Collection of Research Bioresources and cultured in RPMI-1640 media (Sigma Chemical) containing 10% FCS, 25 U/mL penicillin, and 25 ng/mL streptomycin (GIBICO). For culture under hypoxic conditions, we used a hypoxic chamber (Juji Field).

Reagents

IGF-1 and interleukin 6 were purchased from PeproTech EC. LY294002 was from EMD Biosciences, Inc. U0126 was from Cell Signaling Technology. Echinomycin, melphalan, and dexamethasone were sourced from Sigma.

Preparation of Cell Lysates and Immunoblotting

Whole cell lysates or nuclear and cytosolic fractions of lysates were prepared as described elsewhere (9). Nuclear extracts were prepared as described previously (18). Lysate proteins were size fractionated by SDS-PAGE and then electroblotted onto polyvinylidene difluoride membranes. The blots were probed with the following antibodies: anti–HIF-1α, anti–BCL-X, and anti–BCL-2 (BD Transduction Laboratories); anti–phospho-AKT (Ser 473), anti-AKT, and anti–β-actin (Cell Signaling Technology); anti–MCL-1, antisurvivin, and anti-general transcription factor IIH (TFIIH; Santa Cruz); anti–HIF-1α (Novus Biologicals). The blots were visualized using a chemiluminescence detection kit (LumiGLO, Cell Signaling).

RNA Preparation and Real-Time PCR

Total cellular RNA was isolated using an RNeasy Mini Kit (Qiagen). Reverse transcription was done using an M-MLV reverse transcriptase kit (Invitrogen), according to the manufacturer's instructions. Quantitative analysis of survivin mRNA was done using predeveloped TaqMan assay reagents (Applied Biosystems). *18S RNA* was used as an internal control for real-time RT-PCR.

Table 1. Clinical features of patients with multiple myeloma

Patient no.	Age/Sex	Disease status, Durie-Salmon stage	Treatment	Paraprotein type
1	58/M	Refractory	VAD, endoxan	BJP, κ
2	69/M	Refractory	MP	IgG, λ
3	73/M	Newly diagnosed, stage II	—	IgM, κ
4	65/M	Newly diagnosed, stage II	—	IgG, κ
5	79/M	Newly diagnosed, stage II	—	IgG, κ
6	78/M	Newly diagnosed, stage II	—	IgG, κ
7	76/F	Newly diagnosed, stage III	—	IgA, κ
8	59/M	Newly diagnosed, stage III	—	IgG, κ

Abbreviations: VAD, vincristine, adriamycin, dexamethasone; MP, melphalan + prednisolone; M, male; F, female; Ig, immunoglobulin; BJP, Bence Jones protein.

Establishment of Stable HIF-1α Knockdown Cells

The pSuper HIF-1α/siRNA vector and empty pSuper vector were generous gifts from Dr. Daniel Chung (Massachusetts General Hospital, Harvard Medical School; ref. 19). To generate stable transfectants, KMM-1 cells were transfected and selected with 0.5 μg/mL puromycin (Sigma) for 14 d, and clones were selected and screened for HIF-1α silencing by immunoblot analysis.

Apoptosis Assay

Cellular apoptosis was determined using an Annexin V–Azami-Green Apoptosis detection kit (Medical and Biological Laboratories), according to the manufacturer's instructions. For primary myeloma cells, apoptotic cells were detected with the Hoechst staining method because of the limited number of cells.

Isolation of Primary Multiple Myeloma Cells and CD19-Positive Normal B Lymphocytes

This study was approved by the institutional review board of the University of Yamanashi. After informed consent, primary multiple myeloma cells were purified from bone marrow aspirates with anti-CD138 magnetic beads (Miltenyi Biotech) in accordance with the manufacturer's protocol. These cells were further analyzed using Wright-Giemsa staining, and a purity of better than 90% was confirmed. Clinical features are summarized in Table 1. CD19-positive B lymphocytes were collected from peripheral blood of healthy volunteers using anti-CD19 magnetic beads (Miltenyi Biotech) and used as normal B lymphocytes.

Published OnlineFirst August 11, 2009; DOI: 10.1158/1535-7163.MCT-09-0150

Immunofluorescence Microscopic Analysis

Immunofluorescence microscopic analysis for HIF-1α was done in accordance with previous reports (9). Images were obtained using an Olympus BX50 microscope (Olympus) and analyzed using the VB-7010 system (KEYENCE).

Luciferase Assay

A luciferase reporter plasmid vector containing hypoxia-responsive elements (20) was a generous gift from Dr. Shinae Kondo (University of Kyoto). The reporter plasmid was introduced into the myeloma cell lines by lipofection with the pRL-TKLuc internal control plasmid (Promega). Forty-eight hours after transfection, the cells were harvested and subjected to dual luciferase assay, according to the manufacturer's instructions (Promega).

Statistical Analysis

Statistical analysis was done using the Student's t test; $P < 0.05$ was considered statistically significant.

Results

HIF-1α Is Constitutively Expressed and IGF-1 Elevates Protein Levels in Multiple Myeloma Cells

Initially, we investigated the expression of HIF-1α by Western blotting in the three kinds of multiple myeloma-derived

