



Role of the hypoxic bone marrow microenvironment in 5T2MM murine myeloma tumor progression

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Background and Objectives. Unlike most other tumors, multiple myeloma (MM) cells have to survive and to grow in a bone marrow (BM) microenvironment which is already hypoxic by nature. BM hypoxia is crucial for normal hematopoiesis. However, how BM hypoxia and MM affect each other is unknown. We addressed this topic in the 5T2MM mouse model.

Design and Methods. Levels of hypoxia in the BM of control and tumor-bearing mice were analyzed by flow cytometric analysis of pimonidazole hypoxyprobe binding and hypoxia-inducible factor 1 α (HIF-1 α) expression. Micro-vessel density was measured by CD31 staining of BM sections for immunohistochemistry. Apoptosis sensitivity of CD45⁺ 5T2MM subsets in hypoxic conditions were analyzed by detection of active caspase-3.

Results. Analysis of control and 5T2MM-diseased mice injected with pimonidazole hypoxyprobe indicated that both normal BM and myeloma-infiltrated BM are hypoxic. However, the hypoxia in the myelomatous BM was significantly decreased. Analysis of HIF-1 α expression, a surrogate marker of hypoxia, also demonstrated significantly lower levels of hypoxia in myeloma-infiltrated BM. HIF-1 α expression was inversely correlated with the micro-vessel density. *In vitro* culture of 5T2MM cells under hypoxic conditions indicated induction of apoptosis in the CD45⁻ MM-fraction, but not in the CD45⁺ 5T2MM cells.

Interpretation and Conclusions. These data suggest that native BM hypoxia is advantageous for the tumor-initiating CD45⁺ 5T2MM cells. Together with the decreased hypoxia in myeloma-infiltrated BM it also indicates that myeloma-associated angiogenesis is functional and permissive for the expansion of CD45⁻ 5T2MM-cells. All together, the data raise the possibility of an important role of BM hypoxia in myeloma tumor progression.

Key words: myeloma, angiogenesis, hypoxia, CD45.

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Hypoxia – a reduction in the normal level of tissue oxygenation – plays a crucial role in the progression of solid tumors. By altering the expression of genes involved in the regulation of angiogenesis, cell survival, glucose metabolism and invasion, hypoxia acts as a selective pressure for specific subsets within the tumor population. Cancer cells which have adapted to hypoxic conditions are therefore highly aggressive and tumor hypoxia has been associated with poor prognosis and resistance to radiotherapy.^{1,2} Hypoxia-inducible factor 1 (HIF-1) is a transcription factor and a master regulator of hypoxia responsive genes in cancers. HIF-1 is a heterodimeric protein consisting of a HIF-1 α and a HIF-1 β subunit. While HIF-1 β is constitutively expressed, the HIF-1 α protein level is highly regulated by oxygen concentration. When the oxy-

genation is sufficient, HIF-1 α is ubiquitinated and degraded in proteasomes. However, under hypoxic conditions HIF-1 α is rapidly stabilized.³ HIF-1 α expression at mRNA level is mainly regulated by oxygen-independent mechanisms³ HIF-1 directly regulates hypoxia-inducible genes by binding to the hypoxia-responsive elements in the promoter regions. Multiple myeloma (MM) is an incurable plasma cell cancer selectively localized in the bone marrow (BM). Unlike other organs, the normal BM microenvironment is hypoxic. This physiological hypoxia is crucial for normal marrow hematopoiesis.⁴⁻⁶ In humans, the tissue oxygen tension in the *in vivo* extracellular compartment is approximately 40 mmHg,⁷ while medullary cavity oxygen pressure has been estimated at 5% oxygen.⁴⁻⁶ In mouse BM the oxygen tension is even lower than 20

mmHg.⁸ Unlike other tumors, MM-cells – and other cancer cells which metastasize to the BM – therefore have to survive and to grow in a microenvironment which is hypoxic by nature. In solid tumors, induction of a tumor vasculature (angiogenic switch) is necessary to meet the increasing oxygen demands of the progressively growing cancer cells.^{9,10} Not all cancer cells have the capacity to induce angiogenesis, a concept called angiogenic heterogeneity¹¹ and the angiogenic switch is preceded by an increase in angiogenic cancer cells (cancer cells with the capacity to induce neovascularization).^{10,12} In a previous work our group demonstrated in the 5T2MM experimental mouse model that in analogy to solid tumors, angiogenic switch precedes the stages of progressive myeloma growth in the BM and that CD45- 5T2MM cells compose the angiogenic population.¹³ In the current work we investigated how BM hypoxia and myeloma cells affect each other.

