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Molecular Imaging Studies on CD133⁺ Hematopoietic Stem Cells From Human Umbilical Cord Blood

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1. Introduction

During the last decade, there has been enormous progress in understanding both multipotent stem cells such as hematopoietic stem cells and pluripotent stem cells such as embryonic stem cells and induced pluripotent stem cells. However, it has been challenging to study developmental potentials of these stem cells because they reside in complex cellular environments and aspects of their distribution, migration, engraftment, survival, proliferation, and differentiation often could not be sufficiently elucidated based on limited snapshot images of location or environment or molecular markers (Jang et al., 2011). Therefore, reliable imaging methods to monitor or track the fate of the stem cells are highly desirable. Both short-term and more permanent monitoring of stem cells in cultures and in live organisms have benefited from recently developed imaging approaches that are designed to investigate cell behavior and function. Transmission electronic microscopy (TEM) and series of noninvasive imaging technologies enable us to investigate cell behavior in the context of a live organism. In turn, the knowledge gained has brought our understanding of stem cell biology to a new level.

Cell based therapeutics are emerging as powerful regimens. To better understand the migration and proliferation mechanisms of implanted cells, a means to track cells in living subjects is essential, and to achieve that, a number of cell labeling techniques have been developed. Nanoparticles, with their superior physical properties, have become the materials of choice in many investigations along this line. Owing to inherent magnetic, optical or acoustic attributes, these nanoparticles can be detected by corresponding imaging modalities in living subjects at a high spatial and temporal resolution. These features allow

implanted cells to be separated from host cells; and have advantages over traditional histological methods, as they permit non-invasive, real-time tracking *in vivo* (Bhird et al., 2011).

Superparamagnetic iron oxide nanoparticles and magnetic resonance imaging provide a non-invasive method to detect and label hematopoietic stem cells. These nanoparticles exhibit unique properties of superparamagnetism and can be utilized as excellent molecular probes for magnetic resonance imaging. Neumaier et al., (2008) related that cell labeling by iron oxide nanoparticles has emerged as a potentially powerful tool to monitor trafficking of a large number of cells in the cell therapy field. Magnetic resonance imaging and iron magnetic nanoparticles are able to increase the accuracy and the specificity of imaging and represent new imaging opportunities in preclinical and translational research (Neumaier et al., 2008).

Superparamagnetic behaviour of the marker used (magnetic fluid), composed of iron oxide (Fe_3O_4), comes from particles where the magnetization is free to re-orient itself due to thermal fluctuations (Sastry et al. 1995), characterizing the presence of electron-dense granules detectable by TEM. As described above, the nanoparticles can also be detected by magnetic resonance techniques, both *in vitro* and *in vivo*. The fundamental state of the free ion (Fe^{3+}) has an electronic configuration of $3d^5$, having all its 5 spins bound ($S=5/2$ and total momentum orbital=null), giving a 6S configuration for the fundamental state, 6 times degenerated, forming 3 pairs of Kramers levels separated by the local electrical field (Stark effect). Since Fe^{3+} is an ion with an odd number of unpaired electrons, the duplet's degeneration can only be possible by a magnetic field and then the electronic paramagnetic resonance (EPR) can be observed.

CD133 antigen is a surface molecule with unknown functions expressed by more primitive $\text{CD}34^+$ hematopoietic progenitor cells (HPCs) (Miraglia et al., 1997). The presence of antigen CD133 on a subset of very primitive HPCs such as $\text{CD}34/\text{CD}133$ cells points to this marker as an attracting tool to isolated human HPCs for biologic studies and clinical purposes (Peichev et al., 2000).

In humans, CD133 is expressed in stem cells (Yin et al., 1997) and in several cellular progenitors (Yin et al., 1997), including those derived from the hematopoietic and nervous systems (Yin et al., 1997; Miraglia et al., 1997). CD133 expression is also detected in other primitive cells (Gehling et al., 2000) and in some tumors, such as retinoblastoma, glioblastoma, and medulloblastoma (Yin et al., 1997; Pfenninger et al., 2007).

Human cord blood has been described as an excellent source of $\text{CD}133^+$ HPCs with a large application potential (Rubinstein et al., 1998; Kuci et al., 2003).

A large number of studies on stem cell biology and HPCs transplantation indicate that umbilical cord blood (UCB) is a suitable source of transplantable human HPCs (Gluckman et al. 1997; Rocha et al., 2001).

BONANNO et al. (2004) described the clinical isolation and functional characterization of cord blood $\text{CD}133^+$ HPCs with high hematopoietic activity and *in vitro* mesenchymal potential (differentiation mesoderm). Thus, the authors suggest that large-scale isolation of $\text{CD}133^+$ cells from human UCB could represent a primary step to again access to a stem pool

useful for therapeutic hematopoietic transplants, stem cell expansion for clinical purposes and pre-clinical research on tissue regenerating therapies.