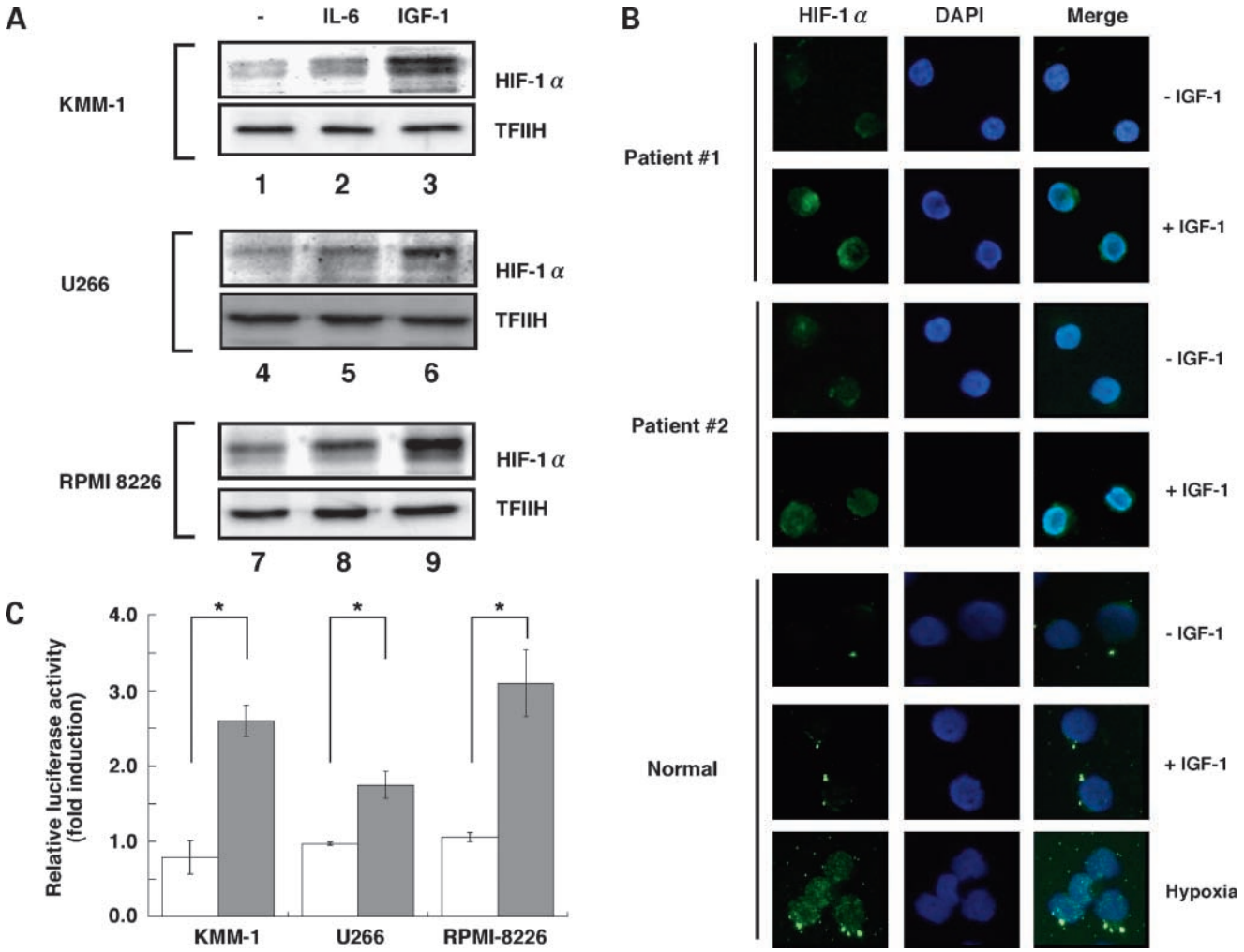

Figure 1. HIF-1α expression in multiple myeloma cells. **A,** after 24 h of serum starvation, the myeloma cell lines were treated with interleukin 6 (50 ng/mL) or IGF-1 (100 ng/mL) for 4 h, and nuclear extracts were prepared for the analysis of HIF-1α protein expression. **B,** $CD138^+$ cells were isolated from bone marrow samples of myeloma patients (patients 1 and 2 in Table 1). $CD19^+$ normal B lymphocytes were purified from the peripheral blood of healthy volunteers. Purified cells were then divided into two groups and cultured with or without IGF-1 (100 ng/mL) for 24 h. Normal B lymphocytes were also cultured under hypoxic conditions (1% O_2) for 24 h. HIF-1α expression was monitored using immunofluorescence microscopy. *Green*, HIF-1α *blue*, 4', 6-diamidino-2-phenylindole nuclear staining. **C,** pGL3-HRE-LUC reporter plasmids were transfected with pRL-TK Luc plasmid into myeloma cell lines. After 24 h in culture, the transfected cells were treated with 100 ng/mL of IGF-1 (*gray column*) or left untreated (*white column*) for an additional 24 h and then harvested to determine luciferase activity. The reporter activities were normalized to an internal control. Each column is the average and SE of the three independent experiments. *, $P < 0.05$.

Published OnlineFirst August 11, 2009; DOI: 10.1158/1535-7163.MCT-09-0150

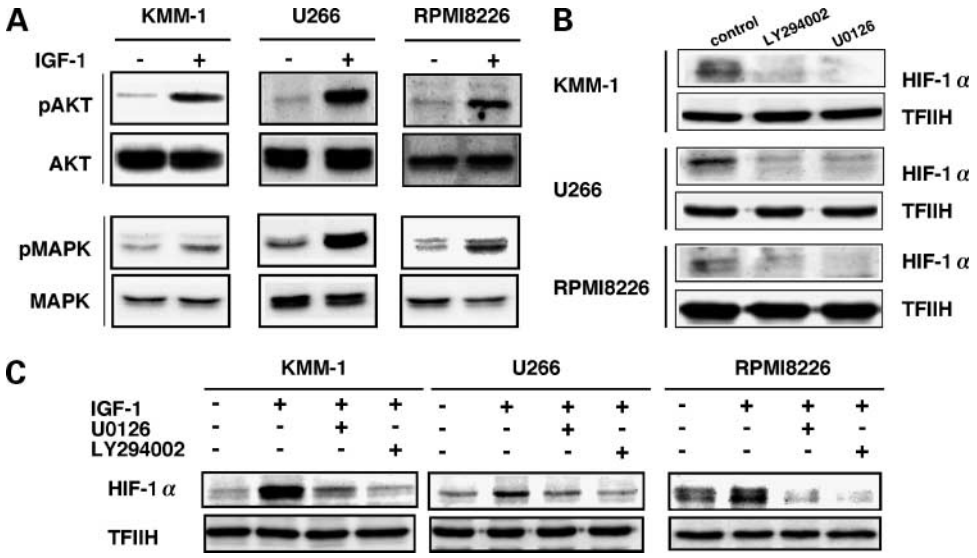

Figure 2. IGF-1 induces HIF-1α through PI3-K and MAPK activation. **A,** myeloma cells were serum starved for 24 h and then stimulated with IGF-1 (100 ng/mL) for 10 min. Whole cell lysates were prepared, and the activation of AKT and MAPK was analyzed by Western blotting using antiphospho antibodies for each protein. **B,** myeloma cells were cultured for 4 h with or without inhibitors (10 μmol/L of LY294002 or 10 μmol/L of U0126) and the expression of HIF-1α was analyzed by Western blotting. **C,** after 24 h of serum deprivation, myeloma cells were left untreated or cultured with U0126 (10 μmol/L) or LY294002 (10 μmol/L) for 1 h. Thereafter, the cells were treated with IGF-1 (100 mg/mL) for 4 h, and HIF-1α expression was monitored by Western blotting.

cell lines: KMM-1, U266, and RPMI8226. As shown in Fig. 1A (*lanes 1, 4,* and *7*), HIF-1α protein was expressed in all three myeloma cell lines under basal culture conditions. By contrast, normoxic HIF-1α expression was not observed in normal bone marrow mononuclear cells (data not shown). Because serum starvation may enhance HIF-1α expression (21), we compared expression of the protein in the presence or absence of serum and found that serum starvation did not enhance HIF-1α expression (Supplementary Fig. S1). We also analyzed HIF-1α expression in primary multiple myeloma cells isolated from bone marrow aspirates from two myeloma patients (patients 1 and 2 in Table 1) using immunofluorescence microscopy. $CD19^+$ B lymphocytes were isolated from healthy volunteers and used as the normal control. Primary myeloma cells expressed HIF-1α protein under basal conditions (Fig. 1B). In contrast, expression of the protein was not recorded in normal B lymphocytes under normoxic conditions, whereas culture of these cells under hypoxic conditions drastically induced expression of the protein. These results suggest that constitutive expression of HIF-1α is specific for multiple myeloma cells.

