The effect of hypoxia and hypoxic preconditioning on human bone marrow mesenchymal stem cells

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Introduction

Stem cells have long been regarded as undifferentiated and capable of indefinite proliferation, self-renewal, and provision of a large number of differentiated cells. Certain cells in postnatal tissues retain their ability to divide and self-renew, and have been categorized based on their differentiation capacity: hematopoietic, bone, epithelial muscle-, neural-, and hepatic stem cells, and so on. Adult stem cells are able to give rise to the cells of their tissue of residence. Mesenchymal stem cells (MSC) were first found in the rodent bone marrow (BM), as non-hematopoietic, multipotent progenitors. MSC are adherent to plastic in culture, capable of colony formation and differentiation into osteoblasts, adipocytes and chondrocytes *in vitro*. Consequently, stromal cells with colony forming and multipotent differentiation capacity were isolated from a perivascular position in many postnatal organs. The main role of MSC is to maintain stem cell niches. A single colony forming unit fibroblast (CFU-F), isolated from the bone marrow, when transplanted ectopically (subcutaneously), can differentiate into all the cell types of a miniature bone-organ (ossicle), is able to recreate the hematopoietic microenvironment (HME) and capable of self-renewal, during serial transplantation. MSC also have been shown to secrete a wide range of pro-angiogenic factors, and have been proposed to have a key role in the support of blood vessel formation and regeneration, given their perivascular position and the still growing body of evidence. Also, cultured MSC were shown to modulate the tissue response to injury by numerous studies. Due to the promising results shown in preclinical models, the relatively easy isolation and culture expansion, the low risk of immune-rejection and the lack of ethical

concerns, MSC have become popular candidates for regenerative cell therapy, and have made one of the fastest transitions from the bench to the clinic. However, MSC therapy has yielded modest, even neutral results in terms of efficacy in the cardiovascular field.

Accordingly, various strategies have been investigated in order to improve the survival and retention of stem cells after injection, for example, hypoxic, hyperoxic and pharmacological preconditioning of MSC. Hypoxia exerts strong effects on MSC, affecting their proliferation, differentiation and migration. However, studies have yielded controversial results, which might be caused by the variation in the conditions that have been tested, including time- span (ranging from minutes to days) and level of hypoxia: from 0.1 to 10% O_2. It has been demonstrated previously that hypoxic preconditioning enhances the therapeutic potential of MSC in applications such as the treatment of cardiac ischemia, critical limb ischemia, traumatic brain injury, and in liver regeneration. The further understanding of MSC biology and response to hypoxia, especially in terms of the secretion of angiogenic factors could be remarkably useful for enhancing the efficacy of MSC-based therapy (Bianco et al 2013). It has been long established that lipids (fatty acids and their derivatives and substances related biosynthetically or functionally to these compounds) are not only essential building blocks of biological membranes and responsible for energy storage in the cell, but also have important regulatory and signaling functions in almost all cellular processes, such as hypoxic signaling (Hannun and Obeid, 2008). To date, little is known about the overall lipidomics of human MSC, or changes in lipid composition in response to stimuli such as hypoxia.

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Aims of the study

The aims of the first set of experiments were (1) to examine the effects of various levels of hypoxia on MSC, and (2) to study the effects of hypoxic exposure prior to their administration to a hypoxic site, which we refer to as hypoxic pre-conditioning (HP). We sought (3) to determine the optimal hypoxia levels and incubation times to promote survival of MSC in an environment of low oxygen and nutrients. In addition, (4) we sought to evaluate the underlying mechanism(s) that promote the survival of MSC and hypothesized, that hypoxic pre-conditioning promotes metabolic adaptations in MSCs that allow increased survival under conditions of limited nutrients and oxygen. To further study the effect of hypoxia on mesenchymal stem cells, our aims were (5) to determine the lipid composition of human bone marrow derived MSC, (6) to identify changes induced by exposure to hypoxia and (7) to evaluate whether these changes have a role in the angiogenic potential of MSC.

Methods

Isolation and culture of MSC

Bone marrow aspirates from human donors were purchased from Lonza (Walkersville, USA) or Stem Express (Placerville, USA) and mesenchymal stem cells were isolated as described previously. For studies with hypoxia, cell cultures were performed in incubators at 37°C with 5% CO_2, humidified atmosphere and dedicated oxygen level (20 (atmospheric), 10, 5 or 1% O_2), as established by replacement with nitrogen injections.

Cell proliferation and cell cycle analysis

During proliferation assays, cells were lifted at the given time points with trypsin treatment and counted using Trypan blue exclusion dye and a hemocytometer. To determine the status in cell cycle, cells were lifted, permeabilized with ethanol, incubated with propidium iodide, and evaluated by flow cytometry.

