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Efficient Intracytoplasmic Labeling of Human Umbilical Cord Blood Mesenchymal Stromal Cells With Ferumoxides

Jae Kwon Lee,*† Man Kyoung Lee,* Hye Jin Jin,* Dal-Soo Kim,* Yoon Sun Yang,* Wonil Oh,* Sung-Eun Yang,* Tae Seok Park,‡ Soo Yeol Lee,‡ Bum-Soo Kim,§ and Sin-Soo Jeun¶

*Medipost Biomedical Research Institute, Medipost Co. Ltd., Asan Institute of Life Science, Seoul, 138-736, Republic of Korea
†School of Science Education (Biology Education), College of Education, Chungbuk National University, Chungcheongbuk-Do, 361-763, Republic of Korea
‡Department of Biomedical Engineering, Kyung Hee University, Seoul, 130-701, Republic of Korea
§Department of Radiology, College of Medicine, The Catholic University of Korea, Seoul, 137-701, Republic of Korea
¶Department of Neurosurgery, College of Medicine, The Catholic University of Korea, Seoul, 137-701, Republic of Korea

Mesenchymal stromal cells (MSCs) are multipotent cells found in several adult tissues; they have the capacity to differentiate into mesodermal, ectodermal, and endodermal tissues in vitro. There have been several reports that MSCs have therapeutic effects in a variety of diseases. Therefore, using a cell labeling technique, monitoring their temporal and spatial migration in vivo, would be useful in the clinical setting. Magnetic resonance imaging (MRI)—tracking of superparamagnetic iron oxide (SPIO)-labeled cells—is a noninvasive technique for determining the location and migration of transplanted cells. In the present study, we evaluated the influence and toxicity of SPIO (ferumoxides) labeling on multiple differentiated MSCs. To evaluate the influence and toxicity of ferumoxides labeling on differentiation of MSCs, a variety of concentrations of ferumoxides were used for labeling MSCs. We found that the cytoplasm of adherent cells was effectively labeled at low concentrations of ferumoxides. Compared with unlabeled controls, the ferumoxides-labeled MSCs exhibited a similar proliferation rate and apoptotic progression. The labeled MSCs differentiated into osteoblasts and adipocytes in an identical fashion as the unlabeled cells. However, chondrogenesis and neurogenesis were inhibited at high concentrations of ferumoxides. Our results suggest the effective concentration for ferumoxides use in tracking MSCs.

Key words: Umbilical cord blood; Mesenchymal stromal cells; Superparamagnetic iron oxide; Ferumoxides; Multilineage differentiation

INTRODUCTION

Currently, intensive research evaluating stem cells for their therapeutic potential is under way worldwide. Although using embryonic- and fetal-derived stem cells may be a viable approach for developing new treatment for incurable diseases (23,25), ethical and moral concerns, as well as the limited availability of stems cells, have prompted the search for alternative stem cell sources. Human umbilical cord blood (hUCB) is conventionally used as an alternative source of stem or stromal cells, hematopoietic stem cells, and MSCs (5,7). More than 3,000 cases of hUCB transplantation have been performed worldwide for patients suffering from a variety of hematologic and genetic disorders (6,13,15, 17,18,24). MSCs are multipotent cells found in several adult tissues, and have the capacity to differentiate in vitro into several mesodermal tissues (bone, cartilage, tendon, muscle, and adipose) and endodermal tissue (hepatocyte) as well as ectodermal tissue (neurons) (28). Moreover, MSCs are not immunogenic; they do not elicit a proliferative response of allogeneic lymphocytes in vitro (19). These characteristics make MSCs potent candidates for the development of cell-based therapeutic strategies. The therapeutic effects of MSCs have recently been reported for bone, joint, and neuronal diseases (22,26). Improving the therapeutic potency and sensitivity of MSC treatment as well as developing a noninvasive technique for tracking MSCs following transplantation is needed.

Among the available methods for tracking cells, magnetic resonance imaging (MRI) of in vivo MSCs labeled

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Address correspondence to Sin-Soo Jeun, Department of Neurosurgery, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea. Tel: +82-2-590-2568; Fax: +82-2-594-4248; E-mail: ssjeun@catholic.ac.kr

with superparamagnetic iron oxide (SPIO) nanoparticles is a powerful technique for monitoring the behavior of these cells. Ferumoxides is a dextran-coated SPIO approved by the US Food and Drug Administration (US FDA) as an MRI contrast agent; it has been shown to be an effective and efficient labeling agent for stem cells and other mammalian cells (10,11).

In this study, we provide data for the first time that shows that ferumoxides can be used to label hUCB MSCs. In addition, we demonstrate that high concentration of ferumoxides can prevent MSCs from differentiating into chondrocytes and neurons.

