Hypoxia Promotes Dissemination and Colonization in New Bone Marrow Niches in Waldenström Macroglobulinemia

Barbara Muz1, Pilar de la Puente1, Feda Azab1, Irene M. Ghobrial2, and Abdel Kareem Azab1

Abstract

Waldenström macroglobulinemia, a rare and indolent type of non–Hodgkin lymphoma, is characterized by widespread lymphoplasmacytic B cells in the bone marrow. Previous studies have shown that hypoxic conditions play a key role in the dissemination of other hematologic malignancies. In this study, the effect of hypoxia was tested on the progression and spread of Waldenström macroglobulinemia. Interestingly, tumor progression correlated with hypoxia levels in Waldenström macroglobulinemia cells and other cells in the bone marrow and correlated with the number of circulating tumor cells in vivo. Mechanistic studies demonstrated that hypoxia decreased cell progression and cell cycle, did not induce apoptosis, and reduced the adhesion between Waldenström macroglobulinemia cells and bone marrow stroma, through downregulation of E-cadherin expression, thus explaining increased egress of Waldenström macroglobulinemia cells to the circulation. Moreover, hypoxia increased the extravasation and homing of Waldenström macroglobulinemia cells to new bone marrow niches in vivo, by increased CXCR4/SDF-1–mediated chemotaxis and maintaining the VLA4-mediated adhesion. Re-oxygenation of hypoxic Waldenström macroglobulinemia cells enhanced the rate of proliferation and cell cycle progression and restored intercellular adhesion between Waldenström macroglobulinemia cells and bone marrow stroma. This study suggests that targeting hypoxic response is a novel strategy to prevent dissemination of Waldenström macroglobulinemia.

Implications: This study provides a better understanding of the biology of dissemination of Waldenström macroglobulinemia and opens new windows for investigation of new therapeutic targets in Waldenström macroglobulinemia based on tumor hypoxia mechanisms. Mol Cancer Res; 13(2); 263–72. ©2014 AACR.

Introduction

Waldenström macroglobulinemia is a rare low-grade B-cell lymphoma characterized by abnormal lymphoplasmacytic cells that overproduce monoclonal immunoglobulins M (IgM) and are spread widely in the bone marrow (1). The Waldenström macroglobulinemia cells infiltrate specifically into the bone marrow niche which implies the critical role of the bone marrow as a supportive microenvironment for the Waldenström macroglobulinemia cells due to the various interactions (2–4). We have previously shown in Waldenström macroglobulinemia that the bone marrow milieu promotes tumor progression including cell proliferation, survival, and drug resistance through production of cytokines, regulation of cell interaction, cell adhesion with stroma and endothelial cells, and cell trafficking (3, 5–7). We have also shown before that the bone marrow microenvironment plays a crucial role in the progression and drug resistance of other hematologic malignancies including multiple myeloma (8–10) and chronic myeloid leukemia (CML; refs. 11, 12).

Hypoxia plays an important role in the progression and dissemination of hematologic malignancies (12–16). Tumor hypoxia activates adaptive transcriptional programs, including HIF1α, that promote cell survival, motility, invasiveness, drug resistance, and neoangiogenesis in multiple myeloma (16, 17), associated with more aggressive tumor (18). We have shown that hypoxia induces egress of multiple myeloma cells from the bone marrow to the circulation and facilitates their homing to the new bone marrow niches in myeloma. These processes involved activation of epithelial-to-mesenchymal transition (EMT) machinery including decreased expression of E-cadherin and increased expression of CXCR4 in multiple myeloma cells (13). However, the role of hypoxia in the progression of Waldenström macroglobulinemia in particular and lymphoma in general was not assessed before. Moreover, the role of hypoxia in the progression of hematologic malignancies aside from cell trafficking including important cell processes, such as proliferation, apoptosis, cell cycle, and cell signaling, was never studied.