Design and Methods

5T2MM myeloma model

5T2 multiple myeloma cells originate from spontaneously developed myeloma in elderly C57BL/KaLwRijHsd mice.^{14,15} The model was initiated and is continued by intravenous injection of 2×10^6 5T2MM BM cells into young (6-10-week old) syngeneic recipients (Harlan, Horst, The Netherlands) as described previously.¹⁶ Tumor-inoculated mice were monitored for myeloma progression by quantification of serum paraprotein concentration.¹³ Animals were used during the quiescent, pre-angiogenic stage or in the angiogenic stage when terminally diseased, as indicated by hind leg paralysis.¹⁷ BM micro-vessel density (MVD) was assessed by CD31 staining for immunohistochemistry as described in detail elsewhere.¹³ All experiments were approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel, Belgium.

Isolation of BM mononuclear cells

HIF-1 α is rapidly degraded under aerobic conditions and rapidly stabilized under hypoxic conditions. Therefore all procedures were performed on ice and in ice-cold medium containing 0.02% sodium azide or at 4°C as described by others.¹⁸ Animals were killed by cervical dislocation. Long bones were immediately dissected and BM cells were immediately flushed out from femora and tibiae. Bone marrow mononuclear cells (BMMNC) were isolated by centrifugation of the cell suspension on Lympholyte M (Cedarlane, Hornby, Ontario, Canada).

Detection of hypoxia with the pimonidazole hypoxyprobe

HypoxyprobeTM-1 (pimonidazole) Kit for the Detection of Tissue Hypoxia (Chemicon International, Inc., Temecula, CA, USA) was used to detect BM hypoxia *in vivo*. Pimonidazole is a chemical component which specifically binds to proteins in hypoxic cells at an oxygen pressure equal to or lower than 10 mmHg. This component is highly diffusible and has no drug delivery limitations.¹⁹ The formed protein adducts are detected by staining with specific monoclonal antibodies. The amount of adducts formed is proportional to the level of hypoxia.²⁰

Normal control mice or tumor-inoculated animals were intravenously injected with 200 mg pimonidazole/kg body weight in phosphate-buffered saline (PBS) according to the manufacturer's instructions. Sixteen hours after the injection animals were killed and BMMNC were obtained as described above. The plasma half-life of pimonidazole in mice is 0.5 hours²⁰ so 16 hours represent 32 half-lives of the circulating marker. This means that at the time of harvesting a negligible amount [$(1/2)^{32}$] of the initial concentration of hypoxyprobe is present in the animal, reducing background binding to a minimum. Pimonidazole adducts in the BMMNC were detected by FACS. First 1×10^6 BMMNC were fixed in 4.5% formaldehyde, 22% (v/v) acetone in FACS flow (Becton Dickinson) for 10 minutes. Subsequently the cells were permeabilized with 1% saponin and 10% bovine serum albumin in FACS flow and stained with HypoxyprobeTM-1 monoclonal antibodies (mouse IgG₁) for 15 minutes. Rat anti-mouse IgG₁- phycoerythrin (IgG₁-PE), (Becton Dickinson, San José, CA, USA) was used as a second step. Isotype-matched irrelevant antibody was used as a negative control. All incubations were performed at room temperature and in the dark. After staining, samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

Expression of HIF-1 α protein

HIF-1 α protein expression was analyzed by flow cytometry as described by others.¹⁸ With this method a strong positive correlation between the amount of HIF-1 α protein levels and hypoxia levels is observed.¹⁸ The cells (1×10^6) were fixed in 4.5% formaldehyde, 22% (v/v) acetone in FACS flow (Becton Dickinson) and permeabilized with 1% saponin and 10% BSA in FACS flow and stained with 18B9 anti-5T2MM idiotype monoclonal antibodies (mouse IgG₁)¹⁶ and with rabbit anti-mouse HIF-1 α antibodies (R&D systems, Minneapolis, MN, USA). Rat anti-mouse IgG₁-PE (Becton Dickinson) was used as a second step to detect