MATERIALS AND METHODS

hUCB MSCs

The hUCB samples were obtained with consent of the mothers. The hUCB MSCs were separated and maintained following the previously described methods (28). hUCB MSCs were cultured with ferumoxides (Feridex I.V., Taejoon, Seoul, Korea) in culture media. After 72 h, the MSCs were washed three times to remove the excess ferumoxides. The labeled cells were incubated for 30 min with 2% potassium ferrocyanide [Perl reagent for Prussian blue (PB) staining] (Sigma, St. Louis, MO, USA) in 1% hydrochloric acid, washed, and counterstained with nuclear fast red (Sigma) to confirm the uptake of ferumoxides into the cytoplasm.

Immunophenotyping of hUCB MSCs

To analyze the cell surface expression of typical marker proteins, hUCB MSCs at the fifth passage were labeled with the following anti-human antibodies: CD45-FITC, CD34-FITC, CD14-FITC, HLA-DR-FITC (Becton Dickinson, San Jose, CA, USA), CD29-PE, CD44-PE, CD73-PE, CD90-PE, CD166-PE, HLA-AB-PE (Pharmingen, Los Angeles, CA, USA), and CD105-PE (Serotec, NC, USA). Mouse isotype antibodies served as the respective controls (Becton Dickinson). Ten thousand cells were measured using a FACScan flow cytometer (Becton Dickinson) and the results were analyzed by CellQuest software (Becton Dickinson).

Multilineage Differentiation

Ferumoxides-labeled MSCs were induced to osteogenic, adipogenic, and chondrogenic differentiation according to a previous study (28) to see whether the labeling ferumoxides had any adverse effect on their multilineage differentiation.

Neurogenic Differentiation

Poly-D-lysine and laminin (Sigma)-coated plate was used to induce neuronal differentiation. Prior to neuronal induction, the hUCB MSCs were pretreated in DMEM, 10% FBS, and 20 ng/ml basic fibroblast growth factor (R&D Systems, Inc., Minneapolis, MN, USA) for 2 days. Neuronal differentiation was induced by neurogenic medium that was composed of DMEM/F12 medium supplemented with 200 μM butylated hydroxyanisole, 25 μg/ml insulin (Sigma), 25 mM KCl, 2 μM valproic acid (Sigma), 10 μM forskolin (Sigma), and 1 μM hydrocortisone (Sigma).

RT-PCR Analysis

The mRNA expression levels of the chondrogenic proteins and neurogenic proteins were compared at steady state in the ferumoxides-unlabeled and -labeled hUCB MSCs by performing RT-PCR. The total RNA was extracted from each differentiated cell by the modified method of Chomczynski and Sacchi (9). The sense primer for NF-M was 5′-TGGGAAATGGCTCGTC ATTT-3′ and the reverse primer was 5′-CTTCATG GAAGCGGC CAATT-3′. The sense primer for MAP2 was 5′-CCTTTTTACAGCCAGATGAC-3′ and the reverse primer was 5′-GTGAAAGTATCCCTTTGCTG-3′. The primers for chondrogenic proteins and GAPDH as well as methods for PCR were previously reported (28).

Western Blotting

Cell extract protein (10 μg) was used to conduct electrophoresis on 4–20% denatured PAGE gels (Bio-Rad Laboratories) and then Western blotting was done as previously described (20). Mouse anti-human tubulin β-III (TBIII) monoclonal antibody (mAb) (Sigma) and mouse anti-human neuronal nuclei (NeuN) mAb were used as a first antibody. The protein bands were detected by chemiluminescence (Amersham Pharmacia, Biotech, NJ, USA).

Cell Death Assays

To determine if ferumoxides labeling resulted in changes in the rates of apoptosis, both unlabeled and labeled cells were collected after 72 h, washed twice with ice-cold phosphate-buffered saline (PBS), and then resuspended in 1 ml of annexin media (Vybrant apoptosis assay kit, Molecular Probes, OR, USA) at 1×10^6 cells/ml. Ten microliters of FITC-labeled annexin V and 2 μl propidium iodide solution were added to 100 μl of cell suspension; this was then kept at room temperature for 15–20 min, and next flow cytometry was performed using a fluorescent-activated cell sorter (FACScalibur, Becton Dickinson). The dead cells were identified by trypan blue exclusion staining.

