HYPOXIA/HYPERCAPNIA PREVENTS IRON-DEPENDENT COLD INJURIES IN CORD BLOOD STEM AND PROGENITOR CELLS

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Running title: Stem cell maintenance in hypothermia

Abstract

Background: Cold-induced cell injuries are associated with an increase in the cellular labile iron pool followed by lipid peroxidation and alteration of mitochondrial function which lead to cell death. Recently, we showed that incubation in a hypoxic/hypercapnic (HH) gas mixture improved the survival of a population of cord blood hematopoietic progenitors and stem cells CD34⁺ in severe hypothermia. In order to explain the underlying mechanism, here we test if this HH-induced cytoprotection in cold conditions is associated with the level of labile iron pool (LIP) and lysosome stability.

Methods: Cord blood CD34⁺ cells were incubated in air (20 %O₂/0.05 % CO₂) or in the hypoxic (5 % O₂)/hypercapnic (9 % CO₂) atmosphere for 7 days at 4°C and analysed.

Results: Incubation in HH condition maintained the day 0 (D-0) level of LIP detected using a bleomycin-dependent method. This was associated with preservation of lysosome integrity and a higher cell survival. Conversely, in the air condition LIP was significantly increased. Also, the presence of a moderate concentration of iron chelator deferoximine improves the conservation of total CD34⁺ cells and committed progenitors in air condition. Pre-treatment of CD34⁺ cells with the lysomotropic agent imidazole induces significant decrease in the lysosomal stability and in all conditions. This is associated with the important decrease of survival of conserved cells and increase in the cellular LIP level.

Discussion: Our study showed that HH gas mixture cytoprotection during hypothermia maintains lysosome stability, which enables preservation of the cellular chelatable iron in the physiological ranges. These findings suggest a way to optimize cell conservation without freezing.

Keywords: CD34⁺ cells; conservation; hypoxia; hypercapnia; hypothermia, iron; lysosomes.

Abbreviations: labile iron pool (LIP); reactive oxygen species (ROS); hematopoietic stem and progenitors cells (HSC/HPC); hypoxic/hypercapnic gas mixture, (HH); propidium iodide (PI); Colony Forming Cell (CFC); deferoxamine (DFX); ethylenediaminetetraacetic (EDTA); bicinchronic acid (BCA); acridine orange (AO); 7-amino-actinomycin (7-AAD); Colony Forming Unit–Spleen (CFU-S).

Introduction

Storage in severe hypothermia (4°C), for more than few days, triggers cellular injuries. It is generally proposed that the primary cause of damage comes from the cold-induced mismatch between ATP supply and demand, leading to a cellular energy deficit. Consequently, the failure of ion-motive ATPases is followed by an impairment of ionic balance. This leads to membrane depolarization and an uncontrolled influx of Ca^{2+} through voltage-gated Ca^{2+} channels, resulting in the activation of Ca^{2+} -dependent hydrolases, and in turn provoking further membrane depolarization, uncontrolled cell swelling and ultimately, cell necrosis [1, 2]. These events are potentiated by the behaviour of plasma membrane lipids in hypothermia which undergo lipid phase transition interfering with membrane function, leading to ions leakage [3].

More recent studies have shown in various cell types that cold-induced cell injuries are associated with an increase in the cellular chelatable labile iron pool (LIP), initiating the formation of reactive oxygen species (ROS) [4-7]. The increase in ROS levels is usually followed by a massive lipid peroxidation, alteration of mitochondrial function and loss of lysosome integrity, which can lead to cell necrosis in hypothermia or apoptosis upon rewarming [4].

Hematopoietic stem and progenitors cells (HSC/HPC) are very sensitive to hypothermia, and their optimal storage conditions (conservation medium, temperature, duration) are critical for the successful stem cell transplantation [8].

Surprisingly, the mechanisms of the cold-induced injuries of HSC/HPC have been poorly explored. Recently, we described a method for prolonged cold storage enabling functional preservation of cord blood (CB) HSC/HPC in the hypoxic/hypercapnic (HH) gas mixture [9, 10]. In order to explain the underlying mechanism, here we test if this HH-induced cytoprotection in cold is associated with the level of LIP and lysosome stability.