In 2007, the same group (BONANNO et al.) related another therapeutic use of CD133⁺ cells from human UCB. They suggested the use of these cells as a treatment for myocardium ischemia due their potential to differentiate into endothelial and cardiomyocyte-like cells *in vitro*. *In vivo* studies showed vasculogenic potential of CD133⁺ cells from UCB in response to ischemia in an animal injury model (Finney et al., 2005; Ma et al., 2005).

CD133⁺ cells can be isolated *in vitro* with monoclonal antibody anti-CD133 bound to magnetic beads that are composed of superparamagnetic nanoparticles (Miltenyi Biotec) and purified by MACS (*magnetic activated cell sorting*).

The low levels of toxicity of the magnetic nanoparticles binding to magnetite (Fe₃O₄) used in the process of cellular selection (MACS - Miltenyi Biotec) can also be observed through studies that used hematopoietic stem cells obtained by the same selection procedure, in transplants of patients with cancer, obtaining satisfactory therapeutic results (Lang et al., 2003; Richel, et al., 2000).

This manuscript presents the study of molecular imaging on CD133⁺ hematopoietic stem cells from human umbilical cord blood.

2. Materials and methods

2.1 Collection and selection of the CD133⁺ hematopoietic stem cells from human umbilical cord blood (UCB)

The collection of the umbilical cord blood was obtained in the Obstetric Centre of the Albert Einstein Israelita Hospital, São Paulo, Brazil, from pregnancies at term (n=5) with written permission from the mother. The collection was performed with sterile equipment through a puncture of the umbilical cord vein, at the moment of birth and after the cord was cut.

Blood bags with a maximum volume of 250mL with 2mL of anticoagulant were used. The volume of the collected blood varied between 70 and 120mL. A sample of 80µL of blood was separated for cell count. The blood was then diluted in 1:2 proportion in a RPMI (Gibco) culture medium and the limpho-mononuclear cells were separated by the Ficoll-Paque™ Plus (Amersham - GE Healthcare) density gradient in a 1:3 proportion (Lehner & Holter, 2002). After 35 minutes in a 400g centrifuge, the fraction of the limpho-mononuclear cells were carefully isolated with the help of a 10mL pipette and washed in RPMI.

The CD133⁺ cell population was purified following the separation protocol by MiniMACS microbeads affinity chromatography using anti-CD133 bound to magnetic beads (Miltenyi Biotec).

The cells were filtrated in 30µm nylon filters and the number of these cells were determined by the cell count in an automatic counter (Coulter), after which they were centrifuged (400g for 5 minutes) and resuspended in the proportion of 300µL of solution for each 10⁸ cells in a PBS solution containing 2mM EDTA and 0.5% BSA (solution 1). For each 10⁸ cells, 100µL of FcR blocker and 100µL of magnetic micro-spheres with CD133⁺ antibodies (6°C) were added. The labelled cells were centrifuged and resuspended in solution 1 to be separated in the chromatography column to isolate only the CD133⁺ cells.

2.2 Flow cytometry

After CD133⁺ cell separation, the cell population was characterized by flow cytometry using the following monoclonal antibodies (Becton Dickinson, San Jose, CA) and (Miltenyi Biotec): CD34 (clone: 581) FITC-conjugated, CD45 (clone: 2D1) PerCP Cy-5.5-conjugated and CD133/2 (clone: AC141) APC-conjugated and the respective isotype controls IgG1 FITC-conjugated, IgG1 PerCP Cy-5.5-conjugated and IgG1 APC-conjugated.

Cells were incubated with antibodies at 4°C, on dark for 30 minutes, and then washed with PBS and fixed with 1% paraformaldehyde. A total of 10⁵ fluorescent cellular events were acquired on the FACSARIA flow cytometry (BD Bioscience) and analyzed by FACSDIVA software. Briefly, the analysis was performed by gating the cell population on forward scatter (FSC) versus side scatter (SSC) followed by gating only the CD45⁺ cells. Within the CD45⁺ cell population, cells were analyzed for expression of CD34 and CD133 markers.

2.3 Transmission Electron Microscopy (TEM)

CD133⁺ cell population (10⁷ cells) was fixed in 0,5% glutaraldehyde in 0,2M cacodylate buffer for two hours at 4°C. Two 15 minute washes in cacodylate buffer followed this process. Post-fixation was performed in 1% osmium tetroxide for one hour at 4°C, followed by another two 15 minute washes in the same buffer. For contrast, the material was immersed in a solution of uranyl acetate in acetone for 30 minutes. After dehydration, the material was embedded in Epon resin diluted in acetone (1:1) and incubated at 4°C with agitation for 24 hours. The material was then transferred to pure Epon resin and incubated at 60° C for 72 hours, until completely polymerized. Semi and ultrathin sections were obtained with the aid of a Porter Blum ultramicrotome. The semithin sections were stained with azur II (1%) and methylene blue (1%). The ultrathin sections were placed on copper grids and stained with uranyl acetate and lead citrate. The grids were studied and photographed in a transmission electron microscope, PHILLIPS CM100.