RESULTS

Immunophenotyping of the hUCB MSCs

The expression of MSC-related cell surface antigens was evaluated on four hUCB MSCs at the fifth passage by flow cytometry (Fig. 1). Similar to MSCs from bone marrow (4), all hUCB MSCs were strongly positive for MSC-related antigens: CD73 (98.7 ± 1.1%), CD105

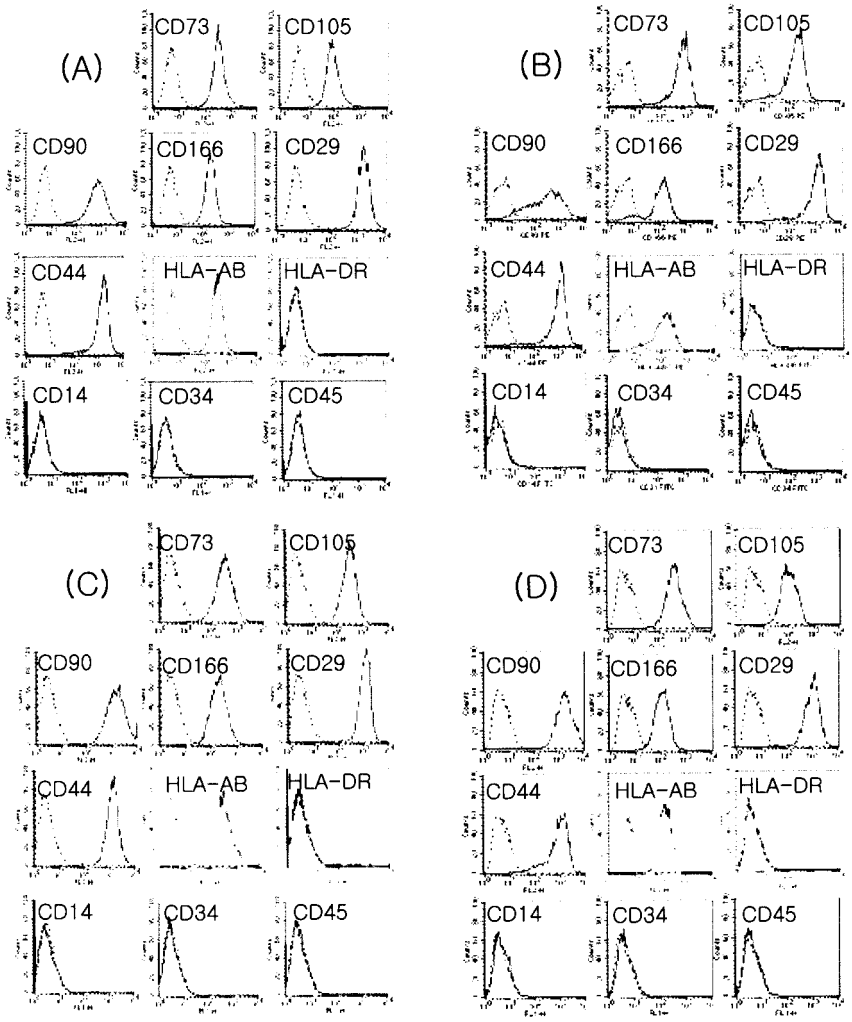


Figure 1. Immunophenotype of the four different hUCB MSCs. MSC-like cells from four different samples of human cord blood at passage 5 were labeled with antibodies against the indicated antigens and they were then analyzed by flow cytometry. The dotted lines indicate the isotype-matched mouse IgG antibody control labeling.