In this study, we focused on the role of hypoxia in dissemination of Waldenström macroglobulinemia cells including the cell–cell interactions in the bone marrow and egress to the circulation, chemotaxis, and homing to new bone marrow niches, adaptation of hypoxic Waldenström macroglobulinemia cells in the new bone marrow milieu, as well as hypoxic effects on proliferation, survival, apoptosis, and cell cycle in Waldenström macroglobulinemia cells.
Materials and Methods

Reagents
Stromal-derived growth factor (SDF-1) for adhesion and chemotaxis assays was purchased from R&D. Transwell plates for chemotaxis assay were obtained from Corning. E-Cadherin blocking antibody and human fibronectin were purchased from EMD Millipore. Total protein concentration for immunoblotting was assessed by Quick Start Bradford dye reagent (BioRad). MTT solution for cell viability assay was purchased from Sigma-Aldrich. Annexin V/propidium iodide (PI) for apoptosis assay and BD BioCoat Human Fibronectin plates for adhesion assay were purchased from BD Biosciences. Cell trackers including calcein-AM, DiO, and DiD were purchased from Invitrogen (Life Technologies). Hypoxia marker pimonidazole hydrochloride (PIM) and its correlating antibody were obtained from HypoxyProbe. A 10× red blood cell (RBC) buffer was obtained from BioLegend.

Cell culture
The Waldenström macroglobulinemia cell lines (BCWM.1, BCWM.1-mCherry, MWCL.1) and HS-5 stromal cells used in this study were a kind gift from Dr. Irene Ghobrial from Dana-Farber Cancer Institute (Boston, MA). Waldenström macroglobulinemia cell lines were cultured in RPMI-1640 (Corning CellGro, Mediatech) supplemented with 10% FBS (Gibco, Life Technologies). 2 mmol/L of L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (CellGro, Mediatech). HS-5 cells were cultured in 20% FBS DMEM (Corning CellGro, Mediatech) containing 1-glutamine and penicillin/streptomycin. Cells were incubated at 37°C under normoxic conditions (21% O₂) in the NuAire Water Jacket Incubator or hypoxic conditions (1% O₂) in the hypoxic chamber from Coy for 24 hours. In some cases, hypoxic Waldenström macroglobulinemia cells were re-oxygenated by exposing them to normal oxygen levels.

Animals
SCID-beige mice (female; 7 weeks old) were obtained from Taconic Farms and Balb/C mice from Charles Rivers Laboratories. Approval for these studies was obtained from the Ethical Committee for Animal Experiments at Washington University in St. Louis Medical School (St. Louis, MO).

Effect of tumor progression on hypoxia in Waldenström macroglobulinemia bone marrow
BCWM.1 cells were genetically engineered to express mCherry fluorescent protein and luciferase (Luc), as described previously (5). Human BCWM.1-mCherry cells were injected into 12 SCID mice intravenously at different concentrations of 0.5 × 10⁶, 1 × 10⁶, and 2.5 × 10⁶ cells per mouse (4 mice per condition) and allowed to grow for 3 weeks. Mice were treated with PIM (100 mg/kg) for 4 hours, sacrificed and their bone marrow and peripheral blood were extracted. Mononuclear cells (MNC) were isolated, washed, fixed, permeabilized, and stained with anti-PIM antibody conjugated to allophycocyanin (APC) antibody. Waldenström macroglobulinemia cells were detected by flow cytometry by gating on mCherry-positive cells, and bone marrow stromal cells were mCherry-negative, and hypoxia in these cells was measured as mean fluorescence intensity (MFI) of APC. Circulating Waldenström macroglobulinemia cells were detected by gating on mCherry-positive cells in the peripheral blood samples after RBC lysis.

Effect of hypoxia on extravasation of Waldenström macroglobulinemia cells
BCWM.1-mCherry cells were exposed to normoxia or hypoxia for 24 hours and each population was injected via tail vein into 4 Balb/C mice (1 × 10⁶ cells per mouse). Fifty microliters of blood was collected from the portal vein at 5, 15, and 30 minutes postinjection, RBCs were lysed, and MNCs were labeled with CD45-APC-Cy7 (BD Biosciences) for 1 hour on ice and analyzed by flow cytometry. The number of circulating Waldenström macroglobulinemia cells was analyzed as the percentage of CD45⁺/mCherry⁺ of the total CD45⁺ population. The absence of these cells from the circulation reflected extravasation of the Waldenström macroglobulinemia cells.

Effect of hypoxia on homing of Waldenström macroglobulinemia cells to the bone marrow
BCWM.1-mCherry cells were first stained with DiD or DiO (5 µL per 1 mL medium) for 2 hours at 37°C, washed, and then exposed to normoxia (1 flash with DiD and 1 flash with DiO) or hypoxia (1 flash with DiD and 1 flash with DiO) for 24 hours. Two suspensions of cells were prepared: normoxic DiD mixed with hypoxic DiO and normoxic DiO mixed with hypoxic DiD. Each cell mix was injected into 3 SCID mice (6 mice in total) at a concentration of 3 × 10⁶ cells per mouse. After 24 hours postinjection, the mice were sacrificed and the MNCs were isolated from the femurs, filtered, stained with DAPI (Sigma-Aldrich), washed and analyzed by the flow cytometry. Viable cells were gated as the population with negative DAPI staining, and the number of mCherry⁺/DiD⁺ or mCherry⁺/DiO⁺ cells was analyzed in each mouse. The number of positive cells reflected the number of Waldenström macroglobulinemia cells that homed to the bone marrow. The numbers of DiD versus DiO cells in each animal were compared, and the results were normalized to the average of the number of normoxic cells in all 6 mice. The purpose of the alternative staining was to eliminate the effect of the different fluorescent labels on the detection sensitivity of the cells in the bone marrow.