2.4 Electron Paramagnetic Resonance (EPR)

In order to corroborate with the referred marking of the CD133⁺ cells bound to iron oxide superparamagnetic nanoparticles the EPR technique was used.

The EPR signal of the iron oxide nanoparticles (iron markers Fe₃O₄) describe a peculiar absorption curve of approximately $g = 2,0$ where the curve area (double integral of the EPR spectrum) is proportional to the concentration of the sample nanoparticles.

To perform a quantitative analysis of the Fe₃O₄ concentration, a calibration curve was first built based on different known concentrations between 2,6µm and 0,6mM.

The sample preparation was obtained from a suspension of 10⁷ stem cells (CD133⁺), bound to iron oxide nanoparticles, in 0,5% glutaraldehyde fixative in a 2µL quartz tube. Following this, the EPR measurements were performed. The methodology of the quantification calibration was already described in previous studies Gamarra et al. (2007); Sadeghiani et al., (2005); Lacava et al., (2002).

The EPR measurements were taken using 20.166mW power, 1,6x10⁵ receiver gain, 4 scanning and the measurements were taken at room temperature. The spectral composition

of EPR absorption was obtained with a Bruker homodyne spectrophotometer, model EMX, operating with a frequency of 9,2 GHz in the band X and modulated to 100 kHz, utilizing one cavity of a rectangular microwave in the operating mode of the dominant cavity TE₁₀₂.

3. Results

3.1 Characterization by flow cytometry of CD133⁺ stem cells from UCB

After selection of CD133⁺ cells with monoclonal antibodies bound to superparamagnetic nanoparticles, flow cytometry analysis revealed that about 82% of the population is comprised by CD34⁺/CD133⁺ cells, most of them expressing CD133 (Figure 1). After this first analysis, the ultrastructural characterization of the CD133⁺ cells was performed.

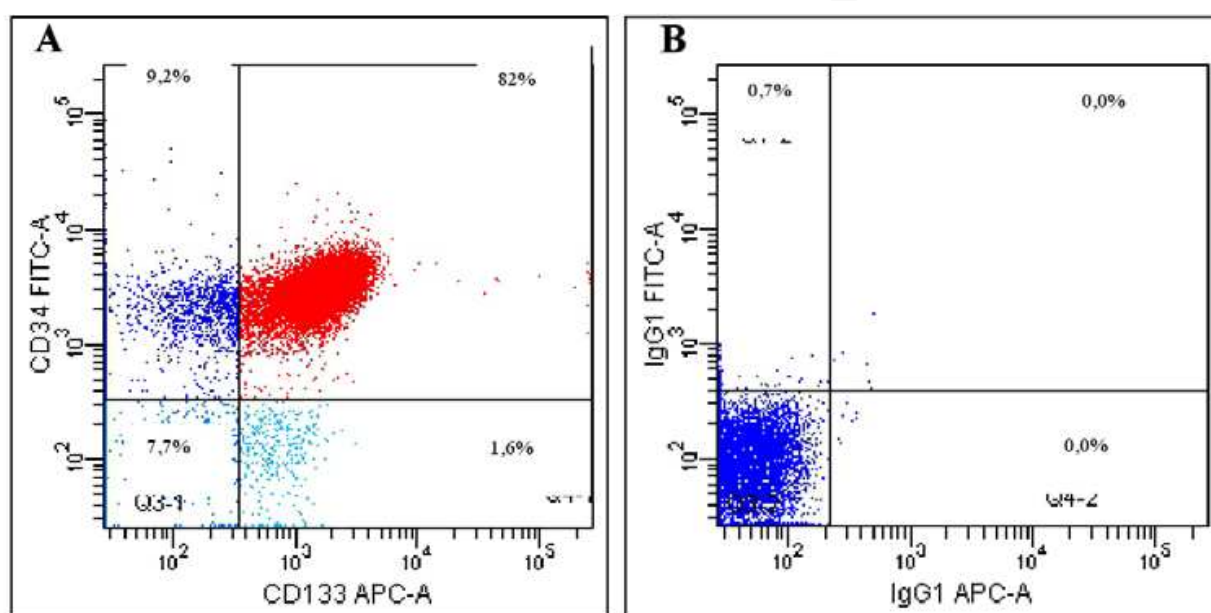


Fig. 1. (A) The Flow Cytometry graph shows that CD133⁺/CD34⁺ (82,0%), CD133/CD34⁺ (1,6%) and CD34⁺CD133⁻ (9,2%). (B) Isotype control graph shows that wasn't unspecific staining.