In vitro cell survival and apoptosis detection

To evaluate survival of MSC in hypoxia and serum deprivation, we first incubated MSC in standard culture medium and 1% O_2, for varying amounts of time (0 (control group), 16, 48 or 96 hours). Then, culture media was changed to MEMα alone (without FBS) and all plates were transferred to 1% O_2 for up to 12 days with no additional media changes. Every three days, cells were counted. To quantify the percentage of dead cells, MSC were cultured on glass cover slips and incubated for 48 hours in standard culture medium in 20% O_2 (control) or 1% O_2 (HP). Then, medium was changed to serum free medium and plates were transferred to 1% O_2 for 9 days with no additional media changes. Cover slips were then incubated in 4 μM Ethidium-homodimer III, images were acquired using a fluorescence microscope, and analyzed for automated counting. For apoptosis detection, cells were lifted using trypsin, stained with phycoerythrin-Annexin V-Apoptosis Detection Kit I and measured by flow cytometry.

In vivo retention study

First, we generated luciferase-expressing MSC using the lentiviral vector with the general form pCCLc-MNDU3-Luciferase-PGK-eGFP-WPRE. Cells were isolated from 3 donors, ad cultured in either 1% or 20% O_2 for 48 hours. For cell administration, immune compromised NOD/SCID-IL2Rγ$^-$/$^-$ (NSG) mice were injected in the medial hamstring muscles with 2 x 10^5 luciferase-expressing MSC in 20 uL of HyStem C (14 animals per group), and cell retention was followed for 28 days using in vivo imaging system (IVIS).

Glucose and lactate measurements

Supernatants were collected every 3 days from MSC cultured under identical conditions to the *in vitro* survival assay, and the glucose and lactate measurements were performed using a Glucose or Lactate Colorimetric assay kit.

Ultrahigh pressure liquid chromatography- quadrupole time-of-flight tandem mass spectrometry (UHPLC- QTOF-MS/MS)

MSC were cultured in either 1% or 20% O_2 for 48 hours, lifted with trypsin, and processed for lipidomics analysis as described by Fiehn and Kind (2007). Lipidomics data were acquired using UHPLC – QTOF MS/MS. Chromatographic separation was followed by electrospray ionization (ESI) in both positive and negative mode; and QTOF-MS/MS. Data were analyzed in a four-stage process. Annotated lipids are abbreviated as: [Lipid class] [total carbon number in the chains]:[total number of double bonds in the chains].

Measurement of DG levels by enzyme-linked immunosorbent assay (ELISA)

MSC were cultured in 20% or 1% O_2, with or without PI-PLC enzyme blocker D609 (50 μM)). MSC in normoxia were also tested in the presence of the hypoxia-mimicking agent Cobalt Chloride (100 μM). After 48 hours, cells were lifted by trypsin treatment, and lipids were extracted as described previously by Bligh and Dyer (1959). To measure DG levels, we used the Human Diacylglycerol ELISA Kit.

Detection of angiogenic factors

MSC were cultured as described for DG measurements, and VEGF, interleukin-8 and angiopoietin-2 protein levels were detected in the supernatant media by the respective ELISA kits. To measure mRNA levels of VEGF, interleukin-8 and angiopoietin-2, total RNA was extracted, reverse transcription, then real time PCR were performed using TaqMan primers/probes.

Wound/scratch assay

Conditioned media of MSC were prepared as described for DG measurements, and stored at -80 ^{o}C. VeraVecs endothelial cells were seeded in plates containing plastic inserts, which leave a homogeneous 500 μm gap in between confluent monolayers of cells. The next day, inserts were removed and media was changed to the conditioned media from MSC. Pictures were taken immediately, and 10 hours after adding conditioned media and analyzed using TScratch software. Wound closure was determined as [open image area after 10h / initial open image area] x 100.

Statistical analysis and presentation of data

All experiments were performed at least three times with MSC derived from different donors. The specific number of biological replicates for each experiment is indicated in the respective figure legend as n. In accordance, results are shown as average with the standard error of the mean (SEM) as error bars. When only two conditions were tested, a Student's t-test was used, where significant differences are denoted as: * $p < 0.05$ and ** $p < 0.005$. In contrast, in experiments where four conditions were tested, analysis of variance (ANOVA) or a Student's t-test with Bonferroni correction were applied, with significant differences from control indicated as * $p < 0.0125$. In lipidomics experiments, values are given as mean +/- SEM. A paired Student t-test was used to determine statistically significant differences established as $p < 0.05$ (see in the respective results sections).