The effect of hypoxia on expression of E-cadherin, CXCR4, and VLA-4 using flow cytometry
BCWM.3 cells (1 × 10⁶) were exposed to normoxic or hypoxic conditions for 24 hours, then aspirated, incubated on ice for 10 minutes, washed with PBS, and stained on ice with primary anti-human CXCR4-APC antibody (BD Biosciences), mouse-anti-human VLA-4 antibody (BD Biosciences), or rabbit-anti-human E-cadherin antibody (Cell Signaling Technologies), followed by staining with secondary anti-mouse or anti-rabbit conjugated with fluoroecein isothiocyanate (FITC) antibody (Cell Signaling Technologies) for 1 hour. The cells were then washed with 1 × PBS and analyzed by flow cytometry.

The effect of hypoxia on cell signaling using immunoblotting
BCWM.1 cells (5 × 10⁶) were exposed to normoxic or hypoxic conditions for 24 hours or re-oxygenated for 24 hours, aspirated, incubated on ice for 10 minutes, washed with 1 × PBS, and lysed. Protein concentration in the cell lysates was normalized and 80 µg of protein was loaded per lane. Electrophoresis was performed using 8% or 10% precast Novex Tris-Clycine gels (Novex) and transferred to a nitrocellulose membrane using iBlot (Invitrogen, Life Technologies). Membranes were blocked with 5% nonfat dry milk in Tris-Buffered Saline/Tween20 (TBST) buffer and incubated with primary antibodies detecting E-cadherin, HIF1α, HIF2α,
Role of Hypoxia in Waldenström Macroglobulinemia

The effect of hypoxia on cell proliferation and viability

Cell growth and cell viability were measured by using Vi-Cell Viability Analyzer (Beckman Coulter) which provides the exact number of viable cells counted and averaged from 100 fields providing the percentile of viable cells. Cell number and viability of BCWM.1 and MWCL.1 cells were registered at time 0 (providing the percentile of viable cells. Cell number and viability of BCWM.1 and MWCL.1 cells were registered at time 0 (providing the percentile of viable cells. Cell number and viability of BCWM.1 and MWCL.1 cells were registered at time 0 (providing the percentile of viable cells. Cell number and viability of BCWM.1 and MWCL.1 cells were registered at time 0 (providing the percentile of viable cells. Cell number and viability of BCWM.1 and MWCL.1 cells were registered at time 0 (providing the percentile of viable cells.

The effect of hypoxia on apoptosis

BCWM.1 and MWCL.1 cells were cultured in normoxic or hypoxic conditions for 24 hours. The cells were then washed twice with ice-cold PBS, and re-suspended in 100 μL Annexin binding buffer. The cells were then stained with 5 μL of Annexin V for 15 minutes followed by staining with 5 μL of PI for 15 minutes at room temperature. A total of 400 μL of 1× binding buffer was added and the cells were analyzed by flow cytometry.

The effect of hypoxia on cell cycle

BCWM.1 and MWCL.1 cells were cultured in normoxic or hypoxic conditions for 24 hours or were re-oxygenated for the next 24 hours. First, the cells were aspirated and kept on ice for 10 minutes, washed twice with ice-cold PBS, and resuspended in 100 μL Annexin binding buffer. The cells were then stained with 5 μL of Annexin V for 15 minutes followed by staining with 5 μL of PI for 15 minutes at room temperature. A total of 400 μL of 1× binding buffer was added and the cells were analyzed by flow cytometry.