18B9 binding. Biotinylated donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA, USA) and streptavidin conjugated to phycoerythrin-cyanin-5 (PE-Cy5, Becton Dickinson) were used as secondary and tertiary reagents, respectively, to detect the binding of HIF-1 α antibodies. For some experiments cell surfaces were stained for CD45 expression with rat anti-mouse CD45-fluorescein isothiocyanate (FITC; clone AMS4508; Biosource International, Camarillo, CA, USA) prior to the intracellular HIF-1 α and anti-5T2MM idiotype staining. Isotype-matched irrelevant antibodies were used as negative controls.

Detection of active caspase-3

BMMNC were isolated from 5T2MM-inoculated mice as described above in medium with no sodium azide. Cells ($5 \times 10^6/4$ mL) in 6-well plates in Dulbecco's modified essential medium (DMEM) supplemented with penicillin-streptomycin, glutamine, minimum essential medium (MEM) (GIBCO, BRL, Belgium) and 10% bovine serum (Hyclone, Utah, Logan, USA), were incubated under hypoxic conditions (7.6 mmHg O₂) at 37°C. To obtain hypoxia, plates were placed in sealed chambers and subjected to repeated vacuum evacuation and injection of nitrogen/carbon dioxide-balanced gas containing 1% oxygen.²¹ Cells were also incubated at conventional conditions (5% CO₂ in air, 37°C) After 24 hours' incubation the cells were harvested and fixed and permeabilized as described above and stained with rabbit anti-active caspase-3-FITC antibodies (anti-active caspase-3-FITC, Becton Dickinson), CD45-biotin and 18B9, anti-5T2MM idiotype antibodies. Streptavidin-PE and rat anti-mouse IgG1-peridinin chlorophyll protein (IgG1-PerCP, Becton Dickinson) was used as a second step for CD45 and 18B9, respectively.

Statistical analysis

StatView 5.0.1 software was used for statistical analyses. Simple regression analysis was performed to calculate correlations. The Mann-Whitney U test was used for comparison of two means. *p* values < 0.05 were considered statistically significant.

Results

Binding of pimonidazole hypoxyprobe to BMMNC in control mice and in 5T2MM-diseased mice

To analyze hypoxia in the BM, animals were intravenously injected with pimonidazole, which has been recognized as a standard for *in vivo* imaging and quantification of hypoxia.^{2,19,22} The BM of both control mice BM and tumor-bearing mice contained hypoxic cells (Figure 1A and Figure 1B, respectively). However, in

myeloma infiltrated BM, which is richly neovascularized,¹³ the level of hypoxia was significantly lower (Figure 1C). No staining occurred in animals that had not received hypoxyprobe (Figure 1A). These data clearly indicate that hypoxia exists in normal and myelomatous BM, but that it is decreased in the latter situation.

Expression of HIF-1 α in BMMNC in control mice and in 5T2MM diseased mice

As mentioned earlier HIF-1 α is highly regulated at a protein level by intracellular oxygen concentration and is therefore also a good marker of hypoxia.²³ HIF-1 α expression in BM cells and 5T2MM cells was assessed by flow cytometric analysis. This method allows quantification of HIF-1 α protein at the single cell level in response to different oxygen concentrations.¹⁸ BMMNC isolated from control mice and from 5T2MM-diseased mice expressed HIF-1 α (Figure 2A and Figure 2B, respectively). The expression level was highest in normal BM (Figure 2C). These data confirm the results obtained with hypoxyprobe, that the level of hypoxia is higher in normal BM than in myeloma-infiltrated BM.

