

***In Vitro* Hypoxic Preconditioning of Bone Marrow Stromal Cells Triggers ERK-Mediated Signaling and Growth of L363 Myeloma Cells**

Celulele stromale ale măduvei hematopoietice preconditionate la hipoxie *in vitro* induc proliferarea celulelor mielomatoase L363 și semnalizarea intracelulară ERK-mediată

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Abstract

Multiple myeloma (MM) remains incurable, therefore the nature of events linked to disease progression still needs to be deciphered. **Objective:** We sought to investigate whether hypoxia [low oxygen (O_2) pressure] and bone marrow (BM) stroma, well-documented factors with substantial role in tumor maintenance, can functionally mimic myeloma microenvironment *in vitro*. **Materials and methods:** L363 MM cells were cultured for 24/72 hours under different settings. Normoxia (21% O_2), extreme (1.5% O_2) and moderate (6.2% O_2) hypoxia and BM stromal cells were tested for their impact on L363 cell viability, proliferation and intracellular phospho(p)-ERK expression, either individually, or in combination. All parameters under study were analyzed by flow cytometry and compared to standard L363 cultures (no stroma, no hypoxia). **Results:** When used as independent factors, neither hypoxia, nor the presence of BM stroma significantly affected the viability, proliferation or expression level of p-ERK in L363 cell cultures (24 hours). Nevertheless, when combined, the viability of L363 cells was restored to the levels recorded in paired standard cultures, proliferation significantly accelerated ($p=0.0083$), and p-ERK expression significantly augmented ($p=0.014$). This last effect was observed only when moderate hypoxia was used in combination with BM stroma. Our findings suggest that hypoxic preconditioning activate the bone marrow stroma and consequently enhance L363 cell growth through ERK-mediated intracellular signaling. **Conclusion:** Suitable combination of culture settings provided substantial growth benefits to MM cells in our study. Hypoxic conditions bring about an indirect magnifying growth signal to L363 cells by promoting a stimulatory stromal microenvironment.

Keywords: multiple myeloma, microenvironment, hypoxia, bone marrow stroma.

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Rezumat

Mielomul multiplu (MM) este incurabil, astfel încât natura evenimentelor legate de progresia bolii rămâne de descifrat. **Obiective:** Am urmărit măsura în care hipoxia și stroma maduvei hematopoietice, factori de întreținere ai neoplaziei bine documentați, pot funcționa *in vitro* în mediul micromediu mielomatos. **Materiale și metode:** Linia celulară mielomatoasă L363 a fost cultivată timp de 24-72 ore în prezenta stromei medulare precondiționată hipoxic de la extrem la moderat (1,5% O₂ sau 6,2% O₂). Efectul individual sau combinat al acestor factori asupra viabilității, proliferării și nivelului intracelular al fosfo(p)-ERK a fost testat prin citometrie în flux. Toți parametrii testați au fost analizați comparativ cu cei obținuți în culturile standard (normoxie, fara stroma). **Rezultate:** Nici unul din acești factori nu a afectat independent viabilitatea, proliferarea sau nivelul p-ERK în culturile de 24 ore. Combinate, hipoxia și stroma au restabilit viabilitatea celulară la nivelul celei din culturile standard, au accelerat semnificativ proliferarea ($p=0,0083$) și au crescut semnificativ nivelul de expresie al p-ERK ($p=0.014$). Acest ultim efect a fost observat doar în cazul condiționării hipoxice moderate a stromei. Rezultatele noastre sugerează ca precondiționarea hipoxică activează stroma și astfel intensifică creșterea celulelor L363 prin intermediul semnalizării intracelulare ERK mediate. **Concluzie:** Condiții de cultură adecvate generează un potențial de creștere important pentru celulele L363. Hipoxia induce un semnal indirect de creștere pentru celulele mielomatoase de linie prin promovarea unui micromediu stromal stimulator.

Cuvinte cheie: mielom multiplu, micromediu, hipoxie, stroma medulară hematopoietică.