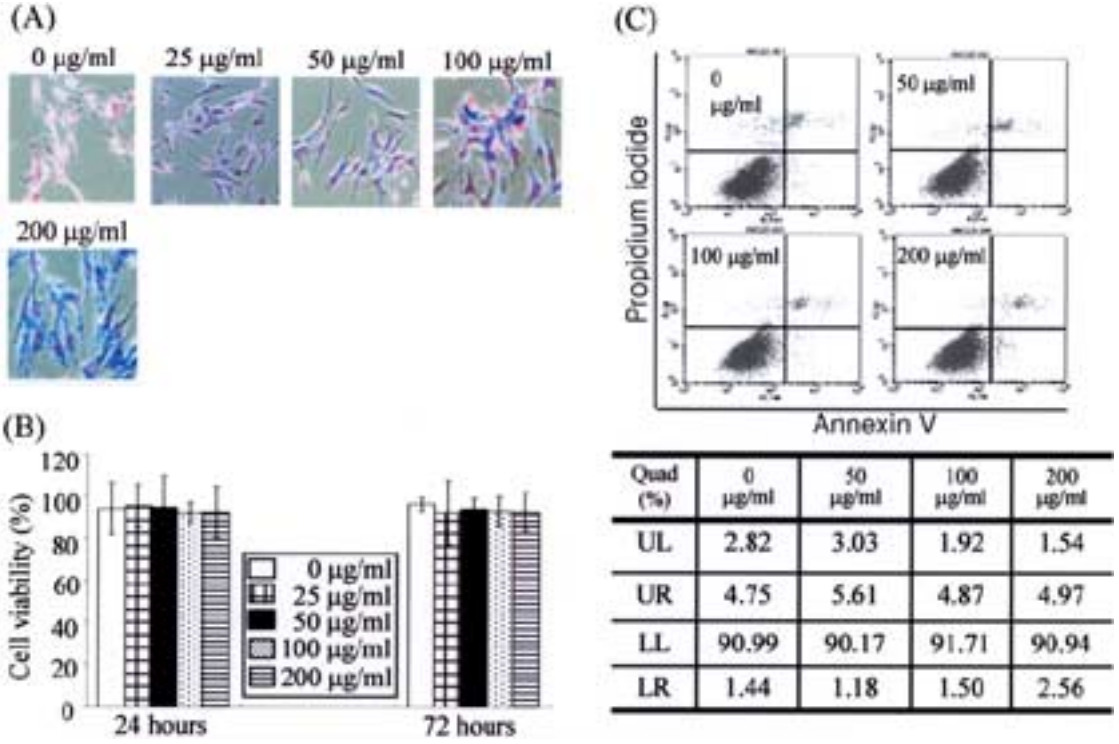
(98.3 ± 1.2%), CD90 (94.4 ± 8.7%), CD166 (98.3 ± 1.26%), CD29 (97.1 ± 3.5%), and CD44 (98.2 ± 1.0%). In addition, these cells were stained positively for HLA class I (HLA-AB; 97.8 ± 2.6) and negatively for HLA class II (HLA-DR). However, the hUCB MSCs did not express the hematopoietic markers: CD14, CD34, and CD45. Among the four hUCB MSCs, we selected one hUCB MSC depending on the capacity for differentiation in each lineage.

Cytotoxic Effect of Ferumoxides

Incubation of the hUCB MSCs with ferumoxides for 72 h showed detectable PB staining that was dependent on the concentration of ferumoxides (Fig. 2A). Labeling efficacy was reproducible at approximately 90–100% of hUCB MSCs. The in vitro culture of hUCB MSCs appeared to be unaffected by ferumoxides because both the labeled and unlabeled MSC cultures grew in a similar fashion as noted by the confluent monolayers. At harvest, trypan blue dye exclusion assay indicated that cell viabilities, of the MSCs, were not changed by ferumoxides labeling [labeled (92–94%) and nonlabeled (95%)] at different points in time (24 and 72 h, respectively) (Fig. 2B). Similarly, there were no significant differences between the labeled and unlabeled hUCB MSC cells in the rate of apoptosis (Fig. 2C).

Osteogenic and Adipogenic Differentiation of Furoximide-Labeled hUCB MSCs

To evaluate the influence of ferumoxides labeling on multilineage differentiation, hUCB MSCs were incubated with ferumoxides for 72 h to induce osteogenic



Quad (%)	0 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
UL	2.82	3.03	1.92	1.54
UR	4.75	5.61	4.87	4.97
LL	90.99	90.17	91.71	90.94
LR	1.44	1.18	1.50	2.56

Figure 2. Labeling of hUCB-MSC using ferumoxides. MSCs were labeled for 72 h with different concentrations of ferumoxides in nondifferentiation media (α-MEM, 10% FBS). (A) PB staining of the labeled MSCs shows an efficient intracellular uptake of particles. (B) Trypan blue dye exclusion assay indicates the cell viability of the labeled cells. The data represent the mean ± SEM, $n = 3$. (C) Apoptosis in the ferumoxides-labeled hUCB MSC. Unlabeled or labeled cells were treated with fluorescent-labeled annexin V and propidium iodide solution and then analyzed by flow cytometry.

and adipogenic differentiation. Both the labeled and unlabeled MSCs showed similar differentiation into the osteogenic and adipogenic lineages when grown in culture with the appropriate differentiation medium. Osteogenic differentiation was determined by von Kossa's and ALP staining (Fig. 3B and C). Adipogenic differentiation was apparent in the unlabeled and labeled hUCB MSCs by cellular accumulation of lipid-rich vacuoles that were stained with Oil Red O (Fig. 3E). The intracytoplasmic ferumoxides was confirmed by PB staining (Fig. 3A and D) in the differentiated cells.