Expression of HIF-1 α by 5T2MM cells during pre-angiogenic and angiogenic myeloma stages

We reported recently that 5T2MM disease progression is characterized by predominantly CD45⁺ MM cells in the early pre-angiogenic stage of the disease followed by expansive outgrowth of CD45⁻ MM cells during the subsequent angiogenic stage.^{13,23} We quantified the level of expression of HIF-1 α , as a measure of hypoxia,^{2,3} during the pre-angiogenic and angiogenic stages (Figure 3A and Figure 3B, respectively). During both disease stages HIF-1 α was expressed by CD45⁺ as well as CD45⁻ 5T2MM cells, but the levels in CD45⁻ 5T2MM cells were significantly higher (Figure 3A and Figure 3B). For all groups (total 5T2MM cells, CD45⁺ and CD45⁻ 5T2MM cells) HIF-1 α expression was consistently higher in the pre-angiogenic stage compared to in the respective group in the angiogenic stage (*p*<0.02 for total 5T2MM cells and for CD45⁻ 5T2MM cells, significance not reached for CD45⁺ 5T2MM cells). Analysis of the BM vascularization indicated an inverse correlation between HIF-1 α expression by the 5T2MM cells and the MVD (Figure 3C). These data clearly demonstrate that 5T2MM cells express HIF-1 α . The HIF-1 α expression decreases during disease progression in parallel with an increase of the MVD, suggesting that the native BM hypoxia level is lowered by myeloma-associated angiogenesis.

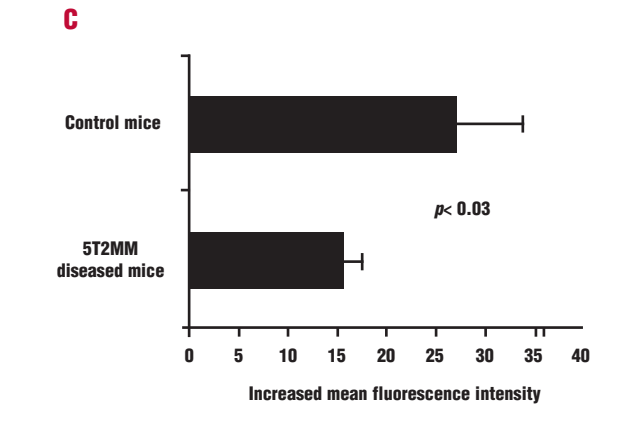
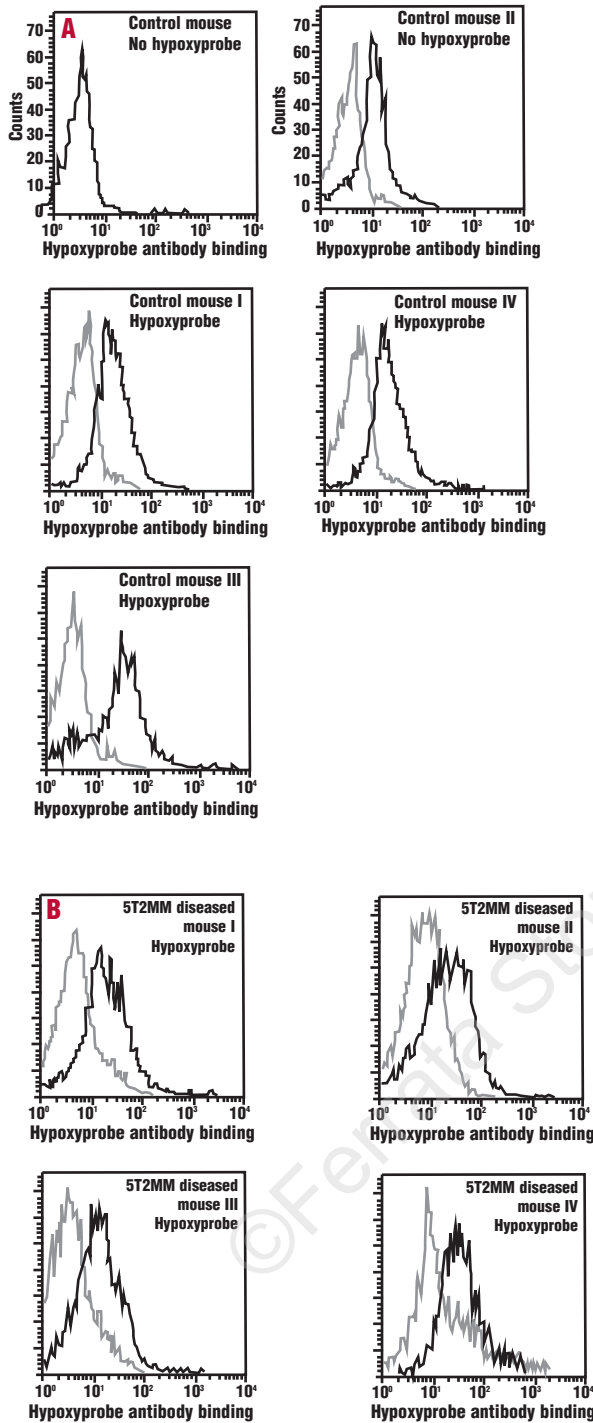