Introduction

Multiple myeloma (MM) is characterized by the unrestrained growth of malignant plasma cells within the bone marrow (BM), attributed to both cell cycle deregulation and loss of apoptotic control. Despite the accessibility of tumor cell systems suitable for testing the *in vitro* effect of various anti-MM drugs, MM cells grow poorly outside their BM microenvironment. Therefore, attempts to explain the nature of tumor environmental events linked to disease progression in MM have raised broad interest, as these would allow a better understanding of the disease and discovery of novel therapeutic candidates. Both BM stroma (1-6) and hypoxia (7-14) have been shown to support the survival of the malignant clone in various cancers. The microenvironment-controlled tumor cell growth is attributed to the frequently reported increased activity within intra-cellular kinase pathways (1, 2, 7, 8, 11, 14, 15). One of the most important regulators of cell growth is the Mitogen Activated Protein (MAP) kinase pathway, where signaling begins at the cell surface and is finally passed to ERK (Extracellular Signaling Regulated Kinase) through intermedi-

ary elements such as Ras, Raf and MEK. Activated [phosphorylated (p)] ERK translocates to the nucleus, activating transcription factors involved in cell cycle regulation (16, 17).

Therefore, we were interested to investigate whether those conditions providing substantial growth benefits to MM cells (such as hypoxia and BM stroma) can be reliably reproduced *in vitro* in order to develop accessible drug testing systems. Here we describe the influence of these two factors on *in vitro* survival, proliferation and the intracellular phosphorylation level of one important component of the MAP kinase signaling pathway, ERK, in L363 MM cells.

Material and methods

Reagents, solutions and antibodies

Para-formaldehyde was stored as a 4% solution in phosphate buffered saline (PBS) at room temperature. Wash buffer was PBS with 1% fetal calf serum (FCS, Gibco) and 0.1% sodium azide. Freezing medium contained 20% FCS and 10% Dimethyl sulfoxide in RPMI-1640 medium (Sigma). Methanol (90%) was stored at -20°C for at least one hour before use as a permeabilization agent. For flow cytometry surface

staining the following fluorochrome- (fluorescein isothiocyanate-FITC and allophycocyanin-APC) conjugated monoclonal antibodies were used: CD45-FITC (BD Bioscience) and CD38-APC (BD Pharmingen). Intracellular staining was performed with the BD Phosflow phycoerythrin- (PE-) conjugated p-ERK(T202/Y204)-PE, specifically directed against phosphorylated threonine (T) and tyrosine (Y). Stock solutions of 200 μ /ml 7-Amino-Actinomycin D (7-AAD) and 1mM Carboxy-Fluorescein-Succinimidyl-Ester, (CFSE, Molecular Probes) were prepared and stored in small aliquots at -20°C.

MM cell culture

L363, a human MM cell line, was maintained as standard cultures in RPMI-1640 medium (Sigma) supplemented with 10% FCS and 1% penicillin-streptomycin (Gibco). BM stroma cells (BMSCs) were obtained based on their selective adherence to culture flasks, as previously described (18). BMSCs were generated from whole BM aspirate of one volunteer according to institutionally approved protocols and legal form of informed consent. Confluent stroma layers were obtained within 4 to 5 weeks. At the time of confluence, stroma cells were detached from the culture flasks using trypsin-EDTA (Gibco) and viably frozen for later use. When assessing the effect on BMSCs on MM cell growth, 5x10⁵ MM cells/mL/well were cultured in 24-well plates upon confluent layers of BMSCs on the bottom of each well.

MM cell culture under normoxic and hypoxic conditions

Hypoxic conditions were generated within a Thermo Scientific Forma Series II Water Jacketed CO₂ incubator (model 3131) having two-stage CO₂ and N₂ gas regulators, with the possibility to set the desired O₂ level in the range of 0–20%. Reduced oxygen tensions were achieved by purging one chamber of the incubator with purified N₂ (Lindegas) to maintain either 1.5% O₂-5% CO₂-93.5% N₂ (extreme hypoxia) or 6.2% O₂-5% CO₂-88.8% N₂ (moderate hypoxia). Cells were cultured 24 and 72 hours

under these two hypoxic conditions in parallel to standard (normoxic) cultures using room atmosphere (*circa* 21% O₂). All three conditions used fully humidified atmospheres at 37°C supplemented with 5% CO₂. Experiments involving L363 cells were performed in triplicates under different culture conditions where hypoxia and BMSCs were used either as independent or as combined factors.

