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

The running title: Role of hypoxia in Waldenström’s Macroglobulinemia

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ABSTRACT

Waldenström's Macroglobulinemia (WM), a rare and indolent type of non-Hodgkin lymphoma, is characterized by widespread lymphoplasmacytic B-cells in the bone marrow (BM). 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 WM. Interestingly, tumor progression correlated with hypoxia levels in WM cells and other cells in the BM, 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 WM cells and BM stroma, through downregulation of E-cadherin expression; thus, explaining increased egress of WM cells to the circulation. Moreover, hypoxia increased the extravasation and homing of WM cells to new BM niches in vivo, by increased CXCR4/SDF-1-mediated chemotaxis and maintaining the VLA4-mediated adhesion. Re-oxygenation of hypoxic WM cells enhanced the rate of proliferation and cell cycle progression, and restored intercellular adhesion between WM cells and with BM stroma. This study suggests targeting hypoxic response is a novel strategy to prevent dissemination of WM.

IMPLICATIONS

This study provides a better understanding of the biology of dissemination of WM and opens new windows for investigation of new therapeutic targets in WM based on tumor-hypoxia mechanisms.
INTRODUCTION

Waldenström’s Macroglobulinemia (WM) 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 (BM) (1). The WM cells infiltrate specifically into the BM niche which implies the critical role of the BM as a supportive microenvironment for the WM cells due to the various interactions (2-4). We have previously shown in WM that the BM 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 BM microenvironment plays a crucial role in progression and drug resistance of other hematologic malignancies including multiple myeloma (MM) (8-10) and chronic myeloid leukemia (CML) (11, 12).

Hypoxia plays an important role in the progression and dissemination of hematologic malignancies (12-16). Tumor hypoxia activates adaptive transcriptional programs, including HIF-1α, that promote cell survival, motility, invasiveness, drug resistance and neoangiogenesis in MM (16, 17), associated with more aggressive tumor (18). We have shown that hypoxia induces egress of MM cells from the BM to the circulation, and facilitates their homing to the new BM 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 MM cells (13). However, the role of hypoxia in the progression of WM 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 WM cells including the cell-cell interactions in the BM and egress to the circulation, chemotaxis and homing to new BM niches,
adaptation of hypoxic WM cells in the new BM milieu, as well as hypoxic effects on proliferation, survival, apoptosis and cell cycle in WM cells.
MATERIALS AND METHODS

Reagents

Stromal-derived growth factor (SDF-1) for adhesion and chemotaxis assays was purchased from R&D (Minneapolis, MN). Transwell plates for chemotaxis assay were obtained from Corning (Manassas, VA). E-cadherin blocking antibody and human fibronectin were purchased from EMD Millipore (St Charles, MO). Total protein concentration for immune-blotting was assessed by Quick Start™ Bradford dye reagent (BioRad, Hercules, CA). MTT solution for cell viability assay was purchased from Sigma-Aldrich (Carlsbad, CA). Annexin V-PI for apoptosis assay and BD BioCoat Human Fibronectin plates for adhesion assay were purchased from BD Biosciences (San Jose, CA). Cell trackers including calcein-AM, DiO and DiD were purchased from Invitrogen (Life Technologies, Grand Island, NY). Hypoxia marker pimonidazole hydrochloride (PIM) and its correlating antibody were obtained from HypoxyProbe (Burlington, MA). 10 x red blood cell (RBC) buffer was obtained from BioLegend (San Diego, CA).

Cell culture

The WM 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). WM cell lines were cultured in RPMI-1640 (Corning CellGro, Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, Grand Island, NY), 2 mmol/L of L-glutamine, 100 U/mL Penicillin and 100 μg/mL Streptomycin (CellGro, Mediatech, Manassas, VA). HS-5 were cultured in 20% FBS Dulbecco’s Modified Eagle’s Medium (Corning CellGro, Mediatech, Manassas, VA) containing L-glutamine, Penicillin/Streptomycin. Cells were incubated at 37°C under normoxic conditions (21% O₂) in the NuAire water jacket incubator (Plymouth, MN) or hypoxic conditions (1% O₂) in the hypoxic chamber from Coy (Grass Lake, MI) for 24hrs. In some cases, hypoxic WM cells were re-oxygenated by exposing them to normal oxygen levels.
Animals

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

Effect of tumor progression on hypoxia in WM 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 (IV) at different concentrations of 0.5, 1 and $2.5 \times 10^6$ cells per mouse (4 mice per condition), and allowed to grow for 3 weeks. Mice were treated with PIM (100mg/kg) for 4hrs, sacrificed and their bone marrow, and peripheral blood were extracted. Mononuclear cells (MNC) were isolated, washed, fixed, permeabilized, and stained with anti-PIM-APC antibody. WM cells were detected by flow cytometry by gating on mCherry positive cells and BM stromal cells were mCherry negative, and hypoxia in these cells was measured as a mean-fluorescent intensity (MFI) of APC. Circulating WM cells were detected by gating on mCherry positive cells in the peripheral blood samples post RBC lysis.