Figure 1. Binding of pimonidazole hypoxyprobe to BMMNC in control mice and in 5T2MM mice. Mice were intravenously injected with hypoxyprobe and after 16h BMMNC were stained for pimonidazole adducts and analyzed by FACS. Panel A illustrates representative histograms obtained from control mice not injected with hypoxyprobe and from all control mice injected with hypoxyprobe (open histograms). Filled histograms illustrate staining with isotype-matched irrelevant antibodies. Panel B illustrates all histograms obtained from 5T2MM-diseased mice injected with hypoxyprobe (open histograms). Filled histograms illustrate staining with isotype-matched irrelevant antibodies. Panel C indicates quantification of the pimonidazole binding. Increase in mean fluorescence intensity compared to control staining is shown. Values represent the mean and SD values of 4 mice.

Caspase-3 activity in CD45⁺ and CD45⁻ 5T2MM cells in hypoxic conditions

Pro-survival as well as apoptotic pathways are activated in cancer cells in response to hypoxia. Cancer cells which cannot adapt to hypoxic conditions undergo apoptotic cell death via activation of caspase-3.² Because the majority of the 5T2MM cells in the early stages of the disease are CD45⁺, we ana-

lyzed whether the hypoxic BM microenvironment exerts a selective pressure on CD45 subsets of 5T2MM cells. For this purpose 5T2MM BM cells were isolated and cultured under normoxic and hypoxic conditions. The presence of active caspase-3 in CD45⁺ 5T2MM cells and in CD45⁻ 5T2MM cells was analyzed by flow cytometry. As shown in Figure 4, caspase-3 activity in CD45⁺ 5T2MM cells was not increased when the cells were incubated in a hypoxic condition rather than in normoxic circumstances. However, in CD45⁻ 5T2MM cells the caspase activity increased significantly under the hypoxic condition. These data indicate that while CD45⁺ 5T2MM cells survive hypoxia, CD45⁻ 5T2MM cells are prone to apoptotic cell death in a hypoxic microenvironment.

Discussion

Unlike most other tumors, MM cells have to survive and to grow in a microenvironment which is already hypoxic by nature. This hypoxic BM microenvironment is crucial for normal hematopoiesis.^{4,6} How BM hypoxia and MM cells affect each other is, however, unknown. In the current work we addressed this question in the 5T2MM mouse model. Injecting the mice with pimonidazole hypoxyprobe indicated that both control BM and