Detection of p-ERK in MM cells by flow cytometry

Frozen aliquots of L363 cells were thawed, washed once in FCS-free PBS, 300 \times g, 5 min, then aliquoted in 50 μ L cell suspensions (up to 1x10⁶ cells) per each FACS tube. Cells were fixed with 1:1 volume of 4% para-formaldehyde, 10 min, 37°C, centrifuged in 2 mL wash buffer, and stained at surface with CD45-FITC (1:25) and CD38-APC (1:50), 1 hour, on ice. After one wash step, cells were permeabilized with 1 mL ice-cold 90% methanol, 30 min, on ice, washed again and stained with p-ERK(T202/Y204)-PE (1:5), 1 hour, on ice. Unstained controls were also prepared and used as negative controls, according to the fluorescence minus one procedure discussed in details elsewhere (19). Data acquisition was performed using a FACSCanto II flow cytometer (BD Bioscience). Up to 10000 events were acquired and saved as data files. The same settings for gains, voltages and spectral overlap corrections were used for all measurements. Data acquisition was accomplished with FACS Diva software (version 6.1) and data analysis with FlowJo software (Tristar Inc.).

MM cell viability assessment by 7-AAD incorporation

Cells were harvested and single cell suspensions were prepared in wash buffer. After one wash step, cells were incubated in 1 μ g/mL 7-AAD, 10 minutes, on ice and percentages of 7-AAD positive (dead) cells were measured by flow cytometry.

CFSE-proliferation assay

Before culture, cell suspensions were

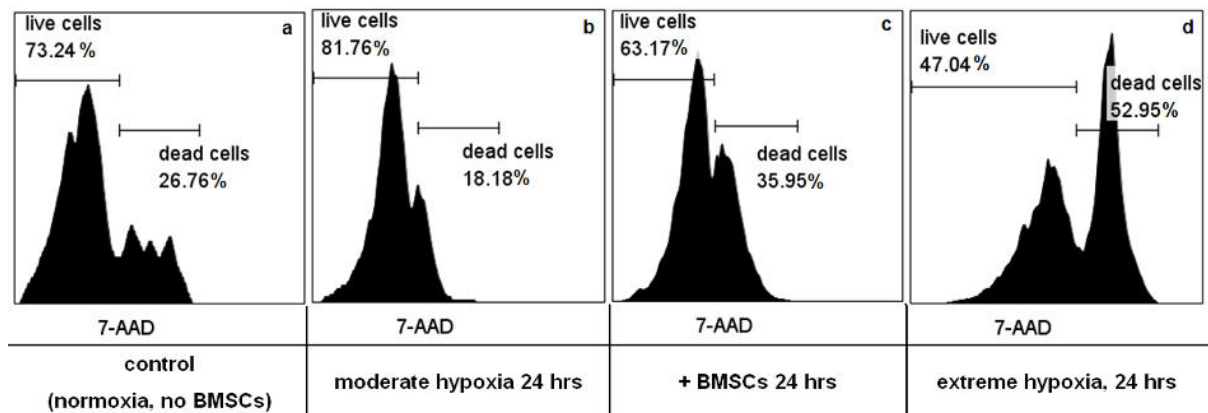


Figure 1. Representative example illustrating the flow cytometry measurement of viable L363 MM cells under different culture settings. L363 MM cells were cultured for 24 hours under different settings. Each culture condition was analyzed for its impact on L363 MM cell viability, either as single factors or as combinations of two factors. Percentages of viable cells were assessed by flow cytometry, based on the segregation of L363 cells in 7-AAD positive (dead) and negative (live) cells. Histograms illustrate one representative analysis example of L363 cells cultured under standard conditions (a), moderate hypoxia (b), BMSCs (c) or extreme hypoxia (d). Data acquisition was performed with FACSDiva software and data analysis with FlowJo software. BMSCs=Bone Marrow Stromal Cells. 7-AAD=7-Amino-Actinomycin D.

prepared as 1×10^7 /mL in PBS. CFSE was added to a final concentration of $1.25 \mu\text{M}$ and then cells were mixed gently and incubated with occasional shaking for 10 minutes at 37°C . To stop the staining and to prevent the CFSE efflux, an equal volume of pre-warmed FCS was added. Cells were washed twice at $300 \times g$, 5 minutes, cultured under specified conditions, then brought to 5×10^6 /mL and analyzed by flow cytometry. As CFSE transported into the cells during incubation binds covalently to cytoplasmic proteins, when efflux completely blocked, the analysis of cell division can be determined by flow cytometry, through the CFSE decreasing intensity. With each cell division, the fluorescent intensity per cell division is reduced 50%, thus providing readout of the mitotic activity within a specific population of cells.