Results

Proliferation and differentiation is inhibited by hypoxia in a dose-dependent manner

We tested the effect of varying levels of hypoxia (1%, 5%, 10% and 20% O_2) on proliferation and differentiation of human BM-MSC, and found that hypoxia caused a decrease in the proliferation and diffentiation of MSC, proportional to the degree of hypoxia, as assessed by proliferation assays, cell cycle analysis and adipogenic and osteogenic differentiation assays. Culture under 1% O_2 had the strongest effect on MSC, while 10% O_2 seemed to only have a mild effect on the proliferation and differentiation of MSC.

Hypoxic pre-conditioning increases survival of MSC *in vitro*

To simulate an environment poor in nutrients and oxygen, cells were transferred to 1% O_2 in serum-free media for 12 days. We observed that for all experimental conditions, no living cells were present after 12 days under serum deprivation and hypoxia. However, MSC that received hypoxic preconditioning for 48 or 96 hours showed an approximately 2-fold increase in survival at 6 days under serum deprivation and hypoxia as compared to control MSC, while 16 hours of hypoxic preconditioning had no effect on survival as compared to controls. To further support these results, MSC were stained with EthD-III or Annexin V, and we found that after 9 days in hypoxia and serum deprivation, 48 hour- HP-MSC showed over a 2-fold reduction in dead cells as compared to controls, and there were significantly less apoptotic cells in the HP-MSC group, than in the control MSC group.

Hypoxic preconditioning enhances retention of MSC *in vivo*

We injected luciferase- expressing MSC intramuscularly into immune deficient mice and followed their retention over time. First, we established a correlation between IVIS- detected luminescence and cell number, generating a standard curve by injecting increasing numbers of MSC into the medial hamstring muscles of immune deficient NSG mice and measured luminescence immediately thereafter. We then addressed cellular retention with and without hypoxic preconditioning using the standard curve to determine the number of retained cells after injection over time. We found that MSC cultured under 20% O_2 prior to injection, are retained for one week after transplantation, but start to decrease rapidly thereafter and are undetectable at

28 days after cell injection. In contrast, we could detect MSC with 48-hour hypoxic preconditioning after 28 days. Cell retention of HP- MSC was also significantly better at day 14 and day 21 after administration as compared to control MSC.

Hypoxic preconditioned-MSC have more glucose available during serum-deprivation *in vitro*

We first cultured cells under 1% O_2 for 0, 16, 48, and 96 hours, and found that glucose levels of supernatant media are significantly higher in MSC cultured in hypoxia for 96 hours, and that lactate levels were increased in MSC cultured in hypoxia as compared to controls. In the next set of experiments, after 3 days in serum deprivation and hypoxia, glucose levels were over 2-fold higher, and lactate levels were significantly lower in supernatant media of 48 and 96 hours HP- MSC, as compared to controls (without HP).

Lipid composition of MSC

MSC from 5 healthy human donors were cultured for 48 hours in 20% or 1% O_2, and processed for total lipid analysis by mass spectrometry. Using a HPLC- ESI- QTOF MS/MS method, a total of 1,965 different molecular ions were detected. 390 ions (21.2%) could be identified as putative lipid species using the most comprehensive lipid database to date, LipidBLAST. From these, phosphatidylcholine species accounted for 76.5% of all identified lipids, followed by sphyngomyelin (11%), phosphatidylethanolamine (2%) and triglyceride (2%) species. Altogether, PC, SM, PE and TG accounted for over

90% of all annotated lipids detected in MSC. The lipid detected most abundantly was PC (18:1/16:0) accounting for 13.1% of all annotated lipids.

Hypoxia induces changes in the lipid composition of MSC and increases the level of diacyglycerols

In order to identify changes in lipid composition of MSC induced by hypoxia, we performed two types of analysis. First, we grouped the detected species by lipid class and performed a Student's t-test to identify significant differences by class between normoxia and hypoxia. We found that TG, DG and fatty acids were significantly increased in MSC exposed to hypoxia by 4.8, 1.8 and 1.3-fold respectively, as compared to MSC in normoxia. 41 lipid species (10.5%) were significantly increased in hypoxic MSC as compared to controls. We found that each of the 7 detected DG species was significantly increased in MSC under hypoxia. We confirmed these results measuring DG levels with ELISA method, in hypoxia and normoxia, and in the presence of the hypoxia mimicking agent $CoCl_2$. To evaluate the possible effects of increased DG levels in MSC in hypoxia, we used D609, an inhibitor of PC-PLC and SMS. We found that addition of D609 (50 μM) reduced DG levels in MSC cultured under hypoxia. Interestingly, D609 did not reduce DG levels in MSC under normoxia.