In addition to constitutive activation, it is well known that growth factors for cancer cells may enhance HIF-1 function in several cancer cell types. To confirm this in our system, we evaluated whether interleukin 6 and IGF-1, both known to be potent growth factors for myeloma cells, enhanced HIF-1α expression in these cells. As shown in Fig. 1A (*lanes 3, 6,* and *9*), IGF-1 significantly induced HIF-1α expression in all myeloma cell lines; by contrast, interleukin 6 exhibited only modest effects on HIF-1α expression levels (Fig. 1A, *lanes 2, 5,* and *8*). In accordance with the results from the established myeloma cell lines, HIF-1α protein was drastically up-regulated after culturing the primary myeloma cells with IGF-1 (Fig. 1B). We also found that IGF-1 failed to induce HIF-1α in normal B lymphocytes (Fig. 1B).

To confirm that IGF-1 not only induces the expression of HIF-1α but also activates HIF-1, we did a reporter assay using a reporter plasmid that contained hypoxia-responsive elements. As shown in Fig. 1C, treatment with IGF-1 significantly increased the transcriptional activity of HIF-1.

Phosphatidylinositol 3 Kinase (PI3-K) and Mitogen-Activated Protein Kinase (MAPK) Pathways Are Involved in Constitutive and IGF-1–Induced HIF-1α Expression in Multiple Myeloma Cells

We explored the molecular mechanisms that induce HIF-1α expression in myeloma cells. Signal transduction molecules, including AKT and MAPK, play important roles in HIF-1α expression under normoxic conditions (22, 23). Both signal transduction molecules play important functions in the growth and survival of multiple myeloma cells (24, 25). We focused our attention on these two signal transduction pathways. We found that both pathways are constitutively activated in multiple myeloma cell lines and that IGF-1 drastically enhanced the phosphorylation of these molecules (Fig. 2A). Based on these observations, we treated the cells with a chemical inhibitor for each signal transduction pathway: LY294002 to inhibit PI3-K and U0126 to inhibit MAPK. Both LY294002 and U0126 reduced the basal levels of HIF-1α expression in all three myeloma cell lines (Fig. 2B). These inhibitors also prevented IGF-1–induced HIF-1α accumulation in the myeloma cell lines (Fig. 2C). These results suggest that constitutive activation of PI3-K and MAPK in myeloma cells is required for basal HIF-1α expression in myeloma

Published OnlineFirst August 11, 2009; DOI: 10.1158/1535-7163.MCT-09-0150

cells and that significant activation of these pathways by IGF-1 can lead to enhanced HIF-1α expression in myeloma cell lines.

Inhibition of HIF-1 Enhances the Sensitivity of Myeloma Cells to Melphalan

We explored the effects of inhibiting HIF-1 functions on the growth and survival of multiple myeloma cells. We established stable HIF-1α knockdown KMM-1 subclones (1 and 12) using siRNA against HIF-1α. In these clones, baseline HIF-1α expression was strongly suppressed (Fig. 3A, comparing *lane 1* to *lanes 3* and *5*); however, expression of the β subunit of HIF-1 was not affected in these cells, indicating that the siRNA specifically blocked HIF-1α expression. In addition, IGF-1 did not induce HIF-1α elevation in the HIF-1α–silencing clones (Fig. 3A, comparing *lane 2* to *lanes 4* and *6*). These results suggest that the siRNA against HIF-1α functions efficiently. Initially, we compared growth of the clones with the growth of the parental cells. Although HIF-1α knockdown did not affect the growth or survival of KMM-1 cells under basal culture conditions (data not shown), these HIF-1α–silencing clones exhibited higher sensitivity to melphalan, one of the most common antimyeloma drugs, at 10 and 25 μmol/L compared with the parental KMM-1 cells (Fig. 3B). These results are consistent with several lines of evidence that the inhibition of HIF-1 functions may sensitize cancer cells to chemotherapeutic agents (26, 27).

HIF-1 Is Required for the Protective Effects of IGF-1 against Melphalan-Induced Apoptosis

Several studies have suggested that IGF-1 plays a key role in drug resistance in multiple myeloma cells and that the inhibition of IGF-1 signals may sensitize myeloma cells to melphalan in a mouse model (28–31). In addition, we found that IGF-1 significantly protects against melphalan-induced apoptosis in parental KMM-1 cells (Fig. 3C). Considering that IGF-1 drastically enhances HIF-1α levels in myeloma cells, we postulated that IGF-1 may exert its protective effects on melphalan-induced apoptosis through HIF-1 activation. To this end, we treated HIF-1α–silencing cells with melphalan in the presence or absence of IGF-1. As expected,