Effect of hypoxia on extravasation of WM cells

BCWM.1-mCherry cells were exposed to normoxia or hypoxia for 24hrs and each population was injected via tail vein into 4 Balb/C mice (1 x $10^6$ cells/ mouse). The 50μL of blood was collected from the portal vein at 5, 15 and 30mins post-injection, RBC were lysed, and MNC were labeled with CD45-APC-Cy7 (BD Biosciences, San Jose, CA) for 1hr on ice, and analyzed by flow cytometry. The number of circulating WM cells was analyzed as the % of CD45+/mCherry+ out of the total CD45+ population. The absence of these cells from the circulation reflected extravasation of the WM cells.
Effect of hypoxia on homing of WM cells to the bone marrow

BCWM.1-mCherry cells were first stained with DiD or DiO (5μL per 1mL medium) for 2hrs at 37°C, washed and then exposed to normoxia (1 flask with DiD and 1 flask with DiO) or hypoxia (1 flask with DiD and 1 flask with DiO) for 24hrs. 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 x 10^6 cells per mouse. After 24hrs post-injection the mice were sacrificed and the MNC were isolated from the femurs, filtered, stained with DAPI (Sigma-Aldrich, Carlsbad, CA), 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 were analyzed in each mouse. The number of positive cells reflected the number of WM cells which homed to the BM. 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 six 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 BM.

The effect of hypoxia on expression of E-cadherin, CXCR4 and VLA-4 using flow cytometry

BCWM.1 cells (1 x 10^6) were exposed to normoxic or hypoxic conditions for 24hrs, then aspirated, incubated on ice for 10mins, washed with PBS, and stained on ice with primary anti-human-CXCR4-APC antibody (BD Biosciences, San Jose, CA), mouse-anti-human-VLA-4 antibody (BD Biosciences, San Jose, CA) or rabbit-anti-human-E-cadherin antibody (Cell Signaling Technologies, Danvers, MA), followed by staining with secondary anti-mouse or anti-rabbit FITC antibody (Cell Signaling Technologies, Danvers, MA) for 1hr. The cells were then washed with 1 x PBS and analyzed by flow cytometry.
The effect of hypoxia on cell signaling using immuno-blotting

BCWM.1 cells (5 x 10^6) were exposed to normoxic or hypoxic conditions for 24hrs, or re-oxygenated for 24hrs, aspirated, incubated on ice for 10mins, washed with 1 x PBS and lysed. Protein concentration in the cell lysates was normalized and 80μg of protein was loaded per lane. Electrophoresis was performed using 8% or 10% precast Novex® Tris-Glycine gels (Novex) and transferred to a nitrocellulose membrane using iBlot (Invitrogen, Life Technologies, Grand Island, NY). Membranes were blocked with 5% nonfat dry milk in Tris-Buffered Saline/Tween20 (TBST) buffer and incubated with primary antibodies detecting E-cadherin, HIF-1α, HIF-2α, pPI3K-P85, pAKT (Ser473), pS6R, pP70S6, Cleaved-Caspase-3, -8, -9, p-Rb, Cyclin D1, Cyclin E1, pCDK2, pCDK6, p21 or α-Tubulin over night at 4°C. The membranes were then washed with TBST for 30mins, incubated for 1hr at room temperature with HRP-conjugated secondary antibody, washed, and developed using Novex ECL Chemiluminescent Substrate Reagent Kit (Invitrogen). Primary and secondary antibodies used for immune-blotting were purchased from Cell Signaling Technologies (Danvers, MA) apart from HIF-2α antibody, which was purchased from Novus Biologicals (Littleton, CO).