Statistical analysis

When appropriate, statistical analysis was performed by means of paired Student's *t* tests. The minimum level of significance was considered at $p < 0.05$. For flow cytometry data, FlowJo-software assisted calculation of median

fluorescence intensity (MFI), median fluorescence intensity ratio (MFIR, as the ratio between MFI of sample versus control), and percentages of positive cells was performed.

Results

L363 MM cell viability is distinctly affected by different culture conditions used

Different culture conditions were analyzed for their impact on L363 cell viability, either as single factors (BMSCs/ moderate hypoxia/ extreme hypoxia), or as combinations of two factors (BMSCs+moderate hypoxia/ BMSCs+extreme hypoxia). Percentages of viable cells were assessed by flow cytometry, according to the 7-AAD negative pattern expressed by L363 cells, as illustrated in *Figure 1* in a representative example of the analysis.

Differences obtained in standard-versus each of the tested conditions were calculated and results of triplicate experiments for each culture condition were displayed as means \pm standard deviation (SD) (*Figure 2*).

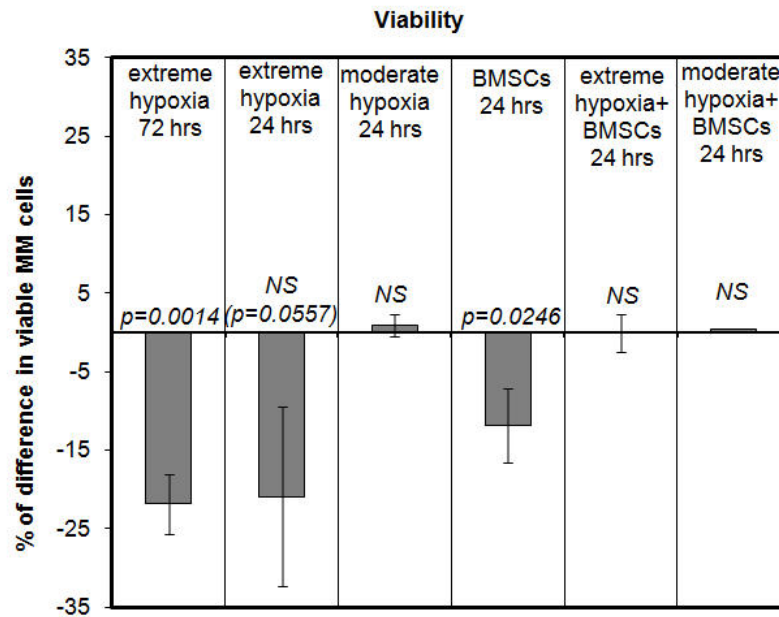


Figure 2. Survival kinetics of L363 MM cells under various culture conditions. Different culture conditions mimicking the BM microenvironment were tested for their impact on L363 MM cell viability, either as single factors (BMSCs alone, moderate hypoxia alone, extreme hypoxia), or as combinations of two factors (BMSCs+moderate hypoxia or BMSCs+extreme hypoxia). Percentages of viable L363 cells grown under these settings, assessed by flow cytometry according to their 7-AAD negative pattern, were provided by the software-assisted statistics of data analysis. Differences obtained in standard- vs. each of the tested conditions were calculated and results of triplicate experiments for each culture condition are here displayed as means +/- SD. Statistically significant differences were achieved by means of paired Student's t tests (p values), and the minimum level of significance was considered when $p < 0.05$. BMSCs=Bone Marrow Stromal Cells. 7-AAD=7-Amino-Actinomycin D. SD=Standard Deviation. NS=not-significant.

Extreme hypoxia decreased the viability of L363 cells after 70 hours of culture in a significant manner ($p=0.0014$), while, after 24 hours, the effect, although still severe, was not significant ($p=0.0557$). L363 cells cultured for 24 hours under moderate hypoxia did not show significant changes in terms of their viability, while the presence of BMSCs led to a significant ($p=0.0246$) decrease of L363 viability. Initially, these results challenged the probity of such culture conditions as *in vitro* reproducible models of the BM environment and contributed to our decision to eliminate the prolonged (72 hours) time culture from further experiments. When BMSCs were used for 24 hours in combination with both types of hypoxia, the viability of L363 was restored to the levels recorded in paired standard cultures (Figure 2).