Material and methods

CD34⁺ cell processing

Purification. CB samples delivered (with the mother's approval) to the Cell Therapy Unit of the French Blood Institute, Bordeaux, rejected for banking, were used in experiments. CD34⁺ cells from umbilical CB were isolated using an immunomagnetic technique (Miltenyi Biotec, Paris, France).

Conservation. CD34⁺ cells (5×10^4 /ml) were plated in Stem α S3 (Stem Alpha SA, Saint Genis, l'Argentière, France), incubated in air (20 % O₂/0.05 % CO₂) or in the hypoxic (5 % O₂)/hypercapnic atmosphere (9 % CO₂) for 7 days at 4°C. All the tests were performed with purified (D-0) and stored CD34⁺ cells after 7 days at 4°C (D-7). In order to obtain the desired gas mixtures, the open flasks with cell suspension were incubated in a O₂ and CO₂ culture chamber equipped with O₂ and CO₂ controllers (PRO-OX and PRO-CO₂, Biospherix, Lacona, NY) for 1h at room temperature then plugged and transferred to 4°C conditions for 7 days. In some variants of the experiment, deferoxamine (DFX) (Sigma Aldrich) was added to the conservation medium. For the estimation of the effect of the lysosomotropic agent on the cell conservation, cells were pre-incubated for 1h at 37°C with 250 mM imidazole (Sigma Aldrich) in Dulbecco's Modified Eagle's medium (Thermo Fischer Scientific) supplemented with 2 % foetal calf serum (HyClone Perbio, Bezons, France) and 10 mM HEPES buffer (Sigma Aldrich), washed and resuspended in conservation medium before transfer to 4°C. Cells were harvested at D-7 and then analysed.

Apoptosis assay

Apoptosis was determined with Annexin V-FITC kit (Beckman Coulter, Roissy, France) following the manufacturer's protocol. Briefly, 10^5 CD34⁺ cells were labelled with Annexin V-FITC solution (AnnV)

and propidium iodide (PI) (10 mg/ml) for 15 min at 4°C, then washed in phosphor buffer saline and analysed with a flow cytometer (Beckton Dickinson FACSCanto II).

Hematopoietic committed progenitor cell determination-Colony Forming Cell (CFC) assay

The committed progenitors CFCs (colony-forming unit–granulocyte, monocyte [CFU-GM] plus burst-forming unit–erythroid [BFU-E]) were assayed as previously described [9]. Briefly, D-0 and D-7 CD34⁺ cells (250 cells/ml and 600 cells/ml, respectively) were seeded in methylcellulose Stem Alpha-1D (Stem Alpha, Stem Alpha Saint Genis l'Argentière, France) supplemented with Recombinant human stem cell factor (SCF), Recombinant human interleukin 3 (IL-3), Recombinant human erythropoietin (EPO) and recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF). After 14 days incubation, CFCs were counted.

Ferritin content measurement

The assay was performed using 10⁵ CD34⁺ cells harvested at D-0, or D-7 conservation in air or HH conditions. Total proteins were extracted with RIPA buffer (50 mM Tris /HCl pH 7.5, 150 mM NaCl, 1 % Triton X 100, 0.5 Na-deoxycholate, 0.1 mM cocktail of protease inhibitors, 1mM ethylenediaminetetraacetic (EDTA); the reagents were purchased from Sigma Aldrich) and quantified with a bicinchronic acid (BCA) (Thermo Fischer Scientific) based method. Ferritin content was determined in protein extracts according to the manufacturer's instructions (Human Ferritin ELISA Kit, Sigma Aldrich). Quantity of ferritin was determined with biotinylated detection antibodies in the presence of horse radish peroxidase conjugated streptavidin. Trimethylbenzidine substrate was added and the intensity of absorbance of the coloured product was measured at 450 nm (Evolis, Biorad).

Lysosome integrity analysis

Lysosome stability was determined by acridine orange (AO, Molecular Probes) staining, as showed previously [11]. Briefly, 10^5 CD34⁺ cells were treated with AO (25 nM) at 37°C for 30 min. To exclude dead cells, 7-amino-actinomycin (7-AAD) was added (BD Bioscience). After rinsing, the step pellet was resuspended in phosphate buffer saline (Lonza, Verviers, Belgium) and analysed with a flow cytometer. Excitation was obtained with argon laser (488 nm) and the acquisition was effected in the phycoerythrin emission spectra using filter 585/40 nm.