The effect of hypoxia on cell proliferation and viability

Cell growth and cell viability was measured by using Vi-Cell Cell Viability Analyzer (Beckman Coulter, Brea, CA) 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 was registered at time 0 (t0), then cells were cultured in normoxia or hypoxia for 24hrs and the measurement was repeated. In addition, cell growth was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Chemicon International), followed by absorbance readout at 570nm using spectrophotometer.
The effect of hypoxia on apoptosis

BCWM.1 and MWCL.1 cells were cultured in normoxic or hypoxic conditions for 24hrs. The cells were aspirated, kept on ice for 10mins, washed twice with ice-cold 1 x PBS and resuspended in 100μL Annexin binding buffer. The cells were then stained with 5μL of Annexin V for 15mins followed by staining with 5μL of propidium iodide (PI) for 15mins at room temperature. 400μL of 1 x 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 24hrs or were re-oxygenated for the next 24hrs. First, the cells were aspirated and kept on ice for 10mins, washed with ice-cold 1 x PBS, fixed with 70% ethanol/PBS, incubated on ice for 30mins, and washed twice. Next, the cells were incubated with 500μL RNase/PBS at the concentration of 10μg/mL for 30mins at 37°C, washed and stained with PI at the concentration of 5μg/mL for 15mins at the room temperature, and analyzed by the 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 x 10³ cells per well in 96-well plates over night in normoxic or hypoxic incubator. Fibronectin (FN) plates were prepared the day before by resuspending 50μg/mL of FN stock solution in 1 x PBS for 5μg/cm² coating, 100μL 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 1μg/mL for 1hr at 37°C and washed with PBS. In some cases BCWM.1 cells were treated with E-cadherin blocking antibody (5μg/mL) for 1hr, or SDF-1 (30nM or 60nM) before plating the cells. The adhesion was conducted on the plates pre-coated with HS-5 or FN for 90mins at 37°C. For fluorescent microscope
analysis of cell adhesion, HS-5 cells were plated in the presence of DiD overnight (5μL per 1mL of medium) and MWCL.1 cells were pre-labeled with calcein-AM (as described above). Non-adherent cells were washed with PBS and adherent cells were measured by detecting the fluorescent intensity signal using fluorescent reader (Ex/Em = 485/520nm) or fluorescent microscope (objective x5).

**Effect of hypoxia on chemotaxis**

The ability of cells to migrate through 8μm pore size of filter was determined using Transwell migration plates (Costar, Corning) according to the manufacturer’s instructions. BCWM.1 or MWCL.1 cells were cultured under normoxia or hypoxia for 24hrs, the cells were plated in the upper chamber and were allowed to transmigrate into the lower chamber containing medium with or without 30nM recombinant SDF-1. After 4hrs 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 three times. Results are shown as mean ± StDev and were analyzed using student t-test or one-way ANOVA for statistical significance, and were considered significantly different for P value less than 0.05.
RESULTS

Progression of WM cells in the BM induces hypoxia in WM cells and in other cells in the microenvironment

We tested the effect of tumor progression on hypoxic phenotype in WM 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 BM of the mice, which was confirmed by the % of mCherry-positive cells in the BM 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 BM and the hypoxia in the WM cells (Figure 1A). We also tested the hypoxic state of other cells in the BM, mCherry-negative population, and we found that these cells were less hypoxic than WM cells, but hypoxic signs were shown at higher tumor burdens (Figure 1B). We tested the effect of tumor hypoxia in the BM on the egress of WM cells in vivo, and found a direct linear correlation between the hypoxia in the BM and the number of circulating WM cells (Figure 1C), which indicates that the mechanism of WM 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 WM cells exposed to hypoxic conditions for 24hrs. We found that after 24hrs of normoxia the BCWM.1 and MWCL.1 cell number doubled; whereas the number of hypoxic cells increased only by approximately 1.3 fold (Figure 2A). The induction of hypoxic response in the WM cells by incubation in 1% O₂ for 24hrs was confirmed by HIF-1α and HIF-2α protein stabilization in BCWM.1 cells (Figure 2B). Furthermore, hypoxia decreased the expression of proteins associated with PI3K signaling pathway, involved in survival and proliferation, including decreased phosphorylation of PI3K-P85, pAKT and pS6R in BCWM.1 cells (Figure 2B). We further studied the cellular mechanism of the decreased proliferation of WM cells in 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 (Figure 2C). These results were confirmed by immune-blotting 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 (Figure 2D).

Then we tested the effect of hypoxia on apoptosis and viability of WM cells. We found that incubation of BCWM.1 or MWCL.1 under hypoxic conditions for 24hrs induced no significant effect on the cell viability of WM cells (Figure 2E). We further investigated the effect of hypoxia on apoptosis of the WM cells by using Annexin V-PI assay and found that BCWM.1 or MWCL.1 cells incubated under normoxic and hypoxic conditions for 24hrs had similarly around 5% of cells in total population undergoing apoptosis (Figure 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 (Figure 2G).