Hypoxia and BMSCs significantly augment L363 cell proliferation

The impact of different culture conditions (single factors or combinations of two factors) on L363 proliferation was analyzed. The influence of extreme hypoxia was not tested. Proliferation rates were assessed by flow cytometry, based on the distribution of CFSE fluorescence. One representative example of the analysis by flow cytometry is illustrated in Figure 3.

Recorded MFI values of CFSE in each culture setting, when subtracted from those of standard cultures, served as a reflection of the differential proliferation rate under different conditions. Differences obtained in standard- versus each of the tested conditions were calculated and results of triplicate experiments for each culture condition

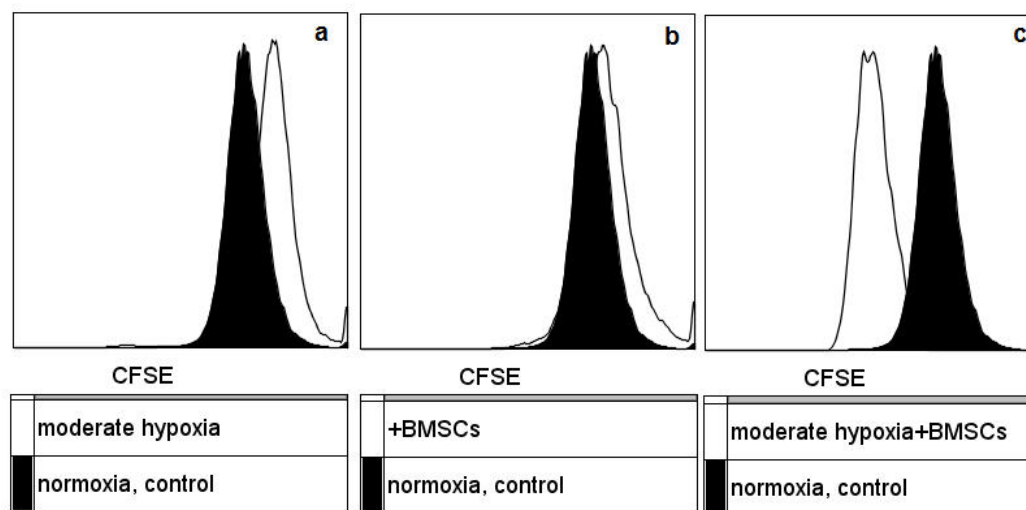


Figure 3. Representative example illustrating the flow cytometry assessment of L363 MM cell proliferation under different culture settings. CFSE-labeled L363 MM cells were cultured for 24 hours under various settings mimicking the BM microenvironment. Proliferation rates assessed by flow cytometry were directly proportional to the descent of CFSE fluorescence within the 24 hours of culture. Histogram overlays display the CFSE fluorescence level of L363 MM cells cultured under standard conditions (solid histograms) vs. the CFSE fluorescence level of L363 MM cells recorded under the presence of moderate hypoxia (a), BMSCs (b) or BMSCs+moderate hypoxia (c). Data acquisition was performed with FACSDiva software and data analysis with FlowJo software. BMSCs=Bone Marrow Stromal Cells. CFSE=Carboxy-Fluorescein-Succinimidyl-Ester.

were displayed as means \pm SD (Figure 4).

Although both moderate hypoxia and the presence of BMSCs used independently slowed down the growth of L363 within 24 hours of culture (Figure 4), none of the single-factor condition tested induced significant change in L363 proliferation rates. Nevertheless, a significant acceleration of L363 growth ($p=0.0083$) was triggered by the combined presence of the two factors for 24 hours (Figure 4), since the CFSE fluorescence levels decreased abruptly in comparison to standard cultures (Figure 3c).

Two hypoxic (extreme versus moderate) culture conditions have opposite outcomes on the phosphorylation level of ERK in L363 MM cells in the presence of BMSCs

Since ERK, a key component within the intracellular MAP kinase pathway, was found to be constitutively activated (phosphorylated) in L363 cells, it was of interest to measure the difference between p-ERK levels

in standard cultures versus each of the culture conditions involved. The phosphorylation level of ERK was investigated by flow cytometry and MFIR values obtained were directly related to the ERK phosphorylation level. In order to avoid any artifactual result, MM cells were gated based on the software-assisted exclusion of non-viable cells and cell doublets, as illustrated in Figure 5.