3.2 Identification and ultrastructural characterization of CD133⁺ stem cells labelled with nanoparticles

Under the electron microscopy, ultrastructural analysis highlighted the presence of electrondense granules in the cell surface. This demonstrates the presence of anti-CD133 monoclonal antibodies bound to superparamagnetic nanoparticles recognizing the CD133 membrane protein (Figures 2A, B, C, D). This does not occur in the cells of the control group (CD133⁻) (Figures 3C, D) since similar electrondense signal surrounding cellular membrane of CD133⁻ cells (control group) were not detected (Figures 3C, D).

Electrondense signals related to superparamagnetic nanoparticles were also observed in the cell cytoplasm, suggesting their internalization through the process of the endocytosis (pinocytosis) of nanoparticles bound to the antibodies (Figures 2C, D; 3A, B). CD133⁺ cells incorporated superparamagnetic nanoparticles through its small cytoplasmic projections forming the pinocytics vesicles, as shown in the figures 2C and 3B.

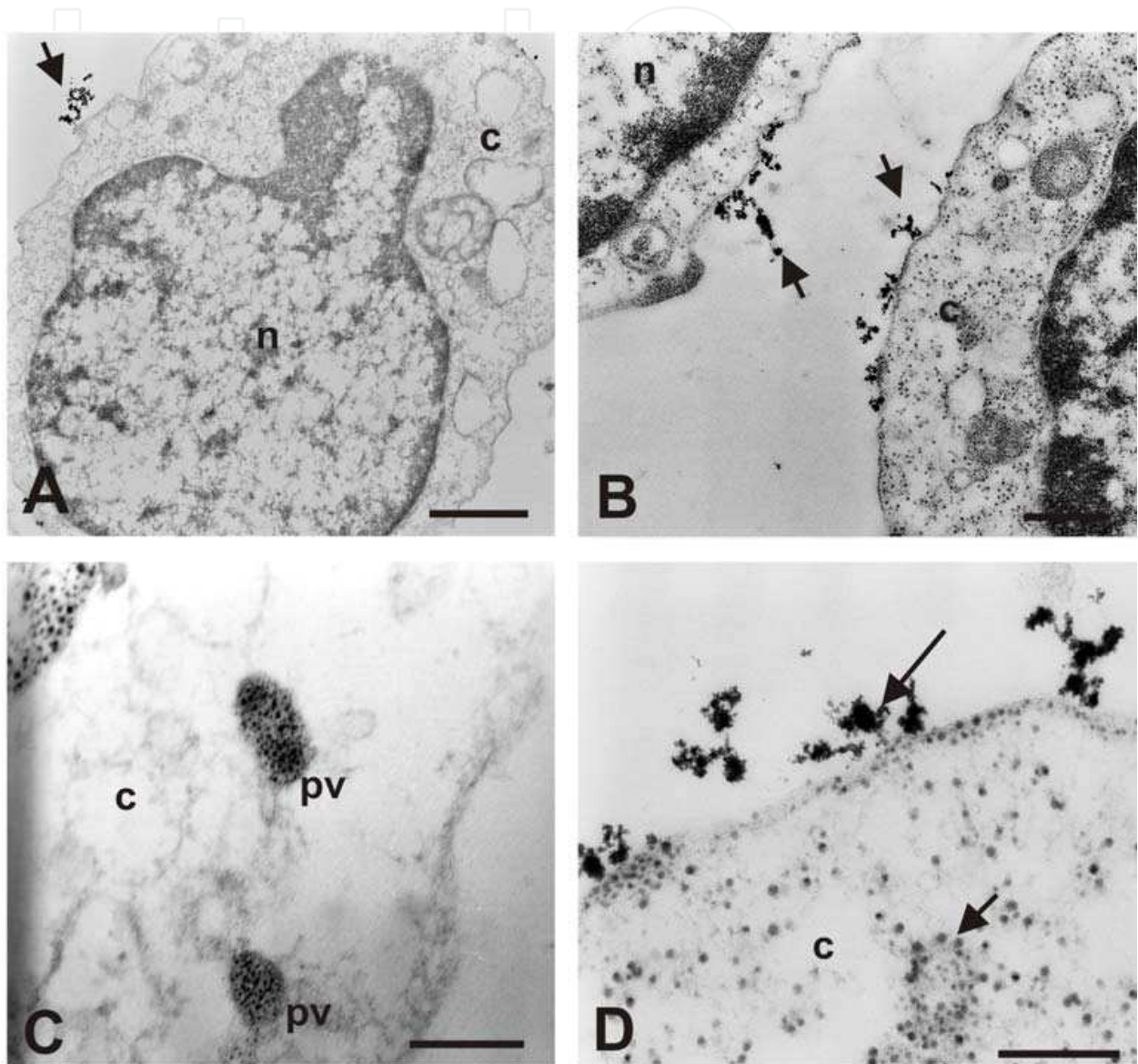


Fig. 2. Transmission electronic microscopy of CD133⁺ stem cells. n = nucleus, c = cytoplasm, arrow = electron-dense granules or magnetic nanoparticles, pv = pinocytotic vesicles. Scale: A) 1 μ m; B) 0,5 μ m; C) 0,25 μ m; D) 0,125 μ m.

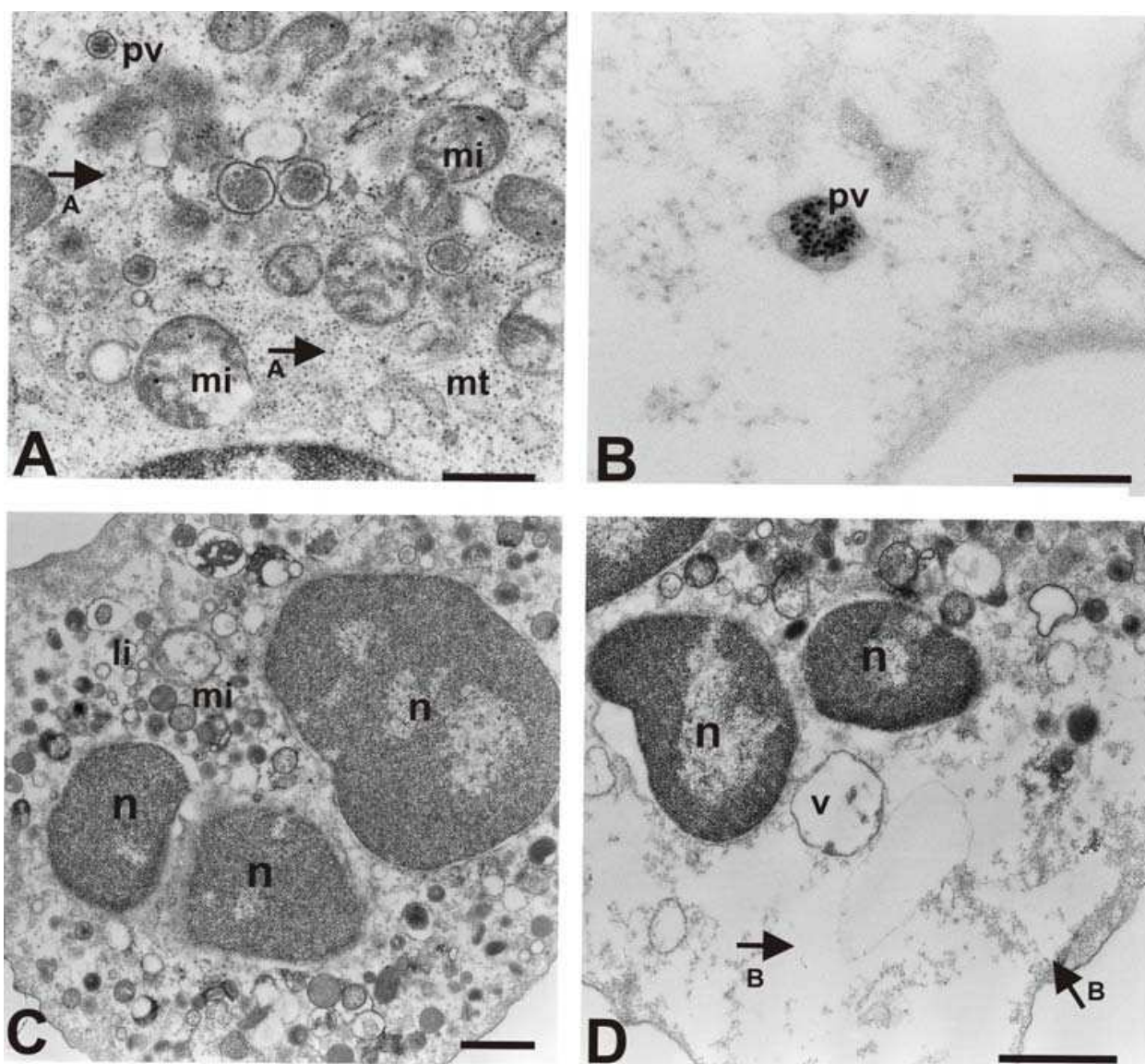


Fig. 3. (A, B) Transmission electronic microscopy of CD133⁺ stem cells. (C, D) Transmission electronic microscopy of CD133⁻ stem cells (control). n = nucleus, mi = mitochondria, mt = microtubules, arrow A = electron-dense granules or magnetic nanoparticles, arrow B = absence of the electron-dense granules or magnetic nanoparticles, pv = pinocytotic vesicles, li = lipids, v = vacuoles. Scale: A) 1 μ m; B) 0,5 μ m; C) 0,25 μ m; D) 0,25 μ m.