Bleomycin-based labile iron determination

LIP was measured using bleomycin-detectable iron assay [12]. All the solutions used in the assay were Chelex 100 (Sigma Aldrich) treated in order to eliminate residual iron. All the reagents were purchased from Sigma Aldrich, it being stated in the article otherwise. CD34⁺ cells, harvested at D-0 and D-7 of conservation, were washed with Hank's Balanced Salt Solution (Thermo Fisher Scientific) and then lysed (TRIS/HCl at 20 mM (pH7.4) with TRITON X-100 1 %. Cell lysates were mixed with 0.25 ml of calf thymus DNA (1 mg/ml), 0.05 ml of 5 mM MgCl₂, 0.025 ml of bleomycin sulphate (1 mg/ml) (Calbiochem), and ascorbic acid (8 mM). After incubation at 37°C for 30 min in a shaking water bath, malondialdehyde is produced proportionally to the quantity of Fe²⁺ ions. The reaction was stopped with 0.5 ml of 0.1 M EDTA. The contents were then mixed with 0.5 ml of 1 % thiobarbituric acid (in 50 mM NaOH) and 0.5 ml 25 % HCl, v/v and incubated at 95°C in order to transform the malondialdehyde into a coloured product. Intensity of absorbance was read at 532 nm (Specord, Analytik Jena). Values were calculated using the 0.10 ml of a 0.25–20 µM FeCl₃ as a standard.

Statistical analysis

The **Wilcoxon-Mann-Whitney** comparison test was used to examine the statistical significance of the differences between the various experimental conditions ($p < 0.05$ was considered statistically significant).

Results

Conservation in HH maintains LIP at the steady-state level

The labile iron pool (LIP) is thought to be involved in causing cell death during hypothermia [5]. In order to examine if HH induces the same phenomenon, we measured bleomycin-dependent LIP in our conditions [13]. Our results showed that a 7-day exposure to HH maintained the day 0 (D-0) level of LIP detected using bleomycin-dependent method. This D-0 content of LIP cells we refer to as the physiological (steady-state) LIP level. Conversely, in the atmospheric air usually used for cell storage at 4°C, cellular LIP was significantly increased compared to D-0 level (Figure 1A).

The presence of an iron chelator in the conservation medium DFX improved the maintenance of CD34⁺ cells in a dose-dependent manner in air condition, indicating that iron dependant injuries underline cell degradation in the cold. However, when in the HH condition - where LIP is maintained in the physiological range - the presence of DFX did not significantly increase total cell maintenance (Figure 1B)

In addition, committed progenitors (CFCs) are significantly better preserved in the presence of a moderate concentration of DFX in air condition. But, stronger iron chelation by a high concentration of DFX abrogates the maintenance of CFCs, **especially erythroid progenitors BFU-E, and this in all experimental conditions**. This implies that iron is indispensable for CFCs survival. In addition, our results suggest that the increase of LIP above this physiological range, as well as its drop below

steady-state level induced by DFX-chelation, abrogates the survival of hematopoietic progenitors (Figure 1C).

Conservation in HH maintains lysosome integrity and ferritin content

Hypothermic cell injuries might be attenuated by the chelation of LIP with a high iron storage protein ferritin-dependant mechanism [13]. In order to determine if the HH condition displays its cytoprotective effect via the ferritin dependant mechanism, we examined cellular ferritin content. Our results showed that at D-7 there is no statistical difference between experimental conditions, with results similar to the ferritin level detected in the D-0 CD34⁺ cells (Figure 2A).

Lysosomes contain redox active iron that could leak into the cytoplasm when the lysosomal integrity is altered. The release of this iron and hydrolytic enzymes may contribute to subsequent cell death and cold-induced cell injuries [14]. In view of this, lysosome integrity upon incubation in hypothermia was analysed by acridine orange (AO), fluorescent dye taken up by proton trapping and retained inside acidic organelles such as lysosomes [11]. After 7 days of incubation in hypothermia and air condition mean fluorescence intensity (MFI) of AO is significantly diminished compared to the D-0 level, indicating important lysosome degradation. By contrast, in HH condition, MFI is similar to D-0 level of CD34⁺ cells, indicating that lysosomes are protected from degradation when hypothermic incubation takes place in the HH mixture (Figure 2B).