Chondrogenic Differentiation of Ferumoxides-Labeled hUCB MSCs

The influence of ferumoxides labeling on chondrogenic differentiation of hUCB MSC was evaluated by Safranin-O stain and RT-PCR. hUCB MSCs, unlabeled or labeled with ferumoxides, were centrifuged to form a pellet micromass, and cultured in serum-free chondrogenic medium. After 4 weeks of differentiation, the intracytoplasmic ferumoxides were confirmed by PB staining (Fig. 4A) in the histological sections. The accumulation of sulfated proteoglycans was visualized by Safranin-O staining. As shown in Figure 4B, the chondrogenic differentiation was interfered with after ferumoxides labeling. On the basis of the Safranin-O staining, incubation of hUCB MSCs for 72 h with 50–100 μg/ml of ferumoxides provides optimum conditions for cell labeling, with no prevention of chondrocyte differentiation compared to unlabeled controls. However, ferumoxides labeling at a high concentration (200 μg/ml) induced abnormal shape of chondrocyte-like lacunae. Consistent with Safranin-O staining results, ferumoxides-labeled cultures, at appropriate concentration (50–100 μg/ml), expressed mRNA of chondrocyte proteins such as aggrecan, collagen type II (Col II), collagen type IX (Col IX), and Sox-IX at 4 weeks; however, the expression of Col IX was decreased depending on the concentration of ferumoxides (Fig. 4C). In addition, Col II and Col IX were not detected, and aggrecan was very weakly detected, in 200 μg/ml ferumoxides-labeled cultures.

Neurogenic Differentiation of Ferumoxides-Labeled hUCB MSCs

Prior to evaluation of the influence on neurogenic differentiation by ferumoxides labeling, we demonstrated neurogenic differentiation in the hUCB MSCs. As

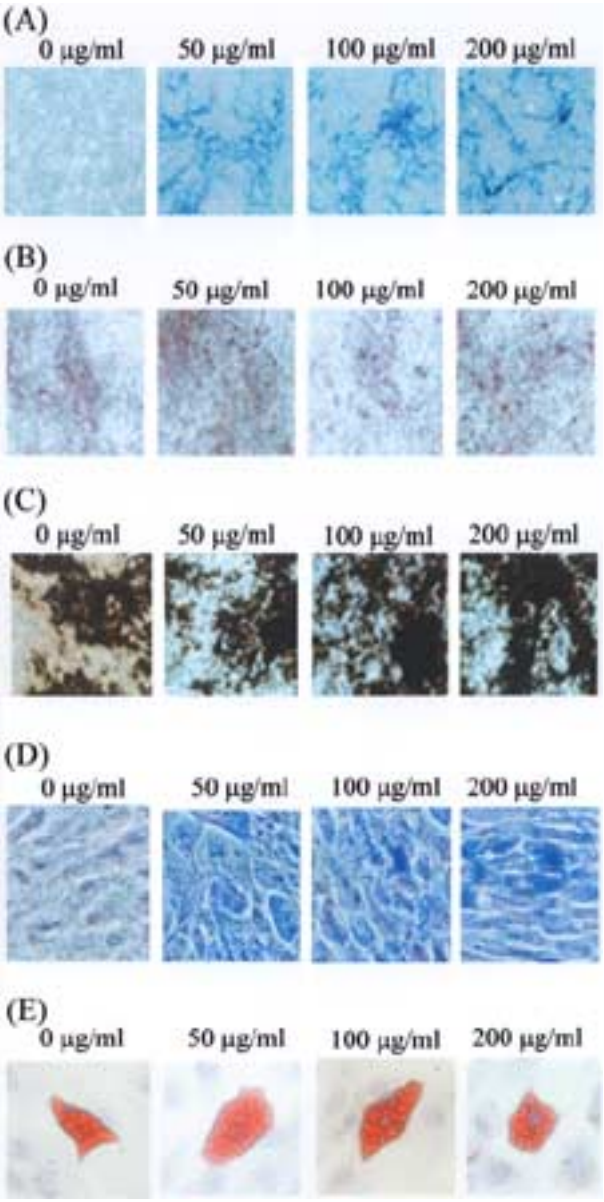


Figure 3. Osteogenic and adipogenic differentiation of the labeled and unlabeled hUCB MSCs. Ferumoxides-labeled and unlabeled MSCs were cultured in nondifferentiation medium or differentiation medium for 4 weeks. PB staining of the labeled hUCB MSCs shows an efficient intracellular uptake of particles into the endosomes (A, D). Osteogenic differentiation was demonstrated by ALP (B) and Von Kossa's staining (C). Adipogenic differentiation was demonstrated by neural lipid accumulation; this was measured by Oil Red O staining (E).

shown in Figure 5A, neurogenic stimulants induced morphological changes towards neuron-like cells; this occurred within 6 h in most of the cells. These cells exhibited round cell bodies and neuron process-like extensions. Neurogenic differentiation altered the pattern of gene expression of neuron-related genes. The expression level of mRNA transcripts for NF-M and MAP2, which are neuronal maturity markers, was increased after 6 h of neuronal induction. However, the level of mRNA for TBIII, which is known as a marker for immature neuronal cells, was not changed during the neuronal induction period (Fig. 5B). As shown in Figure 5C, Western blot analysis showed that the expression of NeuN, which is a mature neuron marker, was increased in the neurogenic-induced MSCs. By contrast, the expression of TBIII was significantly diminished.