Different culture conditions were analyzed for their impact on p-ERK level in L363 cells, either as single factors (BMSCs alone, moderate hypoxia alone, extreme hypoxia alone), or as combinations of two factors (BMSCs + moderate hypoxia or BMSCs + extreme hypoxia). Differences between MFIR obtained in standard- versus each of the tested conditions were calculated and results of triplicate experiments for each culture condition were displayed as means of differences between paired experiments \pm SD (Figure 6).

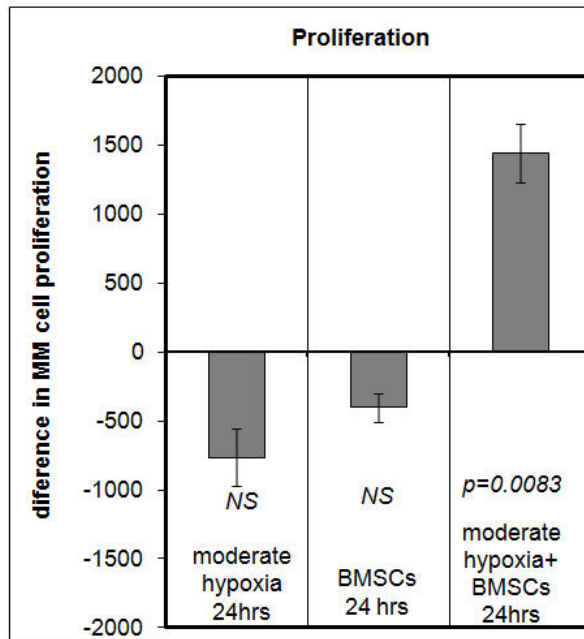


Figure 4. Proliferation kinetics of L363 MM cells under various culture conditions. The 24 hour impact of single factors (moderate hypoxia or BMSCs) or combinations of two factors (BMSCs+moderate hypoxia) on the *in vitro* proliferation of L363 MM cells was comparatively assessed. MFI values of CFSE from standard cultures were subtracted from those of each culture setting and differences obtained in triplicate experiments for each culture condition are here displayed as means \pm SD. Statistically significant differences were achieved by means of paired Student's t tests (p values), and the minimum level of significance was considered when $p < 0.05$. BMSCs=Bone Marrow Stromal Cells. CFSE=Carboxy-Fluorescein-Succinimidyl-Ester. MFI=Mean Fluorescence Intensity. SD=Standard Deviation. NS=not-significant.

Although p-ERK slightly decreased under extreme (Figure 6a) or moderate (Figure 6b) hypoxia, while in the presence of BMSCs it remained constant, none of the single-factor conditions tested induced significant modulation of p-ERK in L363 cells. Conditions based on a combination of two factors induced opposite effects on p-ERK modulation: moderate hypoxia + BMSCs significantly increased the level of p-ERK ($p=0.014$) (Figure 6b), while extreme hypoxia + BMSCs led to an important (though not-significant, $p=0.051$) decrease of p-ERK (Figure 6a). Potential grounds for this apparent discrepancy are discussed below.

Discussions

Our ability to develop tumor cell culture systems resembling the *in vivo* microenvironment holds important clinical and pre-clinical applications. Once those factors with critical role in tumor maintenance have been identified, they need to be tested under appropriate *in vitro* settings that mimic conditions with substantial growth benefits to tumor cells.

In MM, the interaction of tumor cells with their BM microenvironment has been described as a critical process linked to tumor progression. Both hypoxia and BM stroma have been shown to support the survival of the malignant clone (7-10, 12). Therefore, we sought to test whether these two factors (used either independently, or in combination) have a favorable impact on MM *in vitro* cell growth (survival and proliferation) and on the ERK – dependent intracellular signaling process.

In our hands, hypoxia and BM stroma had a combined favorable influence on MM cells, as the presence of both factors enhanced L363 cell viability, accelerated cell growth by increasing proliferation, and increased the phosphorylation level of ERK, a key player in one of the most important growth-/survival-signaling pathway. None of the factors tested had the expected beneficial effect on L363 cell growth when used independently. Similarly, none of the changes induced by single-factor settings were significant, except for the drastic decline in L363 cell viability mediated by BMSCs in 24 hours and by extreme hypoxia (1.5% pO_2) at any time points tested. Although previous studies report an undoubtful tumor-sustaining role of BMSCs in MM, our results are only apparently conflicting.