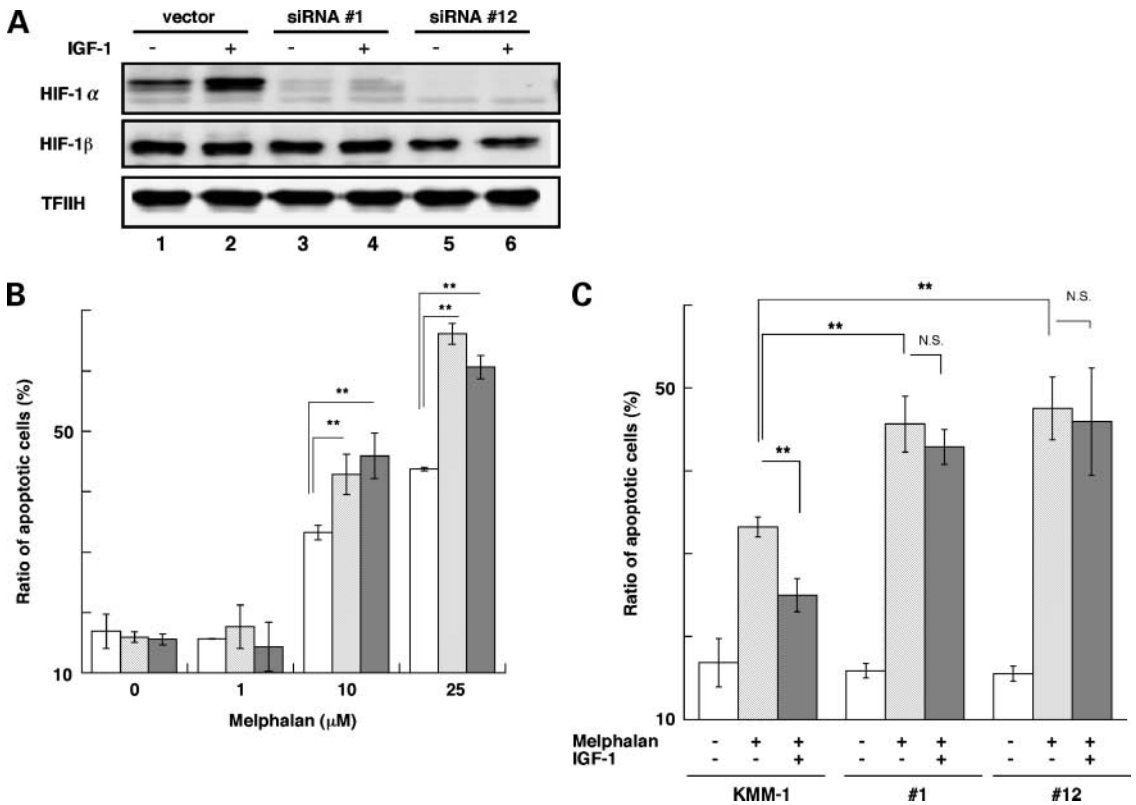

Figure 3. Stable HIF-1α–silencing KMM-1 cells showed increased sensitivity to melphalan-induced apoptosis. **A,** two independent stable HIF-1α knockdown KMM-1 cell lines were established. Parental KMM-1 cells and two HIF-1α knockdown clones (1 and 12) were cultured without serum for 24 h and then stimulated with IGF-1 (100 ng/mL) for 4 h. Nuclear extracts were prepared, and the expression levels of HIF-1α and β-subunit were examined by Western blotting. The membrane was reprobed with an anti-TFIIH antibody. **B,** parental KMM-1 and two independent HIF-1α knockdown clones were cultured with various concentrations of melphalan for 24 h, and Annexin V staining was done to analyze the percentage of apoptotic cells. Each column is the average of three independent experiments and their SEs. *White column*, parental KMM-1; *shaded column*, clone 1, and *black column*, clone #12. **, $P < 0.01$. **C,** parental KMM-1 cells and HIF-1α knockdown clones were cultured with 10 μmol/L melphalan with or without 24 h of IGF-1 pretreatment. Annexin V staining was used to detect apoptotic cells. Average numbers of apoptotic cells in three independent experiments are presented as columns with SE. *White columns*, control; *shaded columns*, melphalan; *gray column*, IGF-1 pretreatment plus melphalan. **, $P < 0.01$. N.S., not significant.

Published OnlineFirst August 11, 2009; DOI: 10.1158/1535-7163.MCT-09-0150

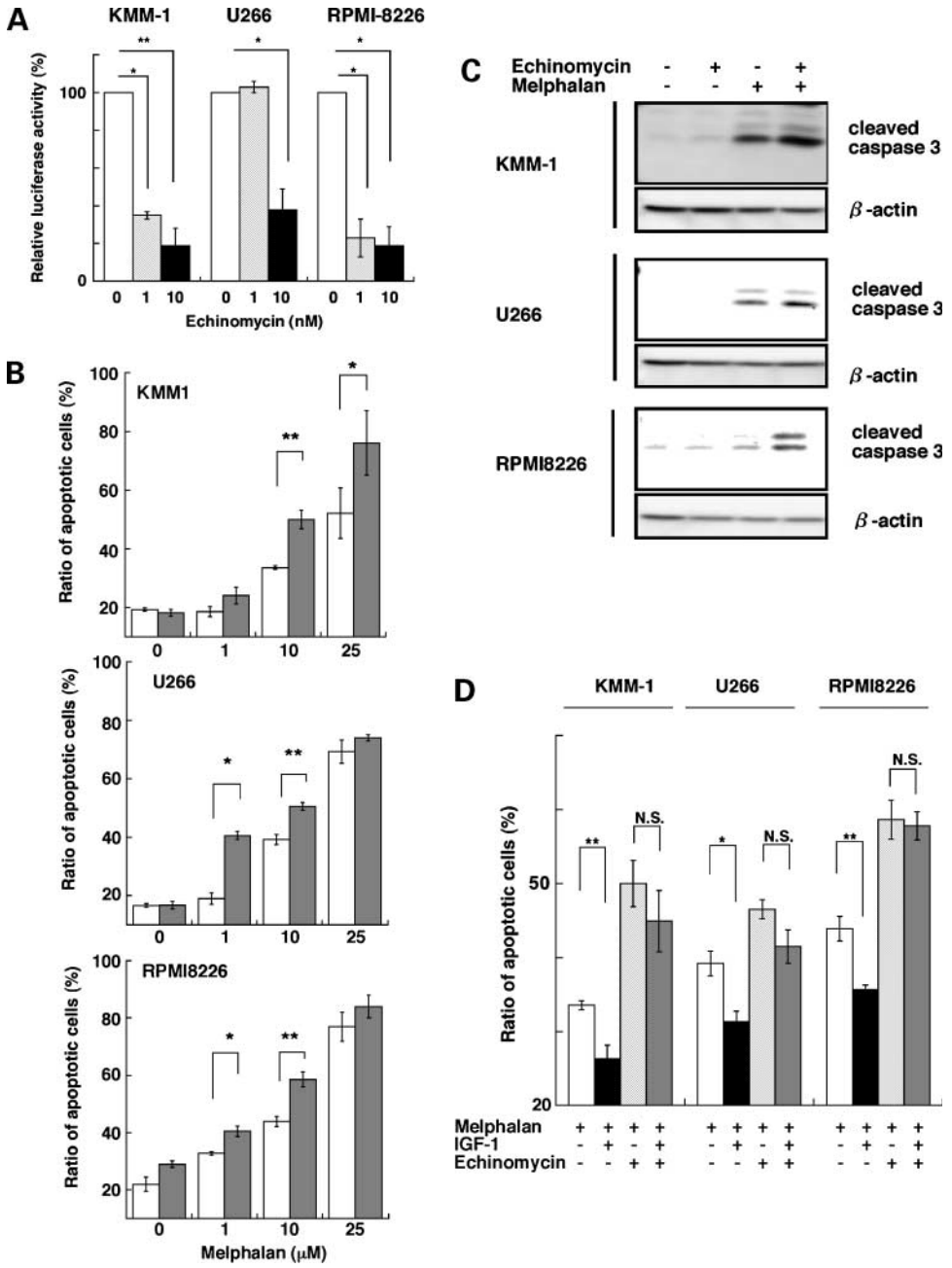

Figure 4. Inhibition of HIF-1 by echinomycin enhances the sensitivity of myeloma cells to melphalan. **A,** pGL3-HRE-LUC reporter plasmids were transfected with the pRL-TK Luc plasmid into myeloma cell lines. The transfected cells were cultured with the indicated concentrations of echinomycin for 48 h and then harvested to determine luciferase activity. The reporter activities were normalized to an internal control. Each column is the average and SEs of three independent experiments. *, $P < 0.05$; **, $P < 0.01$. **B,** each myeloma cell line was cultured with the indicated concentrations of melphalan either with (*gray column*) or without (*white column*) echinomycin (1 nmol/L for KMM-1 and RPMI8226, 10 nmol/L for U266) for 24 h, and the ratio of apoptotic cells was monitored by Annexin V staining. The average of the percentages of apoptotic cells from three independent experiments is shown together with the SE. *, $P < 0.05$; **, $P < 0.01$. **C,** KMM-1, U266, and RPMI8226 cell lines were cultured with echinomycin (1 nmol/L for KMM-1 and RPMI8226, 10 nmol/L for U266), melphalan (10 μmol/L), or echinomycin plus melphalan for 24 h, and total cell lysates were then prepared. Caspase-3 activation was examined by Western blotting using an anti–cleaved caspase-3 antibody. As an internal control, the membrane was reprobed with an anti–β-actin antibody. **D,** myeloma cells were cultured with or without IGF-1 (100 ng/mL) for 24 h, then treated with melphalan (10 μmol/L) or melphalan plus echinomycin (1 nmol/L for KMM-1 and RPMI8226, 10 nmol/L for U266) for an additional 24 h. The ratio of apoptotic cells was analyzed by flow cytometry using Annexin V staining. The columns are average ratios of apoptotic cells ±SEs of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

IGF-1 failed to protect the HIF-1α knockdown cells from melphalan-induced apoptosis (Fig. 3C), suggesting that HIF-1α up-regulation is a prerequisite for the anti-apoptotic effect of IGF-1 in myeloma cells.