The effect of hypoxia on adhesion to stromal cells and fibronectin

Monolayer of HS-5 stromal cell line was prepared by plating 3 × 10⁵ cells per well in 96-well plates overnight in normoxic or hypoxic incubator. Fibronectin plates were prepared the day before by resuspending 50 μg/mL of fibronectin stock solution in 1× PBS for 5 μg/cm² coating. 100 μL was added per well in a 96-well plate, and the plate was stored in 4°C. BCWM.1 and MWCL.1 cells were cultured in normoxia or hypoxia or were re-oxygenated, pre-labeled with calcein AM at the final concentration of 1 μg/mL for 1 hour at 37°C and washed twice with PBS twice. Next, the cells were incubated with 500 μL RNase/PBS at the concentration of 10 μg/mL for 30 minutes at 37°C. washed and stained with PI at the concentration of 5 μg/mL for 15 minutes at the room temperature, and analyzed by flow cytometry.

The effect of hypoxia on chemotaxis

The ability of cells to migrate through 8-μm pore size of filter was determined using Transwell migration plates (Costar) according to the manufacturer’s instructions. BCWM.1 or MWCL.1 cells were cultured under normoxia or hypoxia for 24 hours, the cells were plated in the upper chamber and were allowed to transmigrate into the lower chamber containing medium with or without 30 nmol/L recombinant SDF-1. After 4 hours of incubation in normoxia or hypoxia, the cells which migrated to the lower chamber were counted using flow cytometry.

Statistical analysis

Experiments were performed in triplicates and repeated at least 3 times. Results are shown as mean ± SD and were analyzed using the Student t test or one-way ANOVA for statistical significance and were considered significantly different for P < 0.05.

Results

Progression of Waldenström macroglobulinemia cells in the bone marrow induces hypoxia in Waldenström macroglobulinemia cells and in other cells in the microenvironment

We tested the effect of tumor progression on hypoxic phenotype in Waldenström macroglobulinemia cells in vivo. Different numbers of BCWM.1-mCherry-Luc cells were injected into mice via tail vein to establish different tumor burdens in the bone marrow of the mice, which was confirmed by the percentage of mCherry-positive cells in the bone marrow by flow cytometry. We further characterized the hypoxic state of the BCWM1-mCherry cells by analyzing the MFI of anti-PIM-APC signal. A direct correlation was found between the tumor burden in the bone marrow and the hypoxia in the Waldenström macroglobulinemia cells (Fig. 1A). We also tested the hypoxic state of other cells in the bone marrow, mCherry-negative population, and we found that these cells were less hypoxic than Waldenström macroglobulinemia cells, but hypoxic signs were shown at higher tumor burdens (Fig. 1B). We tested the effect of tumor hypoxia in the bone marrow on the egress of Waldenström macroglobulinemia cells in vivo and found a direct linear correlation between the hypoxia in the bone marrow and the number of circulating Waldenström macroglobulinemia cells (Fig. 1C), which indicates that the mechanism of Waldenström macroglobulinemia cell entry to the circulation is regulated by hypoxia.

Hypoxia reduces cell proliferation and causes cell cycle arrest with no effect on apoptosis

We investigated the effect of hypoxia on proliferation, cell cycle, viability, and apoptosis of Waldenström macroglobulinemia cells exposed to hypoxic conditions for 24 hours. We found that after 24 hours of normoxia, the BCWM.1 and MWCL.1 cell number doubled; whereas the number of hypoxic cells increased only by approximately 1.3-fold (Fig. 2A). The induction of hypoxic response in the Waldenström macroglobulinemia cells by incubation in 1% O₂ for 24 hours was confirmed by HIF1α and HIF2α protein stabilization in BCWM.1 cells (Fig. 2B). Furthermore, hypoxia decreased the expression of proteins associated with PI3K signaling pathway,
Mol Cancer Res; 13(2) February 2015

...hypoxia by looking at the cell cycle status. Hypoxia increased the number of BCWM.1 and MWCL.1 cells in the G1 phase and decreased DNA synthesis (S) and mitotic (G2/M) phases of cell cycle (Fig. 2C). These results were confirmed by immunoblotting which showed that hypoxia reduced the expression of proteins associated with the G1–S transition including pRb and pCDK6 and protein associated with the S–G2 transition including cyclin E (Fig. 2D).

Then we tested the effect of hypoxia on apoptosis and viability of Waldenström macroglobulinemia cells. We found that incubation of BCWM.1 or MWCL.1 under hypoxic conditions for 24 hours induced no significant effect on the cell viability of Waldenström macroglobulinemia cells (Fig. 2E). We further investigated the effect of hypoxia on apoptosis of the Waldenström macroglobulinemia cells by using Annexin V/PI assay and found that BCWM.1 or MWCL.1 cells incubated under normoxic and hypoxic conditions for 24 hours had similarly around 5% of cells in total population undergoing apoptosis (Fig. 2F). We also tested the activation of intrinsic and extrinsic apoptotic pathways and found no difference in expression of cleaved caspase-9 (intrinsic pathway), cleaved caspase-8 (extrinsic pathway), or cleaved caspase-3 (which converge both pathways) in BCWM.1 cells (Fig. 2G).