Influence of Ferumoxides on Neurogenic Differentiation of hUCB MSCs

The presence of intracytoplasmic ferumoxides was confirmed by PB staining (Fig. 6A) in differentiated cells. As shown in Figure 6A, ferumoxides labeling did not prevent neuron-like differentiation at any concentration within 6 h. However, after 72 h, cells labeled with high concentrations of ferumoxides (200 μg/ml) showed

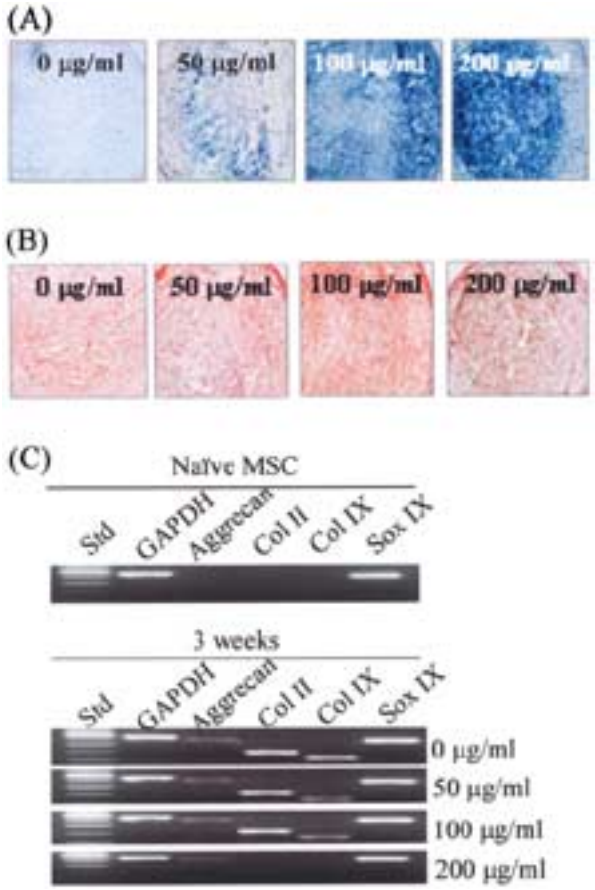


Figure 4. Chondrogenic differentiation of hUCB MSCs. MSCs exposed to ferumoxides induced chondrogenic differentiation. After 4 weeks of induction, the uptake of ferumoxides was visualized by PB staining (A) and the accumulation of sulfated proteoglycans was visualized by Safranin-O staining (B). (C) RT-PCR was performed for chondrogenic differentiation. All samples showed a similar signal for GAPDH.

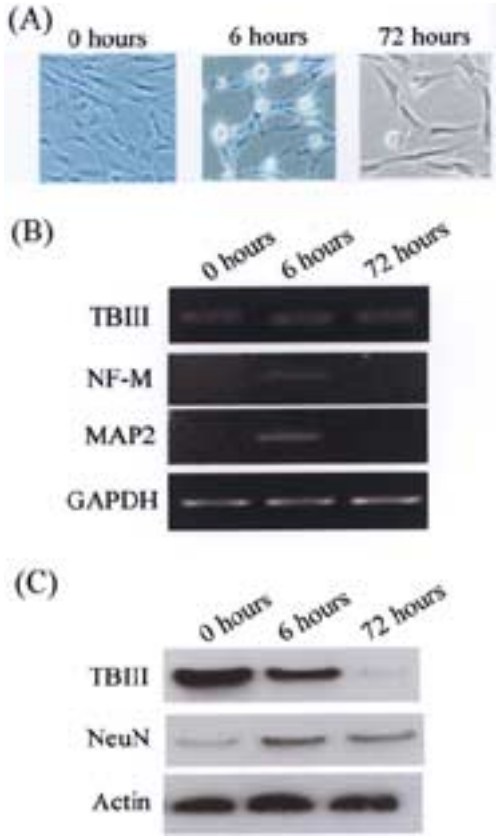


Figure 5. Neurogenic differentiation of hUCB-MSCs. The ferumoxides-exposed MSCs were cultured in nondifferentiation or neurogenic media for 6 and 72 h. (A) The morphological changes were observed via a phase-contrast microscope. (B) Total RNA was extracted and analyzed by RT-PCR for TBIII, NF-M and MAP2. (C) Western blot analysis of TBIII and NeuN was performed with cell extracts from unlabeled or labeled hUCB MSCs for the indicated times.