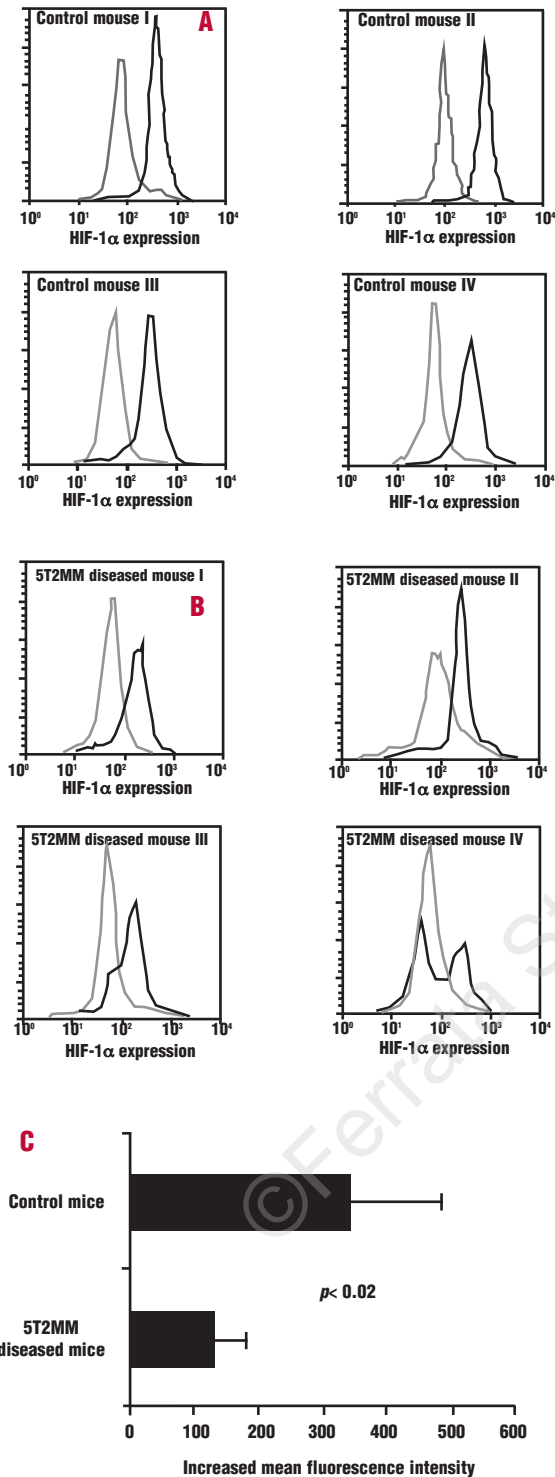


Figure 2. Expression of HIF-1 α in BMMNC in control mice and in 5T2MM-diseased mice. Expression of HIF-1 α was analyzed by FACS staining and analysis as described in Design & Methods. **Panel A.** Histograms obtained from BMMNC isolated from 4 control mice. Filled histograms illustrate staining with isotype-matched irrelevant antibodies. **Panel B.** Histograms obtained from 4 5T2MM-diseased mice. **Panel C** indicates quantification of the HIF-1 α expression. Increase in mean fluorescence intensity compared to control staining is shown. Values represent the mean and SD values of 4 mice.