Hypoxia regulates Waldenström macroglobulinemia cell–cell adhesion contributing to cell egress to the bone marrow

To examine the mechanism by which hypoxia contributes to cell egress from the bone marrow, we investigated the adhesion of BCWM.1 and MWCL.1 cells to the bone marrow stromal cells (BMSC) and also to each other, in vitro. Incubation of BCWM.1 or MWCL.1 in hypoxia reduced their adhesion to a BMSC monolayer by 50% and 25%, respectively (Fig. 3A). Adhesion results were confirmed by using fluorescent microscopy as shown on a representative image of MWCL.1 cell line (Fig. 3B). The decrease in adhesion of Waldenström macroglobulinemia cells was mediated by reduced expression of E-cadherin in hypoxia as demonstrated by immunoblotting and flow cytometry (Fig. 3C). To further confirm that this effect was mediated by E-cadherin, BCWM.1 cells were incubated with E-cadherin blocking antibody for 1 hour before plating onto stromal cells. We observed a significant reduction of adhesion of normoxic WM cells to HS-5 cells due to blocking of E-cadherin while no difference in adhesion in hypoxia (Fig. 3D). Similarly, we found that the interaction between BCWM.1 cells was reduced and the size of clumps formed by BCWM.1 cells exposed to hypoxia was visibly smaller than these formed in normoxia (Supplementary Fig. S1A and S1B). Treatment with E-cadherin blocking antibody in normoxia significantly decreased the size of clumps but had no additional effect in hypoxia (Supplementary Fig. S1A and S1B). Together, these data suggest that the Waldenström macroglobulinemia cells are losing cell–cell interactions between each other and with the bone marrow stroma due to hypoxia, which promotes cell dissemination.

Hypoxia facilitates extravasation and homing of Waldenström macroglobulinemia cells to the bone marrow niche by increased chemotaxis and maintaining VLA-mediated adhesion in response to SDF-1

We have tested the effect of hypoxia on the ability of Waldenström macroglobulinemia cells to extravasate and home to bone marrow niche. The extravasation of Waldenström macroglobulinemia cells from the circulation to the bone marrow was...
monitored by looking at the number of circulating BCWM.1-mCherry cells at 5, 15, and 30 minutes after intravenous injection. As soon as 5 minutes postinjection, there were 2.5-fold more normoxic cells than hypoxic cells observed in the circulation, meaning that hypoxic cells extravasated faster than normoxic cells (Fig. 4A). The homing of Waldenström macroglobulinemia cells to bone marrow niches was tested by injection of labeled hypoxic and normoxic BCWM.1-mCherry cells to mice and direct

Figure 2.
Hypoxia decreases Waldenström macroglobulinemia (WM) cell proliferation, cell cycle, and viability without any effect on cell apoptosis in vitro. The effect of hypoxia (1% O2, 24 hours) on proliferation of WM cells after 24-hour incubation normalized to 0 hour (A); expression of HIF1α, HIF2α, PI3K pathway proteins, and α-tubulin (loading control) detected by immunoblotting in BCWM.1 (B); cell cycle analysis after PI staining analyzed by flow cytometry in WM cell lines (C); expression of cell cycle-related proteins in BCWM.1 (D); viability of WM cells measured by trypan blue staining showing percentage of viable cells (E); cell apoptosis of WM cells based on Annexin V/PI staining and flow cytometry analysis showing the percentage of apoptotic cells which are Annexin-V-positive and PI-positive (F); and apoptosis-related proteins in BCWM.1 with α-tubulin (loading control) detected by immunoblotting (G). H, hypoxia; N, normoxia.
Muz et al.

Figure 3.
Hypoxia regulates cell adhesion in an E-cadherin-dependent manner. The effect of hypoxia (1% O2, 24 hours) on BCWM.1 and MWCL.1 cell adhesion to a monolayer of BMSCs (HS-5) normalized to normoxic cells as measured by fluorescent reader of adherent calcine-AM-positive Waldenström macroglobulinemia (WM) cells (A) and as shown by fluorescent microscope (scale bar represents 1 μm) and representative pictures of adherent calcine-positive MWCL.1 cells (green) adherent to DiD-positive stromal cells (red; B). E-cadherin protein expression in BCWM.1 shown by immunoblotting with α-tubulin (loading control) and on a histogram analyzed by flow cytometry (C). The effect of E-cadherin blocking antibody (5 μg/mL) on BCWM.1 cell adhesion in normoxia or hypoxia normalized to normoxic untreated cells (D). H, hypoxia; N, normoxia; anti-E-cad Ab, anti-E-cadherin blocking antibody.