cytotoxic effects with neurogenic induction. To further investigate the cytotoxic effect of ferumoxides, we performed the trypan blue dye exclusion assay. Trypan blue dye exclusion assay indicated that 200 μg/ml ferumoxides labeling decreased (50%) cell viability during neurogenic induction (Fig. 6B). The results of the Western blot analysis showed that the expression of NeuN was decreased by labeling with 100 μg/ml ferumoxides compared with 0 (Fig. 5C), 25 or 50 μg/ml ferumoxides labeling (Fig. 6C). These results imply that hUCB-MSCs can differentiate into mature neuron-like cells, and that ferumoxides labeling over 100 μg/ml can prevent neuronal differentiation.

DISCUSSION

Several approaches for ferumoxides labeling of human stem cells have been reported in the medical literature (3). Ferumoxides is a US FDA-approved agent that can be used as stable nanoparticles capable of in vivo cell tracking by labeling through endosomal capture. Ferumoxides are negatively charged SPIO that do not attach to the cell membrane without modification of the nanoparticle surface charges. Recently, various methods have been reported to label adult stem cells with ferumoxides. Poly-L-lycine (PLL) has been used as a labeling agent for human stem cells and other mammalian cells (1,2); PLLs have been reported to be efficient agents for endosomal incorporation of magnetic particles. However, PLL–ferumoxides labeling interfered with chondrogenesis of bone marrow-derived MSC (16);

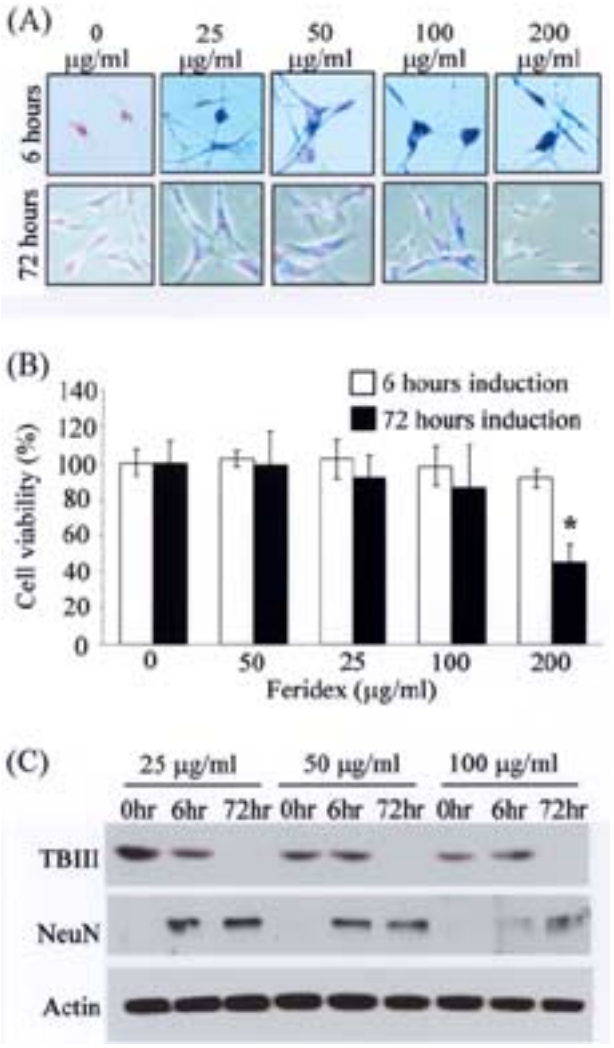


Figure 6. Influence of ferumoxides on neurogenic differentiation of hUCB MSCs. (A) The uptake of ferumoxides was confirmed by PB staining. (B) Trypan blue dye exclusion assay indicated cell viability of the labeled cells. The data represent the mean ± SEM, $n = 3$. $^*p < 0.05$ compared to nontreated controls. (C) Western blot analysis of the cell extracts using TBIII and NeuN antibodies.