Echinomycin, A Small-Molecule Inhibitor of HIF-1, Increases Melphalan-Induced Apoptosis in Myeloma Cells

Considering that HIF-1 inhibition may be a new strategy to increase the efficacy of melphalan and to overcome IGF-1–induced drug resistance in myeloma cells, we decided to explore the effects of echinomycin, which blocks the DNA binding activity of HIF-1 (32). First, inhibition of HIF-1 function by echinomycin was confirmed by a reporter assay with a vector containing hypoxia-responsive elements. We found that 1 nmol/L of echinomycin was sufficient to block HIF-1 activity in KMM-1 and RPMI8226 cells (Fig. 4A). By contrast, 10 nmol/L of the inhibitor was required to block HIF-1 function in U266 cells (Fig. 4A). Although it has been reported elsewhere that echinomycin enhances HIF-1α levels under normoxic conditions in the human HepG2 hepatoma cell line (33), we failed to observe HIF-1α elevation in the cell lines used in this study (data not shown). Based on these

Published OnlineFirst August 11, 2009; DOI: 10.1158/1535-7163.MCT-09-0150

observations, we used the inhibitor at 1 nmol/L for KMM-1 and RPMI8226 cells and at 10 nmol/L for the U266 cells throughout the study. We treated each myeloma cell line with echinomycin and with various concentrations of melphalan for 24 hours, and we monitored the ratio of apoptotic cells by using Annexin V staining. Consistent with siRNA silencing of the KMM-1 cells, treatment with echinomycin alone did not induce apoptosis in myeloma cell lines (Fig. 4B). However, echinomycin significantly enhanced melphalan-induced apoptosis in these cells (Fig. 4B). Similar results were obtained using a caspase-3 activation assay. Echinomycin alone did not significantly increase the level of caspase-3 cleavage in myeloma cells during the observation period. However, it drastically enhanced melphalan-induced caspase-3 cleavage (Fig. 4C). Importantly, the apoptosis-enhancing effects of echinomycin were not observed in HIF-1α–silencing clones (Supplementary Fig. S2). This result indicates that the inhibitor exerts its apoptosis-enhancing effects specifically through inhibition of HIF-1 activity. We also examined whether the inhibitor inhibited the anti-apoptotic effects of IGF-1 on melphalan-treated cells. As illustrated in Fig. 4D, IGF-1 pretreatment significantly reduced melphalan-induced apoptosis. However, adding echinomycin completely canceled the IGF-1 anti-apoptotic effect against melphalan-induced apoptosis in these cell lines. These results suggest that HIF-1 is an attractive agent to increase the antimyeloma efficacy of melphalan. Because we know that IGF-1 also decreases the response of myeloma cells to other cytotoxic chemotherapeutic reagents, that is, doxorubicin (31), we investigated whether the inhibitor also enhanced the response of myeloma cells to doxorubicin. We found that echinomycin also enhanced apoptosis after doxorubicin treatment and abrogated the protective effects of IGF-1 (Supplementary Fig. S3). In contrast, the inhibitor did not show these effects when treated with dexamethasone (data not shown).

Echinomycin Sensitizes Primary Myeloma Cells to Melphalan

To test the relevance of our findings, we used $CD138^+$ primary myeloma cells harvested from six patients at diagnosis (patients 3–8 in Table 1). The cells were cultured under several different conditions for 24 hours, and apoptotic cells were identified by Hoechst 33342 staining (Fig. 5). Consistent with the results from myeloma cell lines, 1 nmol/L echinomycin did not promote apoptosis in primary myeloma cells. However, melphalan induced apoptosis at significant levels in all purified myeloma cells. Furthermore, the addition of echinomycin enhanced the cytotoxic effect of melphalan against primary myeloma cells (Fig. 5). We also analyzed whether treatment with echinomycin enhanced the toxicity of melphalan to normal B lymphocytes. Consistent with the finding that HIF-1 is not activated in normal B lymphocytes (Fig. 1B), the inhibitor did not show apoptosis-enhancing effects on these cells. Together, these findings indicate that our hypothesis that the inhibition of HIF-1 function enhances the sensitivity of myeloma cells to melphalan may also apply to primary myeloma cells.

HIF-1 Induces the Anti-Apoptotic Protein Survivin in Myeloma Cells

We tried to identify downstream molecules that are directly involved in the anti-apoptotic effects of HIF-1 in myeloma cells. Under physiologic and pathologic conditions, HIF-1 regulates numerous anti-apoptotic genes,

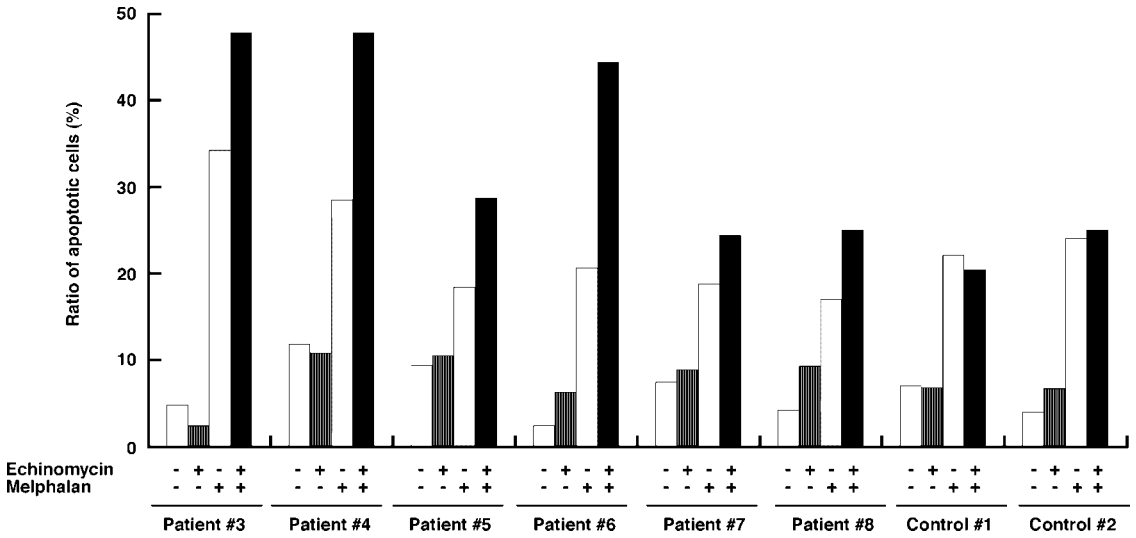

Figure 5. Echinomycin enhances melphalan-induced apoptosis in isolated primary $CD138^+$ myeloma cells to suggest a schematic model of the HIF-1 role in myeloma cells. Primary $CD138^+$ myeloma cells were isolated from bone marrow samples from multiple myeloma patients 3 through 8, as shown in Table 1. $CD19^+$ normal B lymphocytes were purified from peripheral blood from healthy volunteers (control 1 and 2). The cells were divided into four different groups and cultured under the indicated conditions for 24 h. Hoechst staining was done to monitor the apoptotic cell ratio. At least 50 cells were examined for each set of conditions, and the ratio of apoptotic cells was calculated and shown as a column. (*control*; *white*, 1 nmol/L echinomycin; *gray*, 10 μmol/L melphalan; *shaded*, 10 μmol/L melphalan plus 1 nmol/L echinomycin; *black*).