Detection of the number of each population in the bone marrow 24 hours postinjection. We found that hypoxic cells homed more than normoxic cells to the bone marrow (2-fold increase), suggesting that hypoxic Waldenström macroglobulinemia cells have enhanced homing capabilities (Fig. 4B).

To explain the rapid extravasation and homing in vivo, we examined the effect of hypoxia on chemotaxis and integrin-mediated adhesion in vitro. We found that hypoxia increased the chemotaxis of BCWM.1 and MWCL.1 cells toward SDF-1 (Fig. 4C). These results were in agreement with hypoxia-induced overexpression of CXCR4 on the surface of BCWM.1 cells, as detected by flow cytometry (Fig. 4D). Moreover, we tested the effect of hypoxia on VLA-4–mediated adhesion of Waldenström macroglobulinemia cells and found that it did not have an effect on the adhesion of BCWM.1 cells to fibronectin and that hypoxic BCWM.1 cells maintained their ability to increase VLA-4–mediated adhesion to fibronectin in response to SDF-1 chemotactic signals (Fig. 4E). These results were confirmed by the finding that hypoxic cells maintained their expression of VLA-4 compared with normoxic cells (Fig. 4F).

Re-oxygenation boosts the proliferation of Waldenström macroglobulinemia cells by increasing cell cycle progression and adhesion augmented by E-cadherin expression

The last step of cell trafficking after homing is recovery in the bone marrow site. Therefore, we investigated the effect of re-oxygenation of hypoxic cells in different cellular aspects including proliferation, cell cycle, and adhesion. We found that re-oxygenation of hypoxic BCWM.1 cells for 24 hours significantly increased the proliferation of Waldenström macroglobulinemia cells (2.4-fold), compared with hypoxic cells in hypoxia (1.5-fold increase), and re-oxygenated MWCL.1 cells (2-fold) compared with hypoxic cells (1.2-fold; Fig. 5A). The phosphorylation of PI3K-associated proteins involved in cell proliferation such as pAKT, p-S6R, and p-P70S6 was increased after re-oxygenation compared with their hypoxia-driven reduction (Fig. 5B).

Analysis of the cell cycle revealed that the re-oxygenated BCWM.1 and MWCL.1 hypoxic cells exited the hypoxia-induced G1 cell-cycle arrest and increased their DNA synthesis phase more than normoxic cells as shown in Fig. 5C. The proteins involved in the cell cycle, including cyclin D1 and p-Rb, were increased after re-oxygenation compared with their hypoxia-driven reduction (Fig. 5D).

Re-oxygenation of hypoxic BCWM.1 and MWCL.1 cells increased cells adhesion to BMSCs (Fig. 5E) due to restored expression of E-cadherin (Fig. 5F). Moreover, re-oxygenation of BCWM.1 improved cell adhesion between BCWM.1 cells and increasing the size of clumps significantly (Supplementary Fig. S1A and S1B).

Discussion
The driving force for the metastatic process in Waldenström macroglobulinemia is not well understood. Accumulating evidence indicates that egress of Waldenström macroglobulinemia cells from one site of the bone marrow to a new site is a complex...
process that involves intercellular interactions with extracellular matrix (3), soluble growth factors (5), endothelial cells (6), and stromal cells (7). Hypoxia is a decreased oxygenation of tissue which was shown to develop due to uncontrollable tumor cell proliferation. Hypoxia was shown to play a key role in metastasis of solid tumors (19) and to promote resistance to therapy by reducing proliferation and inducing cell cycle arrest (20). It has been shown by a direct *in vivo* measurement that the bone marrow is a hypoxic tissue and the oxygen tension is low even in the highly vascular regions ranging between 1.5% and 4.2% O₂ (21). In case of hematologic malignancies, including multiple myeloma and CML, hypoxia was shown to promote progression and dissemination of the disease (12, 13, 15, 16). Lymphomas were found to show hypoxic features, based on the expression of main endogenous hypoxic marker, HIF1α. Constitutive stabilization of HIF1α was found in the majority of patients with diffuse large B-cell lymphoma and follicular lymphoma (22), as well as in mantle cell lymphoma (18).