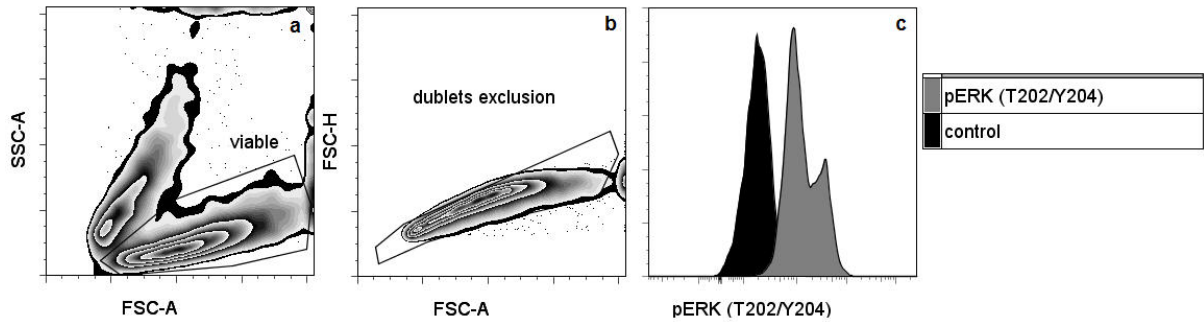


Figure 5. Representative example illustrating the flow cytometry detection and measurement of p-ERK (T202/Y204) within L363 cells. Flow cytometric analysis of p-ERK(T202/Y204) was performed in viable MM cells, identified by exclusion of apoptotic cells (cells with low FSC values and variable SSC) on a FSC/SSC graph (a) and by the software-assisted exclusion of cell doublets on a FSC-Area vs. FSC-Width graph (b). The histogram overlay (c), displays the expression level of p-ERK (black histograms) vs. appropriate negative controls (grey histograms). Data acquisition was performed with FACSDiva software and data analysis with FlowJo software.