Published OnlineFirst August 11, 2009; DOI: 10.1158/1535-7163.MCT-09-0150

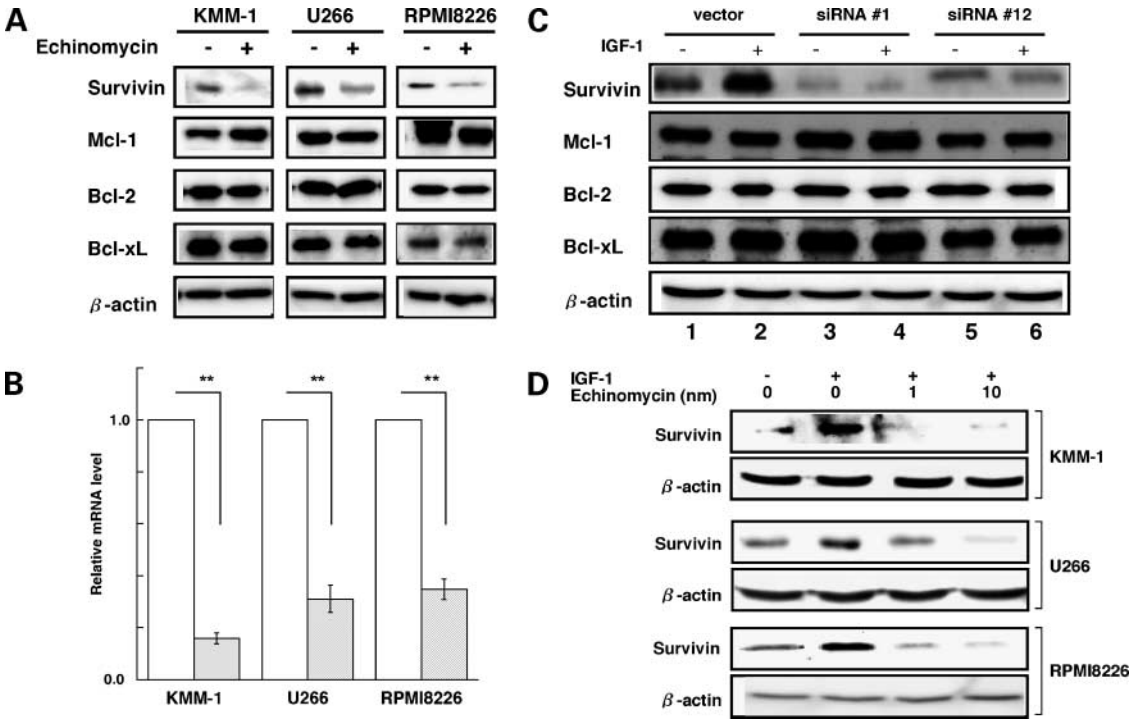

Figure 6. Survivin is a target of HIF-1 in multiple myeloma cells. **A**, multiple myeloma cells were cultured with or without echinomycin (1 nmol/L for KMM-1 and RPMI8226 cells and 10 nmol/L for U266 cells) for 24 h, and total cell lysates were prepared. Expression rates of survivin, MCL-1, BCL-xL, and BCL-2 were analyzed by Western blotting. **B,** myeloma cells were cultured with or without echinomycin for 12 h, and total RNA was prepared. The mRNA level of *survivin* was analyzed by quantitative RT-PCR. Each column is an average of *survivin* mRNA levels normalized to *18S rRNA* (internal control) ±SE of three experiments (*white columns*, without echinomycin; *shaded columns*, with echinomycin). **, $P < 0.01$. **C,** the parental KMM-1 cells and two HIF-1α knockdown clones were serum starved for 24 h and subsequently cultured with 100 ng/mL IGF-1 for 24 h. Total cell lysates were prepared, and Western blotting was done to analyze survivin, MCL-1, BCL-xL, and BCL-2 expression. As an internal control, the membrane was reprobed with an anti–β-actin antibody. **D,** after 24 h of serum starvation, myeloma cells were treated with the indicated concentrations of echinomycin for 1 h and then cultured with IGF-1 for an additional 24 h. Whole cell lysates were prepared for analysis of the survivin expression level.

including Bcl-2 family proteins (e.g., MCL-1) and survivin, a member of the inhibitor of apoptosis protein family (34). In addition, these proteins are known to be essential for myeloma cell survival. Thus, we focused our attention on these proteins as downstream effectors. As shown in Fig. 6A, echinomycin significantly suppressed the expression of survivin, whereas levels of MCL-1, BCL-2, and BCL-xL proteins were not affected in the three myeloma cell lines that we tested. Consistent with the down-regulation of survivin at the protein level, echinomycin suppressed *survivin* expression at the mRNA level (Fig. 6B), suggesting that survivin is transcriptionally regulated by HIF-1. To confirm this notion, we examined the expression of survivin in stable HIF-1α–silencing KMM-1–derived clones (1 and 12). In agreement with our previous results, basal levels of survivin were decreased in the HIF-1α–silencing clones (Fig. 6C, comparing *lane 1* to *lanes 3* and *5*), whereas expression levels of MCL-1, BCL-2, and BCL-xL were not altered. IGF-1–induced survivin expression was abrogated in stable HIF-1α–silencing clones (Fig. 6C, *lanes 2*, *4*, and *6*). In addition, IGF-1 treatment clearly enhanced survivin expression, and echinomycin significantly blocked IGF-1–dependent survivin expression (Fig. 6D).

Discussion

HIF-1 was originally identified as a master transcription factor that regulates cellular hypoxic responses (2). In addition to these primary functions, evidence suggests that this transcription factor plays a central role in cancer biology (3, 7). Recent studies have suggested that HIF-1 is also involved in the pathophysiology of multiple myeloma. Using a myeloma animal model, Asosingh et al. (35) showed that oxygen levels in the bone marrow microenvironment regulate microvessel density in bone marrow, a significant characteristic of the disease. They found that HIF-1 contributes to this process by enhancing vascular endothelial growth factor production in myeloma cells. Colla et al. (36) reported that reduced expression of the tumor-suppressor gene known as *ING-4* (inhibitor of growth family member 4) was correlated with enhanced angiogenesis in myeloma cells. Interestingly, they also showed that inhibition of ING-4 by siRNA induced HIF-1 activation and the subsequent production of angiogenic cytokines in myeloma cells. Although these studies also reported that HIF-1 is expressed in established murine and human myeloma cell lines, our study provides the first evidence that HIF-1α accumulates in primary $CD138^+$ myeloma cells under normoxic conditions.