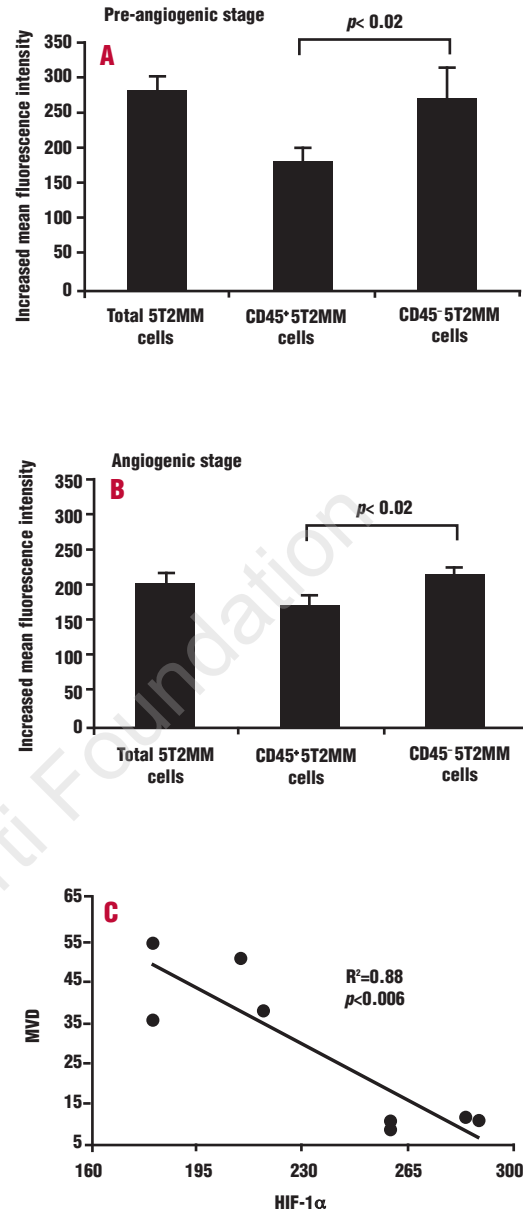


Figure 3. Expression of HIF-1 α by 5T2MM cells during pre-angiogenic and angiogenic stages of myeloma. **Panel A** illustrates quantification of HIF-1 α expression during the pre-angiogenic stage. **Panel B** illustrates quantification of HIF-1 α expression during the angiogenic stage. Increase in mean fluorescence intensity compared to control staining is shown. Values represent the mean and SD values of 4 mice. **Panel C** illustrates the correlation between BM MVD (number of blood vessels/0.2 mm²) and HIF-1 α expression. Each point represents data from one animal. The correlation is based on data obtained from 4 mice in the pre-angiogenic stage and 4 in the angiogenic stage.

myeloma-infiltrated BM are hypoxic. However, the level of hypoxia in myelomatous BM was much lower. These results were confirmed by the pattern of HIF-1 α expression. These data indicate that during 5T2 myeloma disease progression BM hypoxia is decreased, implying that BM oxygenation is

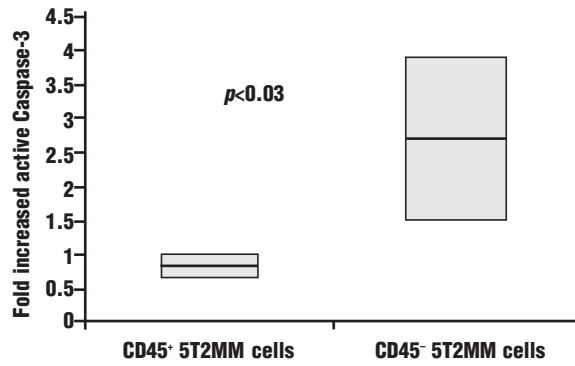


Figure 4. Caspase-3 activity in CD45⁺ and CD45⁻ 5T2MM cells in hypoxic conditions. 5T2MM cells were isolated from the BM and incubated in normoxic and hypoxic conditions. After 24h the cells were stained for 5T2MM, CD45 and active caspase-3 and analyzed by FACS. The figure illustrates the fold increase of active caspase-3 in the hypoxic condition compared to normoxia. Mean (bar) and range values of 4 mice are illustrated.

increased during progression of myeloma. Early stages of the disease are characterized by a pre-angiogenic stage of dormant tumor progression followed by an angiogenic stage of progressive tumor growth.¹³ At the time of clinical presentation, the BM of 5T2MM mice is richly vascularized as in the human situation.¹³ The functional role of angiogenesis in solid tumors is well recognized.¹⁰ Although, in analogy to solid tumors,^{10,11} angiogenic switch,^{13,24,25} angiogenic heterogeneity¹³ and connection of the tumor vasculature to the peripheral blood circulation²⁶ have been reported in MM, the role of these newly formed blood vessels is still subject of debate. Our data of lessened hypoxia suggest that oxygenation in myeloma-infiltrated BM is increased. In addition, the level of HIF-1 α expression was inversely correlated with the level of angiogenesis; there was higher expression during the pre-angiogenic stage than during the angiogenic stage. All together, these data add strength to the hypothesis that myeloma-associated angiogenesis is functional. During the dormant, pre-angiogenic stage a majority of the 5T2MM cells have a CD45⁺ phenotype, are poorly proliferative, but highly invasive and resistant to dexamethasone-induced apoptosis.²³ Analysis of active caspase-3 indicated that a hypoxic microenvironment supports the survival of these cells, but not that of CD45⁻ 5T2MM cells. The majority of the 5T2MM population is CD45⁻ at clinical presentation, in analogy to the human situation.²³ These cells are inoculated in naive mice for continuation of the model; nevertheless, the 5T2MM cells detected in the early stages are predominantly CD45⁺.²³ Previous work from our group