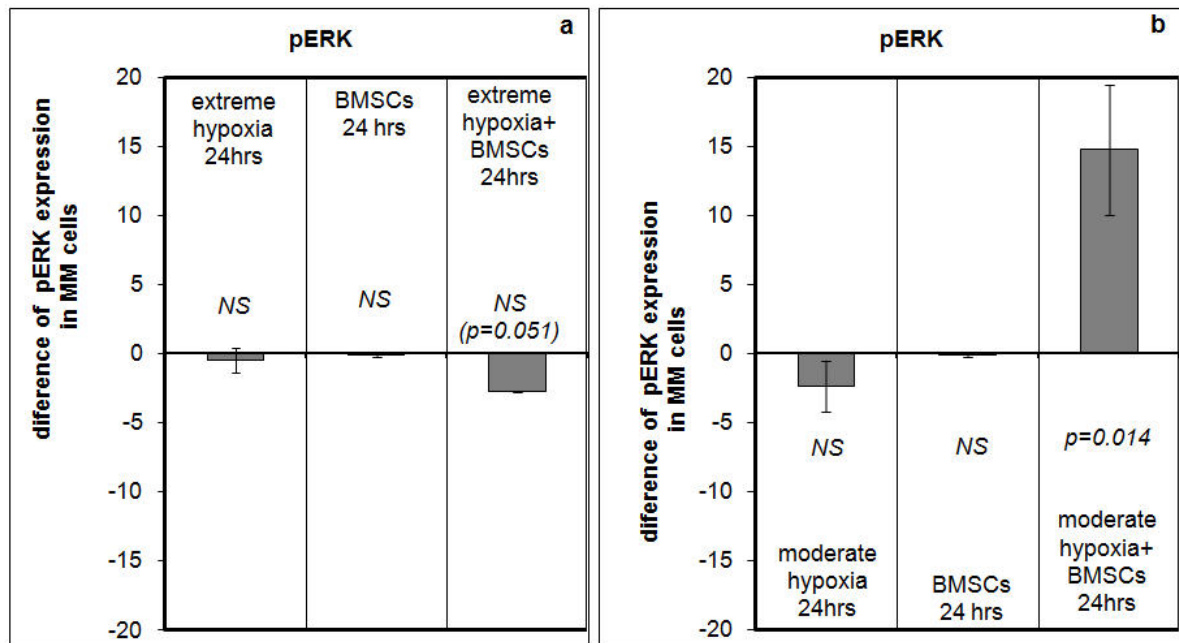


Figure 6 The impact of various culture conditions mimicking the BM microenvironment on the activation (phosphorylation) level of p-ERK within L363 MM cells. Different culture conditions were analyzed for their 24 hour impact on p-ERK level in L363 cells. The influences of two factor combinations [BMSCs+extreme hypoxia (a) or BMSCs+moderate hypoxia (b)] were comparatively analyzed. The phosphorylation level of ERK was measured by flow cytometry as MFIR values. Difference between MFIR obtained in standard- vs. each of the tested conditions were calculated. Graphs display the results of triplicate experiments for each culture condition as means \pm SD. Statistically significant differences were achieved by means of paired Student's t tests (p values), and the minimum level of significance was considered when $p < 0.05$. MFIR=Mean Fluorescence Intensity Ratio. SD=Standard Deviation. NS=not-significant.

The inhibitory effect of MM cell growth conferred by BMSCs after 24 hours found by us may be explained by the fact that L363 cells show a slower growth rate during the first culture period, with a more accelerated course from day 3 to day 6, while BMSCs confer the highest growth advantage to MM cells only after day 6 (18). Additionally, in co-culture systems some competition for the same nutrients may occur, hence, variable period of time for adjustment may be required. However, our results showing the unfavorable influence of extreme hypoxia, both on short, or on long term, couldn't be reasonably explained and, initially, called into question the proper relevance of such culture conditions.

When combinations of two factors were tested in 24 hour cultures, the viability of L363 (negatively affected by hypoxia) was restored to standard levels by the co-presence of BMSCs (Figure 2). Concordantly, significant acceleration of L363 proliferation was observed under the combined influence of BMSCs and hypoxia (Figure 4).

The analysis of p-ERK expression level in L363 generated the most intriguing finding of our study, as, depending on the type of hypoxia used in combination, opposite p-ERK modulation was triggered in L363 cells. In the presence of BMSCs, moderate hypoxia significantly increased, while extreme hypoxia severely lowered the level of p-ERK in L363 cells (Figure 6). Such differential kinetics suggests an indirect effect of moderate hypoxia on L363 growth. Under moderate (but not extreme) hypoxia, BMSCs may become activated themselves and then may secrete hypoxia-induced growth factors and/or express functional receptors, becoming an efficient growth support for MM cells. Similarly, extreme hypoxia may not have a stimulatory effect on BMSCs.

Our findings are supported also by studies showing that the degree of hypoxia in BM tissues is different from that in solid organs. Although most tumors have much lower oxygenation than in the corresponding tissue of

origin (20), what stand true for solid neoplasias may be questionable for hematological malignancies, including MM. Conventional studies use very low O₂ conditions when investigating hypoxia as a tumor supporting factor either in non-hematological (20, 21) or in hematological (2, 7, 11, 22) malignancies. Whether the BM itself is a hypoxic environment has long been speculated, and only few studies clearly indicate an overall BM O₂ pressure ranges from 6 to 7.2% in healthy subjects (23) and in patients with non-hematological (24) or hematological (22) malignancies.

In **conclusion**, taken together, all these data imply a need to reconsider the degree of reduced O₂ when developing *in vitro* tumor cell culture systems based on settings mimicking the BM natural environment. Such systems, once appropriately optimized, may further be translated into accessible drug testing systems with significant clinical and preclinical relevance.

Acknowledgements

This study was made possible by financial support from the Romanian Ministry of Education and Research, The National University Research Council (Consiliul National al Cercetarii Stiintifice din Invatamantul Superior), research grant PN-II-ID-PCE-2007, code 1181/2007.

Conflicts of interest: none.

Abbreviations

7-AAD = 7-Amino-Actinomycin D,
APC = allophycocyanin,
BM = bone marrow,
BMSCs = BM stroma cells,
CFSE = Carboxy-Fluorescein-Succinimidyl-Ester,
ERK = Extracellular Signaling Regulated Kinase,
FCS = fetal calf serum,
FITC = fluorescein isothiocyanate,
MAP = Mitogen Activated Protein,
MFI = median fluorescence intensity,

MFIR = median fluorescence intensity ratio,
 MM = multiple myeloma,
 NS = not-significant,
 P = phosphorylated,
 PBS = phosphate buffered saline,
 PE = phycoerythrin,
 SD = Standard Deviation,
 T = threonine,
 Y = tyrosine.

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