3.3 Electron Paramagnetic Resonance (EPR) of the superparamagnetic nanoparticles labelled of CD133⁺ stem cells

To clearly show the expression of CD133 antigenic marker of the cell population under study, an EPR spectrum was first performed only with monoclonal antibodies anti-CD133 bound to magnetic nanoparticles composed of superparamagnetic nanoparticles. A resonance in $g=2.0$ (Figure 4) was observed, derived from Fe³⁺ spins that interact with themselves, but with a supermagnetic behavior that characterizes the presence of agglomerates (Gamarra et al., 2005; Hseih et al., 2002; Sharma & Waldner, 1977). This signal consists of a strong absorption, while the large width is attributed to the strong interaction between Fe³⁺ ions (Fe³⁺ ion cluster agglomerated by a strong exchange interaction).

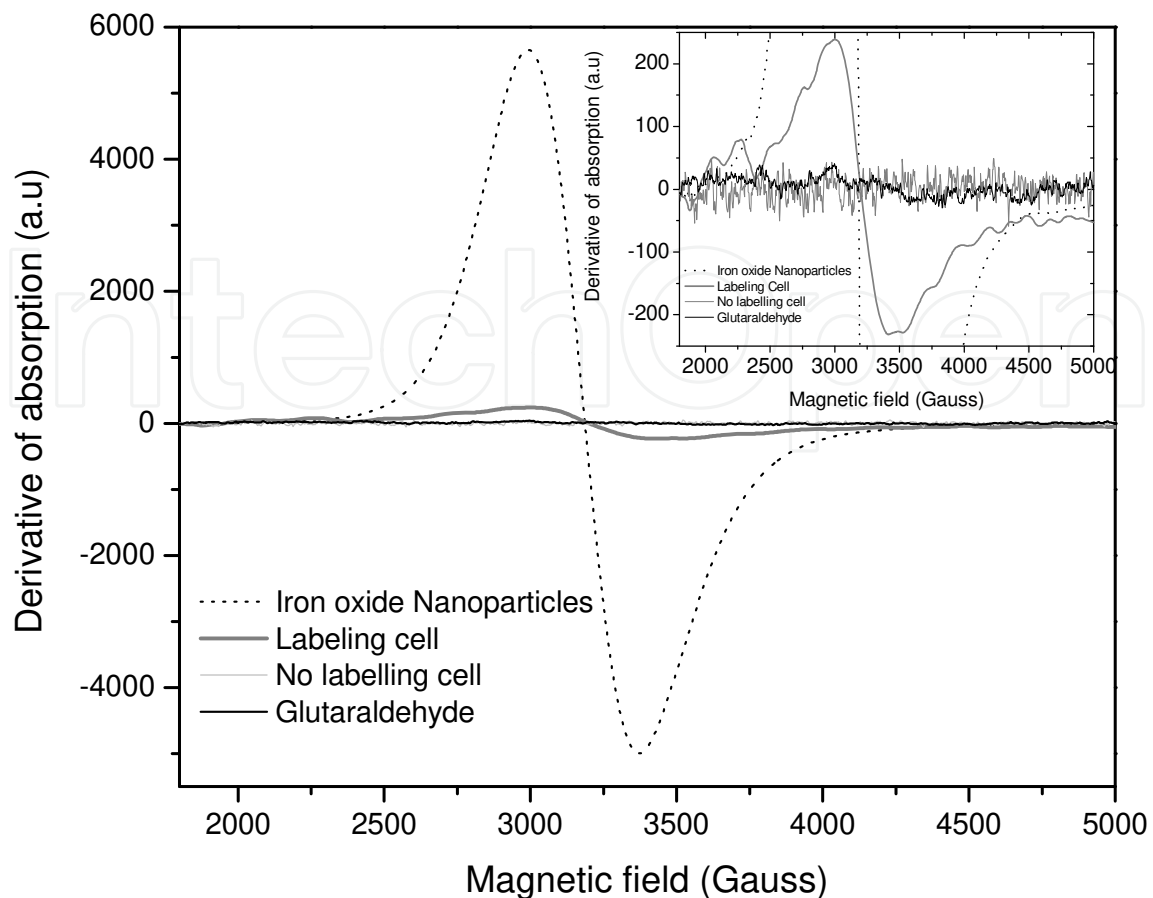


Fig. 4. EPR spectra, showing the resonance absorption curve of the superparamagnetic nanoparticles, labelled cells (CD133⁺), unlabelled cells (CD133⁻) and glutaraldehyde 0,5% fixative. The spectra are shown in the inset.