**Hypoxia regulates WM cell-cell adhesion contributing to cell egress to the BM**

To examine the mechanism by which hypoxia contributes to cell egress from the BM, we investigated the adhesion of BCWM.1 and MWCL.1 cells to the BM stromal cells (BMSCs) and also to each other, in vitro. Incubation of BCWM.1 or MWCL.1 in hypoxia reduced their adhesion to a BMSCs monolayer by 50% and 25%, respectively (Figure 3A). Adhesion results were confirmed by using fluorescent microscopy as shown on a representative image of MWCL.1 cell line (Figure 3B). The decrease in adhesion of WM cells was mediated by reduced expression of E-cadherin in hypoxia as demonstrated by immune-blotting and flow cytometry (Figure 3C). To further confirm that this effect was mediated by E-cadherin, BCWM.1 cells were incubated with E-cadherin blocking antibody for 1hr prior 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 (Figure 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 that these formed in normoxia (Supplementary Figure 1A, 1B). Treatment with E-cadherin blocking antibody in normoxia significantly decreased the size of clumps, but had no additional effect in hypoxia (Supplementary Figure 1A, 1B). Together, this data suggests that the WM cells are losing cell-cell interactions between each other and with the BM stroma due to hypoxia, which promotes cell dissemination.

**Hypoxia facilitates extravasation and homing of WM cells to the BM 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 WM cells to extravasate and home to BM niche. The extravasation of WM cells from the circulation to the BM was monitored by looking at the number of circulating BCWM.1-mCherry cells at 5, 15 and 30mins post IV injection. As soon as 5mins post injection, there were 2.5-fold more normoxic cells than hypoxic cells observed in the circulation, meaning that hypoxic cells extravasated faster than normoxic cells (Figure 4A). The homing of WM cells to BM niches was tested by injection of labeled hypoxic and normoxic BCWM.1-mCherry cells to mice and direct detection of the number of each population in the bone marrow 24hrs post-injection. We found that hypoxic cells homed more than normoxic cells to the BM (2-fold increase), suggesting that hypoxic WM cells have enhanced homing capabilities (Figure 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 towards SDF-1 (Figure 4C). These results were in agreement with hypoxia-induced over expression of CXCR4 on the surface of BCWM.1 cells, as detected by flow cytometry (Figure 4D). Moreover, we tested the effect of hypoxia on VLA-4-mediated adhesion of WM 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 (Figure 4E). These results were confirmed by the finding that hypoxic cells maintained their expression of VLA-4 compared to normoxic cells (Figure 4F).

**Re-oxygenation boosts the proliferation of WM 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 BM 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 24hrs significantly increased the proliferation of WM cells (2.4-fold), compared to hypoxic cells in hypoxia (1.5-fold increase), and re-oxygenated MWCL.1 cells (2-fold) compared to hypoxic cells (1.2-fold) (Figure 5A). The phosphorylation of PI3K-associated proteins involved in cell proliferation such as pAKT, p-S6R and p-P70S6 were increased after re-oxygenation compared to their hypoxia-driven reduction (Figure 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 Figure 5C. The proteins involved in the cell cycle including cyclin D1 and p-Rb, were increased after re-oxygenation compared to their hypoxia-driven reduction (Figure 5D).

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

The driving force for the metastatic process in WM is not well understood. Accumulating evidence indicates that egress of WM cells from one site of the BM to a new site is a complex process that involves inter-cellular 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% O$_2$ (21).

In case of hematological malignancies, including multiple myeloma (MM) and chronic myeloid leukemia (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, HIF-1$\alpha$. Constitutive stabilization of HIF-1$\alpha$ was found in the majority of patients with diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (22), as well as in mantle cell lymphoma (18).

Targeting hypoxia in solid tumors is a one of the current approaches to prevent cancer angiogenesis, metastasis and drug resistance, and it became also an emerging strategy in hematological malignancies. Anti-tumor 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, blocking HIF-1$\alpha$ 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). Since hypoxia is one of the features of WM progression and spread, it would be a reasonable target to improve patient survival.
In this study, we focused on the role of hypoxia in the dissemination and progression of WM. We examined the hypoxic state of tumor cells in vivo in the xenograft mouse model of WM. Tumor progression induced hypoxic conditions in WM cells and to a lower extent in the BM microenvironment; the higher the tumor involvement in the BM the higher the hypoxic conditions observed. These results were consistent with the development of hypoxic conditions in the tumor cells and the BM microenvironment in MM and CML animal models (12, 13). In addition, we tested the correlation between the hypoxic conditions in the BM and the number of circulating WM cells. A direct correlation was found between the hypoxic conditions in the BM and the number of circulating WM 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 MM and WM are more dependent on the BM for their progression, while CML is less BM-dependent due to its circulating nature (8-10).