Published OnlineFirst August 11, 2009; DOI: 10.1158/1535-7163.MCT-09-0150

In addition to the baseline activation of HIF-1, IGF-1 clearly enhances HIF-1α protein levels in established myeloma cell lines and in primary $CD138^+$ myeloma cells. These results suggest that HIF-1α lies downstream of the IGF-1 signaling pathway in myeloma cells. This is supported by the previous report that inhibition of the IGF-1 receptor suppresses HIF-1 activity in the human myeloma cell line MM-1S (31). IGF-1 plays important roles in the pathophysiology of multiple myeloma; it induces growth not only of established multiple myeloma cells but also of primary myeloma cells from patients *in vitro* (24, 25). This cytokine confers multiple drug resistance on myeloma cells (25, 30). Importantly, clinical studies have revealed that increases in serum IGF-1 levels may be correlated with poor outcomes of multiple myeloma (37). IGF-1 is also known to activate HIF-1 in several cancer cell lines. Fukuda et al. reported that exposing colon cancer cells to IGF-1 leads to activation of HIF-1 and results in vascular endothelial growth factor secretion (22). They proposed a model whereby IGF-1 regulates protein synthesis of the α-subunit of HIF-1 through the activation of the PI3-K/AKT and MAPK pathways (22). Elevation of HIF-1α by IGF-1 was also reported in retinal epithelial cells, and PI3-K and MAPK were also shown to be involved in this process (23). In accordance with these observations, our study found that inhibitors for PI3-K and MAPK can suppress IGF-1–induced HIF-1α elevation. In solid tumors, HIF-1 is believed to be one of the most important molecular targets, and numerous reagents that target HIF-1 have been introduced in this field (8). In addition to solid tumors, our results suggest that HIF-1 may be an attractive molecular target in multiple myeloma. Stable silencing of HIF-1α by siRNA caused increased sensitivity to melphalan and loss of the IGF-1 protective effects against apoptosis. Moreover, we used a pharmacologic inhibitor, echinomycin, to block HIF-1 functions. The inhibitory function of this reagent is confirmed by *in vitro* and *in vivo* studies (38). In agreement with studies using siRNA against HIF-1α, echinomycin clearly sensitized myeloma cells to conventional chemotherapeutic agents and abrogated the protective effect of IGF-1 on melphalan-induced apoptosis. Inhibition of HIF-1 also enhanced the efficiency of doxorubicin, another key chemotherapeutic reagent for multiple myeloma (Supplementary Fig. S3). In addition to strategies that directly block HIF-1 function (i.e., siRNA, specific pharmacologic inhibitors), recent studies highlight the way in which several new-generation antimyeloma reagents already used in clinical settings exhibit anti–HIF-1 activity. Bortezomib is one of the most important new-generation agents in the treatment of myeloma (39). Although the precise molecular mechanism remains unclear, bortezomib inhibits the transcriptional activity of HIF-1 in cervical cancer cells (40), breast cancer cells (41), and myeloma cells (16). Inhibitors of heat shock protein 90 are also effective in controlling multiple myeloma (17). Heat shock protein 90 is a molecular chaperone that stabilizes numerous proteins. Interestingly, Issacs et al. (42) reported that heat shock protein 90 regulates HIF-1α stability. Our preliminary observations also suggest that 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin, a heat shock protein 90 inhibitor that exhibits antimyeloma effects (43), clearly reduces basal and IGF-induced HIF-1α expression levels in myeloma cell lines. Although the pathway remains speculative at this stage, HIF-1 may be an important molecular target of heat shock protein 90 inhibitors in multiple myeloma. Bortezomib or heat shock protein 90 inhibitors may enhance the cytotoxicity of conventional chemotherapeutic agents, including melphalan through the inhibition of HIF-1 transcriptional activity.

Finally, we also explored the mechanism by which HIF-1 inhibition sensitizes myeloma cells to melphalan. Although HIF-1 regulates dozens of genes, we chose to focus on the expression of anti-apoptotic proteins that are known to be important for myeloma cell survival. We found that echinomycin or siRNA against HIF-1α specifically blocked both basal and IGF-1–induced survivin levels. Survivin is widely expressed in many types of established myeloma cell lines and contributes to cell survival (28, 44). Romagnoli et al. (44) showed that siRNA-mediated inhibition of survivin expression sensitizes myeloma cells to conventional antimyeloma agents, including melphalan. Furthermore, Peng et al. found that HIF-1 regulates survivin expression in breast cancer cells (34). Although we did not show direct evidence that survivin mediates a HIF-1 survival effect in myeloma cells, survivin may serve as a downstream molecular target of this HIF-1 inhibition strategy. If so, the combination of antimyeloma agents and a survivin inhibitor may be a promising therapeutic strategy for multiple myeloma.

In summary, this is the first study to show the relevance of HIF-1 in multiple myeloma cells. Inhibition of HIF-1 function enhanced the antimyeloma cytotoxicity of melphalan and also abrogated the survival effects of IGF-1 against melphalan-induced apoptosis in myeloma cells. Our results suggest that the inhibition of HIF-1 function can be used as an attractive therapeutic approach to overcome the unfavorable biology of myeloma cells and improve patient outcomes in multiple myeloma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Mirai Iwaya, Mariko Ozawa, Hidemi Yamazoe, and Naoko Enya for their technical assistance.

References

1. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC. Advances in biology of multiple myeloma: clinical applications. Blood 2004;104:607–18.

2. Semenza GL. Life with oxygen. Science 2007;318:62–4.

3. Rankin EB, Giaccia AJ. The role of hypoxia-inducible factors in tumorigenesis. Cell Death Differ 2008;15:678–85.

4. Zundel W, Schindler C, Haas-Kogan D, et al. Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev 2000;14:391–6.

5. Pollard PJ, Spencer-Dene B, Shukla D, et al. Targeted inactivation of fh1 causes proliferative renal cyst development and activation of the hypoxia pathway. Cancer Cell 2007;11:311–9.

Published OnlineFirst August 11, 2009; DOI: 10.1158/1535-7163.MCT-09-0150

6. Selak MA, Armour SM, MacKenzie ED, et al. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-α prolyl hydroxylase. Cancer Cell 2005;7:77–85.

7. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003; 3:721–32.

8. Semenza GL. Evaluation of HIF-1 inhibitors as anticancer agents. Drug Discov Today 2007;12:853–9.

9. Kirito K, Fox N, Komatsu N, Kaushansky K. Thrombopoietin enhances expression of vascular endothelial growth factor (VEGF) in primitive hematopoietic cells through induction of HIF-1α. Blood 2005;105: 4258–63.

10. Jung JE, Lee HG, Cho IH, et al. STAT3 is a potential modulator of HIF-1-mediated VEGF expression in human renal carcinoma cells. FASEB J 2005;19:1296–8.

11. Xu Q, Briggs J, Park S, et al. Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways. Oncogene 2005;24:5552–60.

12. Belaiba RS, Bonello S, Zahringer C, et al. Hypoxia up-regulates HIF-1α transcription by involving phosphatidylinositol 3-kinase and nuclear factor κB in pulmonary artery smooth muscle cells. Mol Biol Cell 2007; 18:4691–7.

13. Rius J, Guma M, Schachtrup C, et al. NF-κB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1α. Nature 2008;453:807–11.