EPR spectrum of the labelled cells (CD133⁺) was then performed, observing a resonance line in $g=2.0$ showing the presence of the magnetic nanoparticles bound to the cells, which were immersed in glutaraldehyde 0,5% fixative. This fixative was removed by centrifugation of the labelled cells and it was analyzed using the EPR spectrum. This spectrum showed the absence of resonance in glutaraldehyde fixative thus evidencing the absence of iron oxide superparamagnetic nanoparticles in the fixative.

As expected, the EPR spectrum of unlabelled cells (CD133⁻), used as negative controls, did not present a resonance line.

4. Discussion

Modern imaging technologies that allow for *in vivo* monitoring of cells in intact research subjects have opened up broad new areas of investigation. In the field of hematopoiesis and stem cell research, studies of cell trafficking involved in injury repair and hematopoietic engraftment have made great progress using these new tools. Multiple imaging modalities are available, each with its own advantages and disadvantages, depending on the specific application. For mouse models, clinically validated technologies such as magnetic resonance imaging (MRI) and positron emission tomography (PET) have been joined by optical

imaging techniques such as *in vivo* bioluminescence imaging (BLI) and fluorescence imaging, and all have been used to monitor bone marrow and stem cells after transplantation into mice. Each modality requires that the cells of interest be marked with a distinct label that makes them uniquely visible using that technology. For each modality, there are several labels to choose from. Finally, multiple methods for applying these different labels are available (Duda et al., 2007).

In this work was described the molecular imaging on CD133⁺ hematopoietic stem cells from human umbilical cord blood through the nanobiotechnological applications. We were able to distinguish CD133⁺ from CD133⁻ cells by TEM, based on their attachment to nanoparticles, as well as by signal analysis of EPR generated by these nanoparticles. Our findings indicate potential nanobiotechnological applications for *in vivo* cell imaging and therapy.

In the hematopoietic system, CD133 is especially expressed in stem cells and progenitor cells found in the bone marrow, peripheral blood and human umbilical cord blood, which was used as the cellular source for this study (Yin et al., 1997; Miraglia et al. (1997). Recent data suggest that CD133⁺ cells, besides having extensive hematopoietic plasticity (Wynter et al., 1998), can be used in different clinical applications, being an alternative in the utilization of CD34⁺ cells, more commonly used in transplant protocols (Handgretinger et al., 2003). CD133⁺ cellular fraction of bone marrow harbors precursors of granulocytes/macrophages and dendritic cells (Yin et al., 1997).

Studies performed by Gehling et al. (2000) demonstrated that CD133⁺ cells have the capacity to differentiate in endothelial cells. After that, other researchers identified an endothelial progenitor (CD133⁺) that co-expresses the receptor for vascular endothelial growth factor (VEGFR) (Gill et al., 2001). They also detected functional responses in these cells during vascular trauma, suggesting that these CD133⁺ endothelial progenitors can be mobilized by VEGF, being useful in repairing vascular tissue injury (Gill et al., 2001).

Powell et al. (2005) related diseases of the coronary arteries with the reduction of fractions from three different sub-types of CD133⁺ circulant cells: CD133⁺CD34⁺; CD133⁺CXCR4⁺ and CD133⁺VEGFR2⁺. These data indicated that the functional meaning of CD133 positive cellular fractions (CD133⁺) transcends the hematopoietic tissue (Bhatia, 2001).

In the present study, after the previous analysis of flow cytometry confirming the significant expression (82%) of the CD133 cell surface antigen (CD133⁺ phenotype) in the stem cells of the human umbilical cord blood (UCB), the analysis of these cells through Transmission Electronic Microscopy (TEM) was performed.

The electron micrographs highlighted the presence of electrondense granules in the cellular membrane, as well as dispersed in the cytoplasm of CD133⁺ cells, which refers to the superparamagnetic nanoparticles bound to anti-CD133 monoclonal antibody. These electronic micrographs described an important ultrastructural finding related to the presence of vesicles that internalize of nanonparticles conjugated to anti-CD133 by the process of pinocytosis for all antibodies tested. We suggest that in our study the pynocytisis occur by receptor-mediated endocytosis. The endocytosis process happens after the antibody biding to the receptor, and then a depression arises on the cell membrane which is followed by the formation of the pinocytic vesicles. These pinocytic vesicles that are surrounded by the cytoplasmic proteins called clatrines, are internalized (Pavon et al., 2010).