In order to mimic the effect of hypoxia at the primary tumor bed in vitro, we used 1% \( \text{O}_2 \), which is believed to be the acceptable level in tumor-induced hypoxia (25). We examined the effect of hypoxia on the cellular functions of WM cells in vitro including proliferation, cell cycle, survival, apoptosis, cell adhesion and cell migration. It was confirmed that 1% \( \text{O}_2 \) in vitro induced hypoxic response in the WM cells including high expression of HIF-1\( \alpha \) and HIF-2\( \alpha \). This is in agreement with our precious findings showing that these in vitro conditions induced hypoxic response in MM 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 to the quiescent tumor cells in the hypoxic core (26). Therefore, we tested the effect of hypoxia on proliferation of WM cells. Hypoxia inhibited the proliferation and decreased proliferative cell signaling pathways such as PI3K in WM 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 due to hypoxia shown by
Annexin-PI assay, and no caspase cleavage or changes in the expression of other pro-apoptotic 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 MM and CML are warranted.

We have previously shown that hypoxia promotes egress of MM and CML cells from the BM through activation of an EMT-like mechanism in which these cells downregulated E-cadherin in the initial stages of egress (12, 13). Hypoxic WM cells showed a similar pattern of behavior of decreased E-cadherin which resulted in a decreased adhesion of WM 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 WM cells was correlated to the hypoxia in the BM, 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 BM niches. It was found that hypoxic WM 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 BM niches. These results are associated with increased CXCR4 expression in hypoxic WM cells which facilitates faster chemotaxis of WM cells towards the chemo-attractant SDF-1. In addition, hypoxia did not change the SDF-1-induced adhesion of WM cells to fibronectin (mediated by integrin VLA-4), as the first step of the process of homing into new BM niches (3). Therefore, the hypoxic cells seem to have improved machinery for homing to new BM niches including enhanced chemotaxis and integrin-mediated adhesion.

The last step of WM dissemination is recovery of cells in the new ‘normoxic’ BM 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 WM cell adhesion to stroma.

In summary, hypoxia plays a critical role in WM progression, dissemination and cell trafficking. This study demonstrated for the first time that hypoxia decreases proliferation in WM 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 WM cells to home from the circulation to new BM sites and that hypoxic WM cells recover and promote a proliferative form of the disease in the new BM site. Further studies are warranted to test targeting hypoxic response in WM cells, through inhibition of HIFs or their downstream targets, as a novel strategy to prevent dissemination of WM. Furthermore, studies are warranted to test the effect of the hypoxia-induced G1-arrest on WM cell sensitivity to chemotherapy, and targeting hypoxic responses in WM cells to overcome drug resistance in WM.
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AUTHORSHIP

Contribution: A.K.A and B.M. designed the study, performed experiments, analyzed, interpreted the data and wrote the manuscript; P.P., F.A. evaluated the data, reviewed and edited the manuscript; I.G. provided the MM cell lines. All authors reviewed and approved the manuscript.
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CML cells disrupts their interaction with the bone marrow microenvironment and sensitizes them to


FIGURE LEGENDS

Figure 1. Progression of WM cells in bone marrow induces hypoxia in WM cells and in stromal cells in the microenvironment and induces WM cell egress into the circulation in vivo.

The effect of tumor progression (% of BCWM.1-mCherry cells) in the bone marrow on their hypoxic status shown as a ratio of mean-fluorescent intensity (MFI) of APC-PIM and MFI-isotype of the mCherry-positive population analyzed by flow cytometry (A). The effect of tumor progression (% of BCWM.1-mCherry cells) in the bone marrow on hypoxic status of the bone marrow stromal cells shown as a ratio of MFI of APC-PIM and MFI-isotype of the mCherry-negative population analyzed by flow cytometry (B).

The effect of tumor hypoxia in the bone marrow (MFI of APC-PIM in BCWM.1-mCherry cells) on the number of circulating tumor cells (% of mCherry+ positive BCWM.1 cells) analyzed by flow cytometry (C). The coefficient of determination ($R^2$) denotes a strong linear association between those variables.