Preservation of lysosome integrity using HH mixture during hypothermia correlates with cell survival and stabilisation of LIP level

In the order to discern the relationship between the HH-mediated maintenance of lysosome integrity and cell survival during hypothermic storage, we pre-treated CD34⁺ cells with a lysomotropic agent

imidazole. Our results showed that imidazole induces a significant decrease in lysosomal stability after 7 days of incubation, and this in a similar manner in air- and HH conditions compared to D-0 (Figure 3A). This alteration of lysosome integrity correlates with the significant increase in LIP level compared to D-0 in both conditions (Figure 3B). Also, it coincides with a high decrease in survival of conserved CD34⁺ cells in either gas mixture (Figure 3C).

Discussion

Our study indicates that cytoprotection in HH against severe hypothermia is mediated by the stabilisation of lysosome integrity, and that iron-dependant cold-induced injuries are critical for the survival of CB CD34⁺ cells. We show here that this issue is valid for total CD34⁺ cell population (consisting mainly of various hematopoietic progenitors and rare stem cells [15]) as well as its CFC fraction.

Lysosomes contain redox-active iron and a wide spectrum of hydrolytic enzymes, which could provoke death when released into the cytoplasm [16]. Also, lysosomes have been identified as being particularly susceptible to hypothermia; a decline in lysosome integrity is consistent with cold-induced loss in cell viability [14]. These observations have been proved for the various cells types and organs [14, 17-19] and here we reported similar data for the hematopoietic progenitors.

In our study, when lysosomal stability is compromised, the cytoprotective effect of HH during hypothermia is significantly abrogated, which correlates with an increase in LIP and a decrease in cell viability (Figure 3).

Our results fit with previously published data showing that one of the consequences of alteration of lysosome integrity on cell survival during hypothermia, is the release of chelatable iron [5, 14, 20]. In presence of the ROS, which were shown to have increased in hypothermic conditions (other authors' and our data [5, 9]), the oxidative iron-dependant Fenton-type reactions can take place [5]. It was

reported that this might result in massive lipid peroxidation which, in turn, attacks the lysosomes but also other cell membrane structures, and leads to cold-induced injuries [16].

Interestingly, our work suggests that LIP, but not oxidative stress, is particularly important in the occurrence of cold-induced injuries and cell death in hematopoietic progenitors. More specifically, we showed recently that HH cytoprotection is ruled out despite a sustained ROS production [9]. Also, in this study, the maintenance of LIP within the physiological range is crucial for the survival of hematopoietic progenitors, since its increment above steady-state level abrogates HH-mediated cell protection in hypothermia (Figure 3).

Thus, we propose that functional preservation of CD34⁺ cells in hypothermia by HH involves stabilisation of the lysosome membrane, which prevents release of LIP that could provoke deadly cold-induced injuries in the presence of hypothermia-produced ROS. This raises the question of how the HH gas mixture stabilises the lysosome membrane in the first place? A possible explanation lies in the fact that hypercapnic condition creates a slightly acidic environment (intra and extra cellular) when in hypothermia, we have shown this previously [9]. In hypercapnic condition CO₂ diffuses across the membrane, enabling a permanent load of carbonic acid and protons in the cytosol. In these conditions, the plasma membrane lipid bilayer is protected from cold-induced injuries [7, 9]. In addition to the plasma membrane, this protecting effect could be applied to all other cellular membrane structures including the lysosome.

Thus, according to our previous work and these findings, we suggest that preservation of CD34⁺ cells during hypothermic storage is mediated by the double action of HH gas mixture: hypercapnia diminishes the occurrence of cold-induced injuries, while hypoxia prevents apoptosis upon rewarming after the transfer to 37°C [9, 10, 21].

Since LIP has a critical role in cell survival during hypothermic incubation of cultured cells, improved cell preservation was accomplished by the utilisation of conservation medium supplemented with

iron chelators [5, 7]. In this study, DFX added in the conservation medium improved cell viability only in the conditions where LIP was above steady-state (D-0) level in CD34⁺ cells (Figure 1).

At 37°C, DFX is internalised by endocytosis and localised exclusively in lysosomes, where it act as a chelator of intralysosomal iron, stabilising the lysosomes [22]. At 4°C endocytosis is blocked, the DFX remains in the extracellular compartment, where it removes the iron from the extracellular space. But, since ~~extracellular~~ labile iron could pass through the membrane, the presence of DFX in the extracellular compartment [23] could diminish intracellular iron too.