moreover, the incubation time of PLL with ferumoxides affects the size of the ferumoxides–PLL complex. Over time, incubation led to the formation of oversize particles for incorporation into the endosomes in cells (3). Protamine sulfate, a US FDA-approved agent, can be used as a labeling agent for ferumoxides. This agent has been used to effectively label a variety of cells with no effect on cell viability, proliferation, and differentiation. Although the use of protamin sulfate has demonstrated labeling efficiency, as a translation ex vivo cell labeling method from the bench to the bedside, the best method for ferumoxides labeling in cells is without any labeling agents. Magnetoelectroporation has been demonstrated to be an alternative method for endosomal labeling with the ferumoxides, without the need for labeling agents (27). With high concentration of ferumoxides used for labeling, magnetoelectroporation-labeled stem cells exhibited an unaltered viability, proliferation, and mitochondrial metabolic rate. In this study, we demonstrated that ferumoxides can be used to label hUCB MSCs efficiently without using other agents. The current method showed a very similar proliferation rate and cytotoxicity between ferumoxides-labeled and unlabeled cells. Although the exact intracellular uptake mechanism for ferumoxides is not known, intracellular incorporated ferumoxides was clearly detected at a variety of concentrations from 25 to 200 μg/ml of fermuoxide as determined by PB staining. This is in sharp contrast to experiments performed by Kostura et al. (16), who reported failure of ferumoxides to label MSCs without labeling agents. However, they obtained their results using a different source of cells, bone marrow-derived MSCs; therefore, their conclusions cannot be compared with our data.

In this study, we used different hUCB MSCs for the differentiation experiments, because we had found that each hUCB MSC has different ability to differentiate into the adipogenic and neurogenic lineages. Bieback et al. (4) reported that hUCB MSC-like cells were difficult to differentiate into adipocytes and required specific culture conditions to differentiate into an adipogenic lineage. Campagnoli et al. (8) and Goodwin et al. (14) suggested that clonal cell isolation and a large number of cell doublings may have caused altered responsiveness in the MSC-like cells towards adipogenic induction. Moreover, the varied capacity to undergo adipogenic differentiation may be explained by the fact that cord blood cells more closely resemble primitive cells, because adipocytes may increase with aging to either occupy excess or support hematopoiesis (12).

Recent studies have indicated that morphological changes and increases in neuronal markers of bone marrow-derived MSCs by simple chemicals are likely the result of cellular toxicity and do not represent neurogenic differentiation (21). However, we used a neurogenic differentiation method by simple chemical to demonstrate the influence of ferumoxides on neurogenic differentiation of hUCB MSCs. As shown in Figure 6B, neurogenic differentiation using simple chemicals did not induce cellular toxicity of hUCB MSCs. The reason for the varied capacity to undergo neurogenic differentiation has not yet been reported. In this study, we noted that the number of cells per square centimeter as well as whether cord blood was a source affecting the differentiating capacity of MSCs. For most of the hUCB MSCs, a cell density of 2,500–3,000/cm^2 resulted in reproducible neurogenic differentiation (data not shown).

In the research on bone marrow-derived MSCs, ferumoxides labeling using PLL (2,10) or protamin sulfate (3) did not show short- or long-term toxicity on multilineage differentiation. By contrast, Kostura et al. has recently reported that labeling bone marrow-derived MSCs with 25 μg/ml of ferumoxides prevented chondrogenic differentiation (16). In the present study, we found a dose-dependent reduction in chondrogenic differentiation with ferumoxides labeling. At 200 μg/ml, the ferumoxides-labeled cultures had abnormal shaped lacunae; these tissues did not express Col II and Col IX mRNA. Although chondrogenic differentiation was blocked by our labeling methods, ferumoxides at a concentration of 50–100 μg/ml showed very clean labeling and a lower cytotoxic level than when using PLL as the labeling agent. We also found that neurogenic differentiation of hUCB MSCs can be blocked by ferumoxides labeling. Incubation for 72 h with a high concentration (200 μg/ml) of ferumoxides resulted in increased cytotoxicity compared with controls (Fig. 6B). Moreover, based on the results of the Western blot analysis, at a concentration above 100 μg/ml ferumoxides decreased the expression of immature and mature neuronal markers (Fig. 6C). However, we confirmed that with the appropriate concentration (25–50 μg/ml) of ferumoxides labeling showed similar differentiation in cells with neuron-like morphology compared to unlabeled cells. These results imply that hUCB MSCs can differentiate into mature neuron-like cells, and ferumoxides labeling over 100 μg/ml can prevent neuronal differentiation.

In conclusion, ferumoxides, without a labeling agent, effectively labeled hUCB MSCs with no or little effect on cell viability and proliferation. However, the multilineage and transdifferentiation capabilities of hUCB MSCs were partially inhibited; these inhibitions were dependent on the concentration of ferumoxides. Further studies are needed to understand the mechanism of inhibition on differentiation. In addition, the appropriate concentration of ferumoxides for clinical application must be evaluated by conducting animal studies. Currently, we cannot conclude whether labeling with ferumoxides induces a positive or negative influence on

hUCB MSC function. However, this is the first report showing the effects of ferumoxides on human cord blood-derived cells without label agents. Our findings may serve as reference information for monitoring the migration of hUCB MSCs in the body, and may help enhance the development of MSC-based strategies for tissue repair.

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