Figure 2. Hypoxia decreases WM cell proliferation, cell cycle and viability without any effect on cell apoptosis in vitro.

The effect of hypoxia (1% $O_2$, 24hrs) on proliferation of WM cells after 24hrs incubation normalized to 0hrs (A); expression of HIF-1$\alpha$, HIF-2$\alpha$, PI3K pathway proteins and $\alpha$-Tubulin (loading control) detected by immuno-blotting 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 % 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 immuno-blotting (G). Abbreviations: N – normoxia, H – hypoxia.

Figure 3. Hypoxia regulates cell adhesion in an E-cadherin-dependent manner.

The effect of hypoxia (1% O₂, 24hrs) on BCWM.1 and MWCL.1 cell adhesion to a monolayer of BM stromal cells (HS-5) normalized to normoxic cells as measured by fluorescent reader of the signal of adherent calcein-AM-positive WM cells (A), and as shown by fluorescent microscope (scale bar represents 1 micrometer) and representative pictures of adherent calcein-positive MWCL.1 cells (green) adherent to DiD-positive stromal cells (red) (B); E-cadherin protein expression in BCWM.1 shown by immune-blotting 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). N – normoxia, H – hypoxia, anti-E-cad Ab – anti-E-cadherin blocking antibody.

Figure 4. Hypoxia promotes extravasation and homing of WM cells to the BM niches.

The effect of hypoxia (1% O₂, 24hrs) on the number of circulating WM cells detected as mCherry-positive cells per 100,000 CD45+ mononuclear cells (MNCs) analyzed by flow cytometry at 5, 15 and 30mins of blood aspiration post-injection (A). The effect of hypoxia (1% O₂, 24hrs) on WM cell homing to the bone marrow shown as the number of BCWM.1-mCherry+ (DiD+ or DiO+ cells) detected in the bone marrow, analyzed by flow cytometry and normalized to normoxic cells (B). The effect of hypoxia (1% O₂, 24hrs) in the presence of SDF-1 (30nM) on BCWM.1 and MWCL.1 cell chemotaxis analyzed by flow cytometry and shown as a % of migrated cells normalized to cells which migrated towards SDF-1 in normoxia (C). The effect of hypoxia on the expression of CXCR4 in BCWM.1 cells shown as histogram analyzed by flow...
cytometry (D). The effect of hypoxia (1% O\textsubscript{2}, 24hrs) with or without SDF-1 (30nM and 60nM) on adhesion to fibronectin (FN) of BCWM.1 cells stained with calcein-AM and analyzed by fluorescent reader and shown as the percentage of adherent cells normalized to normoxic untreated cells (E). The effect of hypoxia on the expression of VLA-4 in BCWM.1 cells shown as histogram analyzed by flow cytometry (F).

**Figure 5. Recovery of hypoxic cells under normoxic conditions.**

The effect of hypoxia (1% O\textsubscript{2}, 24 and 48hrs) and re-oxygenation (hypoxia for 24hrs followed by normoxia for 24hrs) on BCWM.1 and MWCL.1 cell proliferation rate analyzed using MTT and shown after normalization to hypoxic cells at 24hrs (A); the expression of proteins associated with PI3K and proliferation shown by immune-blotting with \(\alpha\)-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 immune-blotting with \(\alpha\)-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 immune-blotting with \(\alpha\)-Tubulin as a loading control (E); H - hypoxia, re-O\textsubscript{2} – re-oxygenation.
Figure 1

A

\[ P = 0.0018 \]

\[ y = 0.052x + 1.2375 \]

\[ R^2 = 0.9688 \]

B

\[ P = 0.0011 \]

\[ y = 0.0157x + 0.7244 \]

\[ R^2 = 0.9367 \]

C

\[ R^2 = 0.8553 \]

\[ P = 0.0082 \]

% of WM cells in BM

% of WM cells in BM

Hygrocida in BM cells

Hygrocida in BM cells

% of WM cells in BM

% of WM cells in BM

(MFI of PIM in mCherry-BCWM1 cells)
Figure 2

E

Viability (% of viable cells)

<table>
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<tr>
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<th>BCWM.1</th>
<th>MWCL.1</th>
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Time (hours)

F

Apoptotic cells (% of total cells)

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<tr>
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<th>MWCL.1</th>
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<tbody>
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<tr>
<td>Hypoxia</td>
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</table>

G

N  H

Cleaved-Caspase-9
Cleaved-Caspase-3
Cleaved-Caspase-8
α-Tubulin

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