Reduction of diacylglycerol levels with D609 affects secretion of angiogenic proteins in MSC

After incubation of MSC for 48 hours in normoxia or hypoxia, with or without D609, supernatant conditioned media were collected and the amount of secreted VEGF, CXCL8/IL-8 and Ang-2 were measured by ELISA. We

observed a significant increase in VEGF secretion in MSC exposed to hypoxia, while addition of D609 (i.e. the reduction of DG levels) caused a decrease in VEGF levels in MSC in both normoxia and hypoxia. In contrast, secreted IL-8 levels were reduced by hypoxia, while addition of the enzyme inhibitor D609 increased IL-8 secretion in MSC in both normoxia and hypoxia. Secreted levels of Ang-2 by MSC were overall very low. However, we detected a slight decrease in Ang-2 secretion in MSC exposed to hypoxia, as compared to controls, while addition of D609 reduced Ang-2 secretion even further in MSC in both normoxia and hypoxia. Overall, these trends are very similar at mRNA levels, measured after incubation for 12 hours in hypoxia. However, only mRNA levels of VEGF and Ang-2 showed a similar trend to the respective secreted protein levels. Our results show that VEGF and Ang-2 are both regulated transcriptionally by hypoxia, however all other differences in mRNA levels in between conditions were not significant.

Reduction of DG levels in MSC limits their potential to induce migration of endothelial cells *in vitro*

To further investigate whether the increase of DG in MSC under hypoxia could exert a functional effect, we prepared conditioned media from MSC cultured under normoxia or hypoxia and in the presence or absence of D609. We then tested whether these conditioned media affected the migration of human endothelial cells *in vitro*, using a wound healing assay. We found that endothelial cells migrated less in the preconditioned media from D609 treated hypoxic MSC than non-treated hypoxic MSC. However, D609 treatment did not cause a difference in the migration-inducing capacity of MSC in normoxia.

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Conclusions

Despite the paramount of data about paracrine factor secretion and beneficial effects on tissue repair in preclinical models, most clinical trials have failed to show robust recovery in cardiovascular disease patients, with the regenerative capacity of MSC somehow 'lost in translation'. Indeed, as discussed above, various factors are possibly responsible for this phenomenon, which highlights the utmost importance of conducting well-designed experiments focused on the nature, basic biology and differentiation potential of MSC. On the other hand, efforts must be aimed at improving the survival and engraftment rates of the injected cells. In my opinion, the combined application of stem cell therapy and various biomaterials could lead to a more successful path in the future.

It has been previously demonstrated that hypoxic preconditioning enhances the therapeutic potential of MSC in applications such as treatment of cardiac ischemia (Hu X, et al. 2008, He A et al 2009), critical limb ischemia (Rosova et al 2008, Huang et al 2014, Leroux et al 2010), traumatic brain injury (Chang et al 2013), and in liver regeneration (Yu J et al 2013). One of the key factors in the hypoxic preconditioning- induced enhancement of tissue repair seems to be the increased retention of MSC in tissues (Hu et al. 2008, Huang et al 2014 Leroux et al 2010), but the underlying mechanisms remain elusive. We tested the effects of varying levels of hypoxia on proliferation and differentiation of human MSC, and observed that hypoxia caused a decrease in the proliferation of MSC, and inhibited their osteogenic and adipogenic differentiation proportionally to the degree of hypoxia. Our conclusion is that the proliferation and differentiation of MSC are inhibited by hypoxia in a

dose-dependent manner, and that the lowest level of oxygen used, 1% O_2 (equivalent to 10 mmHg O2 in the culture media) exerted the strongest inhibitory effect on MSC, especially during osteogenic differentiation.

We also addressed the effect of HP on the survival of cells upon new exposure to hypoxia, and found 1% oxygen is an optimal level for preconditioning, and established an optimal duration of preconditioning: in our experiments, 16 hours of HP did not have a profound effect on survival, while 48 and 96 hours of HP transitorily promoted cell survival to a similar degree in an *in vitro* environment with limited oxygen and nutrients. Importantly, 48 hours of HP significantly increased cell retention after *in vivo* transplantation. Overall, our results strongly encourage, at both the clinical and research level, pre-incubation of MSC in hypoxia prior to transplantation to enhance their retention. We also investigated the underlying mechanism(s) that promote the survival of hypoxic preconditioned MSC and found that glucose levels are significantly higher in MSC cultured in hypoxia for 96 hours, suggesting that under this condition MSC use up less glucose as compared to normoxic controls. After 3 days in serum deprivation and hypoxia, glucose levels were over 2-fold higher in supernatant media of 48 and 96 hours hypoxic preconditioned MSC, as compared to controls (without hypoxic preconditioning). We propose that, under limited glucose availability, hypoxic preconditioned MSC survive for a longer period of time due to a lower rate of glucose consumption, thereby allowing more glucose availability over a longer period of time.