Targeting hypoxia in solid tumors is one of the current approaches to prevent cancer angiogenesis, metastasis, and
drug resistance, and it became also an emerging strategy in hematologic malignancies. Antitumor activity of the hypoxia-activated pro-drug (TH-302) was demonstrated in combination with bortezomib which improved survival of mice with myeloma (14). TH-302 also delayed onset and progression of acute myeloid leukemia (AML) xenograft model (23). Moreover,

Figure 5.
Recovery of hypoxic cells under normoxic conditions. The effect of hypoxia (1% O2, 24 and 48 hours) and re-oxygenation (hypoxia for 24 hours followed by normoxia for 24 hours) on BCWM.1 and MWCL.1 cell proliferation rate analyzed using MTT and shown after normalization to hypoxic cells at 24 hours (A); the expression of proteins associated with PI3K and proliferation shown by immunoblotting with α-tubulin as a loading control (B); cell cycle analysis of BCWM.1 and MWCL.1 after PI staining analyzed by flow cytometry (C); expression of proteins associated with cell cycle in BCWM.1 cells shown by immunoblotting with α-tubulin as a loading control (D); adhesion of re-oxygenated BCWM.1 and MWCL.1 cells normalized to hypoxic cells (F); expression of E-cadherin in BCWM.1 shown by immunoblotting with α-tubulin as a loading control (E). H, hypoxia; re-O2, re-oxygenation.
blocking HIF1α signaling, which is constitutively expressed in mouse lymphoma and human AML stem cells, preferentially eliminated cancer stem cells abrogating colony-forming unit activity and tumor initiation (24). Because hypoxia is one of the features of Waldenström macroglobulinemia progression and spread, it would be a promising target to improve patient survival.

In this study, we focused on the role of hypoxia in the dissemination and progression of Waldenström macroglobulinemia. We examined the hypoxic state of tumor cells in vivo in the xenograft mouse model of Waldenström macroglobulinemia. Tumor progression induced hypoxic conditions in Waldenström macroglobulinemia cells and to a lower extent in the bone marrow microenvironment; the higher the tumor involvement in the bone marrow, the higher the hypoxic conditions observed. These results were consistent with the development of hypoxic conditions in the tumor cells and the bone marrow microenvironment in multiple myeloma and CML animal models (12, 13). In addition, we tested the correlation between the hypoxic conditions in the bone marrow and the number of circulating Waldenström macroglobulinemia cells. A direct correlation was found between the hypoxic conditions in the bone marrow and the number of circulating Waldenström macroglobulinemia cells. Similar results were observed in myeloma; however, no such correlation was found in CML; we hypothesize that the reason for these observations is that both multiple myeloma and Waldenström macroglobulinemia are more dependent on the bone marrow for their progression, whereas CML is less bone marrow-dependent due to its circulating nature (8–10).

To mimic the effect of hypoxia at the primary tumor bed in vitro, we used 1% O2, which is believed to be the acceptable level in tumor-induced hypoxia (25). We examined the effect of hypoxia on the cellular functions of Waldenström macroglobulinemia cells in vitro including proliferation, cell cycle, survival, apoptosis, cell adhesion, and cell migration. It was confirmed that 1% O2 in vitro induced hypoxic response in the Waldenström macroglobulinemia cells including high expression of HIF1α and HIF2α. This is in agreement with our previous findings showing that these in vitro conditions induced hypoxic response in multiple myeloma and CML cells (12, 13).

It is well established in solid tumors that hypoxia regulates tumor cell proliferation; highly proliferative cancer cells are neighboring blood vessels, compared with the quiescent tumor cells in the hypoxic core (26). Therefore, we tested the effect of hypoxia on proliferation of Waldenström macroglobulinemia cells. Hypoxia inhibited the proliferation and decreased proliferative cell signaling pathways such as P3K in Waldenström macroglobulinemia cells. This also led to induction of G1 cell cycle arrest shown in DNA staining with PI as well as inhibition of cell cycle signaling. Despite the inhibition of proliferation, no apoptosis was observed because of hypoxia shown by Annexin/PI assay, and no caspase cleavage or changes in the expression of other proapoptotic proteins was observed. These are the first results to show the effect of hypoxia on proliferation and cell cycle in hematologic malignancies; further experiments to examine the effect of hypoxia on proliferation of other hematologic malignancies including multiple myeloma and CML are warranted.