14. van Uden P, Kenneth NS, Rocha S. Regulation of hypoxia-inducible factor-1α by NF-κB. Biochem J 2008;412:477–84.

15. Emerling BM, Weinberg F, Liu JL, Mak TW, Chandel NS. PTEN regulates p300-dependent hypoxia-inducible factor 1 transcriptional activity through Forkhead transcription factor 3a (FOXO3a). Proc Natl Acad Sci U S A 2008;105:2622–7.

16. Shin DH, Chun YS, Lee DS, Huang LE, Park JW. Bortezomib inhibits tumor adaptation to hypoxia by stimulating the FIH-mediated repression of hypoxia-inducible factor-1. Blood 2008;111:3131–6.

17. Mitsiades CS, Mitsiades NS, McMullan CJ, et al. Antimyeloma activity of heat shock protein-90 inhibition. Blood 2006;107:1092–100.

18. Kirito K, Uchida M, Yamada M, Miura Y, Komatsu N. A distinct function of STAT proteins in erythropoietin signal transduction. J Biol Chem 1997;272:16507–13.

19. Mizukami Y, Li J, Zhang X, Zimmer MA, Iliopoulos O, Chung DC. Hypoxia-inducible factor-1-independent regulation of vascular endothelial growth factor by hypoxia in colon cancer. Cancer Res 2004;64: 1765–72.

20. Ogura M, Shibata T, Yi J, et al. A tumor-specific gene therapy strategy targeting dysregulation of the VHL/HIF pathway in renal cell carcinomas. Cancer Sci 2005;96:288–94.

21. Fang Y, Sullivan R, Graham CH. Confluence-dependent resistance to doxorubicin in human MDA-MB-231 breast carcinoma cells requires hypoxia-inducible factor-1 activity. Exp Cell Res 2007;313:867–77.

22. Fukuda R, Hirota K, Fan F, Jung YD, Ellis LM, Semenza GL. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. J Biol Chem 2002;277:38205–11.

23. Treins C, Giorgetti-Peraldi S, Murdaca J, Semenza GL, Van Obberghen E. Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. J Biol Chem 2002;277:27975–81.

24. Ge NL, Rudikoff S. Insulin-like growth factor I is a dual effector of multiple myeloma cell growth. Blood 2000;96:2856–61.

25. Qiang YW, Kopantzev E, Rudikoff S. Insulinlike growth factor-I signaling in multiple myeloma: downstream elements, functional correlates, and pathway cross-talk. Blood 2002;99:4138–46.

26. Li L, Lin X, Shoemaker AR, Albert DH, Fesik SW, Shen Y. Hypoxia-inducible factor-1 inhibition in combination with temozolomide treatment exhibits robust antitumor efficacy *in vivo*. Clin Cancer Res 2006;12: 4747–54.

27. Liu F, Wang P, Jiang X, et al. Antisense hypoxia-inducible factor 1α gene therapy enhances the therapeutic efficacy of doxorubicin to combat hepatocellular carcinoma. Cancer Sci 2008;99:2055–61.

28. Mitsiades CS, Mitsiades N, Poulaki V, et al. Activation of NF-κB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. Oncogene 2002;21:5673–83.

29. Oh SH, Jin Q, Kim ES, Khuri FR, Lee HY. Insulin-like growth factor-I receptor signaling pathway induces resistance to the apoptotic activities of SCH66336 (lonafarnib) through Akt/mammalian target of rapamycin-mediated increases in survivin expression. Clin Cancer Res 2008;14: 1581–9.

30. Xu F, Gardner A, Tu Y, Michl P, Prager D, Lichtenstein A. Multiple myeloma cells are protected against dexamethasone-induced apoptosis by insulin-like growth factors. Br J Haematol 1997;97:429–40.

31. Mitsiades CS, Mitsiades NS, McMullan CJ, et al. Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. Cancer Cell 2004;5:221–30.

32. Kong D, Park EJ, Stephen AG, et al. Echinomycin, a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity. Cancer Res 2005;65:9047–55.

33. Vlaminck B, Toffoli S, Ghislain B, Demazy C, Raes M, Michiels C. Dual effect of echinomycin on hypoxia-inducible factor-1 activity under normoxic and hypoxic conditions. Febs J 2007;274:5533–42.

34. Peng X-H, Karna P, Cao Z, Jiang B-H, Zhou M, Yang L. Cross-talk between epidermal growth factor receptor and hypoxia-inducible factor-1{α} signal pathways increases resistance to apoptosis by up-regulating survivin gene expression. J Biol Chem 2006;281:25903–14.

35. Asosingh K, De Raeve H, de Ridder M, et al. Role of the hypoxic bone marrow microenvironment in 5T2MM murine myeloma tumor progression. Haematologica 2005;90:810–7.

36. Colla S, Tagliaferri S, Morandi F, et al. The new tumor-suppressor gene inhibitor of growth family member 4 (*ING4*) regulates the production of proangiogenic molecules by myeloma cells and suppresses hypoxia-inducible factor-1 α (HIF-1α) activity: involvement in myeloma-induced angiogenesis. Blood 2007;110:4464–75.

37. Standal T, Borset M, Lenhoff S, et al. Serum insulinlike growth factor is not elevated in patients with multiple myeloma but is still a prognostic factor. Blood 2002;100:3925–9.

38. Cairns RA, Papandreou I, Sutphin PD, Denko NC. Metabolic targeting of hypoxia and HIF1 in solid tumors can enhance cytotoxic chemotherapy. Proc Natl Acad Sci U S A 2007;104:9445–50.

39. Richardson PG, Barlogie B, Berenson J, et al. A phase 2 study of bortezomib in relapsed, refractory myeloma. N Engl J Med 2003;348: 2609–17.

40. Birle DC, Hedley DW. Suppression of the hypoxia-inducible factor-1 response in cervical carcinoma xenografts by proteasome inhibitors. Cancer Res 2007;67:1735–43.

41. Kaluz S, Kaluzova M, Stanbridge EJ. Proteasomal inhibition attenuates transcriptional activity of hypoxia-inducible factor 1 (HIF-1) via specific effect on the HIF-1α C-terminal activation domain. Mol Cell Biol 2006; 26:5895–907.

42. Isaacs JS, Jung YJ, Mimnaugh EG, Martinez A, Cuttitta F, Neckers LM. Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 α-degradative pathway. J Biol Chem 2002;277: 29936–44.

43. Chatterjee M, Jain S, Stuhmer T, et al. STAT3 and MAPK signaling maintain overexpression of heat shock proteins 90{α} and {β} in multiple myeloma cells, which critically contribute to tumor-cell survival. Blood 2007;109:720–8.

44. Romagnoli M, Trichet V, David C, et al. Significant impact of survivin on myeloma cell growth. Leukemia 2007;21:1070–8.

Published OnlineFirst August 11, 2009; DOI: 10.1158/1535-7163.MCT-09-0150

Molecular Cancer Therapeutics

Inhibition of hypoxia-inducible factor-1 function enhances the sensitivity of multiple myeloma cells to melphalan

Yongzhen Hu, Keita Kirito, Kozue Yoshida, et al.

Mol Cancer Ther 2009;8:2329-2338. Published OnlineFirst August 11, 2009.