With our method, the DFX is added to the conservation medium during the preparation phase dedicated to obtaining the desired gas mixture, which is done at room temperature. At this temperature, DFX could slowly enter the intracellular compartment by endocytosis but could not be trafficked to the lysosomes[24]. Thus, with this method, the DFX could capture the iron from the intra as well as the extracellular environment.

On the other hand, our results showed that a decrease below steady-state level of LIP with the highest dose of DFX is not compatible with the survival of **committed** hematopoietic progenitors in either gas mixtures (Figure 1C). These results were expected since it has been shown in the model of anemic Belgrade laboratory (b/b) rat [25], that severe deficiency of intracellular iron provoked by the mutation of the iron transport protein Nramp2 [26] blocks the proliferation of the hematopoietic progenitors (in this case Colony Forming Unit–Spleen, CFU-S) [27]. This was completely overpassed by the treatment of b/b rats with a high dose of molecular iron or a non-toxic dose of hemin [25].

With our method, incubation in HH preserves functional CB stem and progenitor cells significantly more efficiently than other models of cold storage without freezing [9, 10]. Here we revealed that lysosome stability and iron-dependant cold-induced injuries are the critical issue for hematopoietic progenitor cold preservation. In this regard, we think that the addition of iron chelators or stabilisers of the lysosome membrane are the way to optimise CB storage survival. Our approach could be an alternative to freezing in clinical practice in some situations. This should enable avoiding

cryopreservation of the hematopoietic grafts intended for transplantation shortly after collection (some cases of autologous and intra-familial cord blood transplantation) or for long-distance transport. Also, it would facilitate organization and improve results of autologous or allogeneic transplantation procedures.

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Conflict of interest: The authors declared that they have no conflicts of interest.

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Figure legends

Figure 1. Chelatable iron-dependant conservation in hematopoietic progenitors in hypothermia. A) Bleomycin-detectable labile iron pool is estimated in CD34⁺ cells at D-0 and D-7 of incubation at 4°C in air or HH gas mixture. The bars are presented as a mean $\pm$ SD of 8 independent experiments. B) Conservation of CD34⁺ cells presented as the average fold change in the number of viable cells (Annexin V/PI⁻) detected in the presence of DFX relative to the number of cells detected in the same experiment without DFX. C) Conservation of the erythroid, granulocyte-monocyte and totals committed progenitors in the presence of the growing concentrations of DFX in air or HH gas mixture. The bars are presented as a mean number of the progenitors relative to D0 level $\pm$ SD of 6 independent experiments. The asterisks indicate a significant difference at $p < 0.01$ (**) (*), Wilcoxon-Mann-Whitney.

Figure 2. Ferritin content and lysosome integrity in CD34⁺ cells during hypothermia. A) Level of ferritin is determined in CD34⁺ cells at D-0 and D-7 of incubation at 4°C in air or HH gas mixture. B) Stability of lysosomes is evaluated by acridine orange (AO) using flow cytometry in CD34⁺ cells at D-0 and D-7 of incubation at 4°C in air or HH gas mixture presented in the histogram. One representative of five independent experiments is shown. AO mean fluorescence intensity (MFI) is presented as the mean $\pm$ SD. The asterisks indicate a significant difference at $p < 0.01$ (**) vs D-0 condition, Wilcoxon-Mann-Whitney test.

Figure 3. Conservation of CD34⁺ in hypothermia pre-treated with imidazole. A) AO staining of lysosomes in CD34⁺ cells at D-0 and D-7 of incubation at 4°C in air or HH gas mixture. B) The bleomycin-detectable labile iron pool is estimated in CD34⁺ cells at D-0 and D-7 of incubation at 4°C in air or HH gas mixture. C) CD34⁺ cell survival after 7 days of incubation at 4°C in air or HH gas mixture. Cell survival is presented as the % of viable cells (Annexin V/PI⁻). The bars are presented as a mean $\pm$ SD of 5 independent experiments. The asterisks indicate a significant difference at $p < 0.01$ (**) and $p < 0.05$ (*), Wilcoxon-Mann-Whitney test. MFI, Mean fluorescence intensity. IMI, imidazole.

Figure 1

A)

B)

C)

Figure 2

Figure 3