We have previously shown that hypoxia promotes egress of multiple myeloma and CML cells from the bone marrow through activation of an EMT-like mechanism in which these cells down-regulated E-cadherin in the initial stages of egress (12, 13). Hypoxic Waldenström macroglobulinemia cells showed a similar pattern of behavior of decreased E-cadherin which resulted in a decreased adhesion of Waldenström macroglobulinemia to stromal cells in vitro, which was further confirmed to be E-cadherin-mediated. These results are in accord with the findings that the number of circulating Waldenström macroglobulinemia cells was correlated to the hypoxia in the bone marrow, in which hypoxic cells are less adhesive to the microenvironment and egress more to the circulation.

The next step of cell dissemination, after egress to circulation, is homing to new bone marrow niches. It was found that hypoxic Waldenström macroglobulinemia cells have the capability to extravasate from the circulation faster than normoxic ones and that the hypoxic cells ended up homing more efficiently into new bone marrow niches. These results are associated with increased CXCR4 expression in hypoxic Waldenström macroglobulinemia cells which facilitates faster chemotaxis of Waldenström macroglobulinemia cells toward the chemoattractant SDF-1. In addition, hypoxia did not change the SDF-1–induced adhesion of Waldenström macroglobulinemia cells to fibronectin (mediated by integrin VLA-4), as the first step of the process of homing into new bone marrow niches (3). Therefore, the hypoxic cells seem to have improved machinery for homing to new bone marrow niches including enhanced chemotaxis and integrin-mediated adhesion.

The last step of Waldenström macroglobulinemia dissemination is recovery of cells in the new “normoxic” bone marrow site, which implies exposure to normal oxygenation (re-oxygenation). Therefore, we tested the behavior of hypoxic cells after re-exposure to normoxic conditions. It was found that the proliferation after re-oxygenation of hypoxic cells induced exit of the G1 arrest and boosted cell proliferation rate. Moreover, the cells restored their E-cadherin expression, which facilitated Waldenström macroglobulinemia cell adhesion to stroma.

In summary, hypoxia plays a critical role in Waldenström macroglobulinemia progression, dissemination, and cell trafficking. This study demonstrated for the first time that hypoxia decreases proliferation in Waldenström macroglobulinemia cells and induces more quiescent cell phenotype. In parallel, hypoxia induces loose adhesion to the primary tumor site to facilitate egress, enhances the ability of Waldenström macroglobulinemia cells to home from the circulation to new bone marrow sites and that hypoxic Waldenström macroglobulinemia cells recover and promote a proliferative form of the disease in the new bone marrow site. Further studies are warranted to test targeting hypoxic response in Waldenström macroglobulinemia cells, through inhibition of HIFs or their downstream targets, as a novel strategy to prevent dissemination of Waldenström macroglobulinemia. Furthermore, studies are warranted to test the effect of the hypoxia-induced G1 arrest on Waldenström macroglobulinemia cell sensitivity to chemotherapy and targeting hypoxic responses in Waldenström macroglobulinemia cells to overcome drug resistance in Waldenström macroglobulinemia.

Disclosure of Potential Conflicts of Interest
A.K. Azab reports receiving commercial research grant from Selexys, Verastem, Karyopharm and Cellworks. No potential conflict of interest were disclosed by other authors.
Molecular Cancer Research

Authors' Contributions
Conception and design: I.M. Ghobrial, A.K. Azab
Development of methodology: B. Muz, I.M. Ghobrial, A.K. Azab
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Muz
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Muz, P. de la Puente, I.M. Ghobrial, A.K. Azab
Writing, review, and/or revision of the manuscript: B. Muz, P. de la Puente, F. Azab, I.M. Ghobrial, A.K. Azab
Study supervision: A.K. Azab

References

Grant Support
This study was supported by a grant from the International Waldenstrom’s Macroglobulinemia Foundation.

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.

Received March 19, 2014; revised August 27, 2014; accepted September 8, 2014; published OnlineFirst September 17, 2014.
Hypoxia Promotes Dissemination and Colonization in New Bone Marrow Niches in Waldenström Macroglobulinemia

Barbara Muz, Pilar de la Puente, Feda Azab, et al.


Updated version  Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-14-0150

Supplementary Material  Access the most recent supplemental material at: http://mcr.aacrjournals.org/content/suppl/2014/09/18/1541-7786.MCR-14-0150.DC1

Cited articles  This article cites 26 articles, 13 of which you can access for free at: http://mcr.aacrjournals.org/content/13/2/263.full.html#ref-list-1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.