indicated that this is partially due to a higher *in vivo* BM migration of CD45⁺ cells, apart from the fact that some CD45⁻ 5T2MM cells can re-express CD45.²⁷ Data in this current work also suggest that the native hypoxic BM microenvironment favors the survival of CD45⁺ myeloma cells. Based on these results we speculate that during the very early stages of the disease, after migration into the BM, only those myeloma cells which express CD45 survive. The native hypoxic BM microenvironment induces apoptotic cell death of the majority of CD45⁻ MM cells. The angiogenic switch decreases the BM hypoxia and allows outgrowth of the CD45⁻ MM cells. The observation that CD45⁻ 5T2MM cells, but not the CD45⁺ 5T2MM cells, secrete vascular endothelial growth factor,¹³ the key angiogenic factor and a HIF-1 α responsive gene is in line with this finding. Moreover, the highest HIF-1 α protein levels were found in the CD45⁻ 5T2MM cells. In solid tumors it has been demonstrated that a hypoxic microenvironment selects tumor populations resistant to apoptosis.²⁸

Cancer stem cells are a very important new paradigm in the field of cancer research.²⁹ There is accumulating evidence that cancer growth and metastasis rely on stem cells.^{30,31} There are indications that cancer stem cells also exist in myeloma and that these myeloma-initiating cells are contained within the CD45⁺ fraction.³² One of the defining characteristics of cancer stem cells is their ability to thrive under hypoxic conditions.^{5,33} These works support our observations that myeloma disease progression in the 5T2MM mouse model is characterized by predominant CD45⁺ cells in the initial stages²³ and that the CD45⁺ 5T2MM cells are resistant to hypoxia. In addition, cDNA array analysis recently indicated that BM micrometastases of human breast cancer have a distinct molecular signature compared to that of lymph node metastases; in particular upregulation of HIF-1 α was observed, indicating an important role of this pathway in early BM metastasis.³⁴ BM hypoxia is also essential for hematopoietic development.^{35,36} The lesser hypoxia during myeloma progression may thus contribute to the suppressed hematopoiesis in myeloma patients. Mice knockout for HIF-1 β , the obligate dimerization partner of HIF-1 α , die *in utero*³⁷ due to an altered BM stromal environment.³⁸ Experiments with knockouts of another member of the HIF-family, HIF-2 α also demonstrated that a less hypoxic BM microenvironment has a different expression profile of stromal cell adhesion molecules, extracellular matrix proteins and proteases.⁶ As reviewed by Subarsky *et al.* several other groups have demonstrated that in solid tumors the composition of the microenvironment is controlled by hypoxia.¹ Thus,

it is very likely that during myeloma disease progression the altered hypoxia induces an altered BM microenvironment which is more supportive for the survival and expansion of the malignant cells.

The fact that HIF-1 α is important for hematopoiesis indicates that it may not be a selective target for MM. On the other hand, our data encourage anti-angiogenic treatment as initial therapy. Decreased angiogenesis could restore the native BM hypoxia and the native BM microenvironment resulting in apoptosis and suppressed proliferation of the MM cells.

Although it has been reported that HIF-1 α plays an important role in hematopoiesis³⁹ to the best of our knowledge no one has been able to demonstrate its expression in the BM at protein level. Two groups have analyzed HIF-1 α expression in normal BM but no staining was observed on paraffin sections.^{40,41} However, recently the technique of detecting HIF-1 α on tissue sections from clinical samples has been questioned,¹⁸ since under aerobic conditions HIF-1 α is rapidly degraded if samples are not processed with special precautions.

In conclusion, the data presented in this work suggest that BM hypoxia may play an important role in

myeloma biology. The native hypoxic BM microenvironment selects the MM-population for tumor-initiating CD45⁺ MM cells. Hypoxia is lessened during myeloma progression, suggesting that myeloma-associated angiogenesis is functional.

The results obtained are from a single mouse model, which is representative of one type of myeloma patient.

Therefore, experiments with other models using human myeloma cells should be performed to confirm these findings. However, most importantly, this work forms a new foundation for further investigations leading to a better understanding of MM-cell interactions with the BM microenvironment and thus also for other cancer types metastasizing to the BM.

IVR, BVC and KV contributed intellectually to the conception of the manuscript and critically revised the final version. KA and HDR contributed equally to this work.

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