Electron Paramagnetic Resonance (EPR) analysis of the CD133⁺ stem cells from UCB detected that the signal (spectrum) generated by the labelled cells comes from the superparamagnetic nanoparticles composed of iron oxide bound to these cells.

For spectrum detection of the EPR of the superparamagnetic nanoparticles it was necessary to perform an earlier study of the qualitative visualization through TEM of these nanoparticles also bound to CD133⁺ stem cells. The micrographies showed that the anti-CD133 antibodies, bound to nanoparticles are bound to the cell surface antigens, thus demonstrating that the stem cell morphology, even after the selection process, was preserved. In this way the information of the analysis of the quantification by EPR of the iron content in the cells was validated (Billotey et al., 2003; Wilhelm et al., 2002), which is necessary for the intensity study of the image signal by magnetic resonance (MRI), to obtain the molecular image.

This findings may guide future research in the study of molecular imaging on CD133⁺ haematopoietic stem cells from human umbilical cord blood, highlighting the immunolocalization of monoclonal antibodies associated with superparamagnetic nanoparticles, as well as the signal detection generated by these nanoparticles (EPR) bound to the cells studied, suggest many biomedical applications using new resources in nanoscience as they become available. The EPR results, together with ultrastructural characterization of immunolocalized nanoparticles, strongly suggest that CD133⁺ cells labelled with nanoparticles can be considered for different nanobiotechnological applications, i.e., matter manipulation in a nanometric scale to help areas such as diagnosis, therapeutics or even bioengineering (Fargeas et al., 2006). This is because these CD133⁺ cells from human UCB could represent a primary step to again access to a stem pool useful for therapeutic hematopoietic transplants, stem cell expansion for clinical purposes and pre-clinical research on tissue regenerating therapies.

Recent developments are revolutionizing the understanding about hematopoietic stem-cell therapy. Over the past three decades, much knowledge has been accumulated to provide a sound basis for the optimal use of this approach in the treatment of hematopoietic cancers, especially acute myelogenous leukemia, Hodgkin's disease and other lymphomas and multiple myeloma (Verlinden et al., 1998; Thomas; Chift, 1999; Negrin et al., 2000; Childs et al., 2000; Sharp et al., 2000). This approach has also been used in the treatment of some solid tumors, most notably breast cancer, for which its value is still in dispute because of poor accrual in randomized trials and because it takes many years to obtain definitive answers, even from well-designed trials (Joshi et al., 2000). Hematopoietic stem-cell therapy is also being tried in sickle cell disease and thalassemia and recently in progressive multiple sclerosis, systemic scleroderma, severe systemic lupus erythematosus, and rheumatoid arthritis with a poor prognosis (Shichishima et al., 1983; Papanikolaou et al., 2011; Alderuccio et al., 2011).

The broadening therapeutic applications of hematopoietic stem cells also reflect an increased understanding of how to modulate the cells of the immune system to minimize both rejection and graft-versus-host disease and to improve the management of the infectious complications of immunosuppression (Verlinden et al., 1998; Sharp et al., 2000).

While much has been studied about hematopoietic stem-cell therapy several questions remain regarding the biology of stem cells in living subjects. For most therapeutic

applications of regenerative medicine, critical issues such as stem cell type and delivery route of stem cells remain to be elucidated. In addition, after cells are transplanted to the living subject, it becomes critical to understand the biology of transplanted cells and how they interact with the microenvironment. Recent developments in molecular imaging modalities may likely permit investigators to answer some of these questions. Furthermore, some of these imaging strategies have the potential to be translated to patients, which makes them plausible to be used in the clinics.

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7. Abbreviations

CD133: CD133 and the alternative tiles PROMININ, PROML1 and AC133 was originally identified as a protein that selectively localized at the apical surface of murine neuroepithelial cells, whereas PROML1 was identified as an antigenic marker (AC133 antigen) in hematopoietic stem cells.

Anti-CD133: monoclonal antibody against the CD133.

CD133⁺: presence of CD133 antigen expression.

CD133⁻: absence of CD133 antigen expression.

8. References

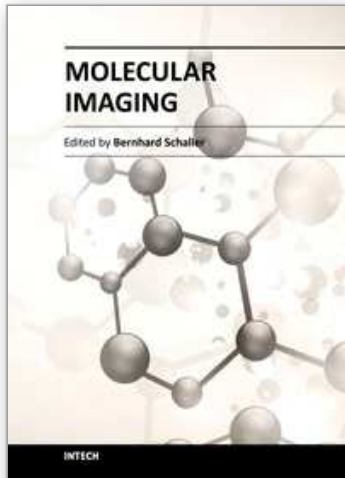
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