Further understanding MSC biology and response to hypoxia, especially in terms of the secretion of angiogenic factors could be remarkably

useful for enhancing the efficacy of MSC-based therapy (Bianco et al 2013). It has long been established that lipids are crucial first and second messenger molecules (van Meer et al., 2008) and it has also been shown that lipids are involved in hypoxic signaling (Ueda et al 1998, Vlassaks et al 2013). Analyzing the lipid composition of MSC using HPLC-QTOF-MS/MS and the LipidBLAST library, we found a strong up-regulation of many lipids in MSC exposed for 48 hours to hypoxia and especially noteworthy was the increase of all detected DG species. To study the effects of increased DG levels in hypoxia, we used D609, to reduce overall DG levels (Adibhatla et al 2012), and evaluated the effect on the angiogenic factors secreted by MSC. In line with previous publications, we observed a significant increase in VEGF secretion in MSC exposed to hypoxia, while D609 caused a decrease in VEGF levels in MSC in both normoxia and hypoxia. In contrast, secreted IL-8 levels were reduced by hypoxia, while addition of D609 increased IL-8 secretion in MSC in both normoxia and hypoxia. Secreted levels of Ang-2 by MSC were overall very low. Altogether, our results suggest that DGs are involved in the secretion of VEGF, IL-8 and Ang-2 in MSC in both normoxic and hypoxic conditions. We also tested whether conditioned media from MSC cultured under either normoxia or hypoxia and in the presence or absence of D609 affect the migration of human endothelial cells in vitro, using a wound healing assay. Our results indicate that conditioned media from MSC cultured under hypoxia promote endothelial cell migration to a similar extent than MSC under normoxia. However, under hypoxia, addition of D609 reduced the migration of endothelial cells, suggesting that the increase of DG in MSC under hypoxia is an important mechanism to alter the angiogenic secretome of MSC. This suggests that the specific signals of MSC promoting migration of

endothelial cells are distinct under normoxia and hypoxia, where under hypoxia signals are more dependent on DG levels. The results of our experiments measuring DG levels in response to hypoxia and D609 also strengthen this argument, since D609 treatment for 48 hours inhibited DG levels in MSC only under hypoxia. Although these are *in vitro* results, cautioning their interpretation, it is interesting to note, that blocking the increase in DG levels in hypoxia seems to have functional consequences in the migration-inducing capacity of MSC.

The changes in lipid composition presented here offer new insight into the changes that MSC undergo under hypoxia, which are important rather from the perspective of the bench, but could impact the therapeutic application at the bedside.

List of publications

Publications related to the dissertation:

1. Beegle J, Lakatos K, Kalomoiris S, Stewart H, Isseroff RR, Nolta JA, Fierro FA. Hypoxic preconditioning of mesenchymal stromal cells induces metabolic changes, enhances survival, and promotes cell retention in vivo. Stem Cells. 2015;33(6):1818-28.
2. Lakatos K, Kalomoiris S, Merkely B, Nolta JA, Fierro FA. Mesenchymal Stem Cells Respond to Hypoxia by Increasing Diacylglycerols. J Cell Biochem. 2016;117(2):300-7.

Publications not related to the dissertation:

3. Kalomoiris S, Cicchetto AC, Lakatos K, Nolta JA, Fierro FA. Fibroblast Growth Factor 2 Regulates High Mobility Group A2 Expression in Human Bone Marrow-Derived Mesenchymal Stem Cells. J Cell Biochem. 2016;117(9):2128-37.
4. Nardai S, Dobolyi A, Skopál J, Lakatos K, Merkely B, Nagy Z. Delayed Gelatinase Inhibition Induces Reticulon 4 Receptor Expression in the Peri-Infarct Cortex. J Neuropathol Exp Neurol. 2016;75(4):379-85.
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6. Lakatos K, Dékány G, Lendvai Zs, Berta B, Molnár L, Becker D, Nagy Z, Merkely B, Skopál J. Adrenaline induced platelet aggregation in patients with coronary artery disease undergoing stent implantation. Cardiologia Hungarica 2012; 42 : 106–111