Long term in vivo MRI tracking of endothelial progenitor cells transplanted in rat ischemic limbs and their angiogenic potential.

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Abstract

Stem cell therapy has been used to repair ischemic tissues in limbs, myocardial infarction and the brain. To understand the mechanism implicated during healing, a contrast agent capable of inducing sufficient MR contrast would be very useful in providing fundamental information related to the migration and incorporation of cells into the ischemic tissue.

An MRI contrast agent composed of dextran and gadolinium chelate was synthesized. Hydroxyl groups of dextran were activated with 1,1'-carbonylbis-1H-imidazole and reacted with propanodiamine resulting in aminated dextran, which was then reacted with mono-N-succinimidyl 1,4,7, 10-tetraazacyclodecane-1,4,7, 10- tetraacetate and FITC, and finally reacted with a gadolinium chloride solution (Dex-DOTA-Gd^{3+}).

Endothelial progenitor cells (EPC) were selected as a stem cell model for MRI tracking. Cells were isolated from bone marrow harvested from femurs and tibias of rats, Dex-DOTA-Gd^{3+} was then introduced in the EPCs by electroporation and intracellular stability and cytotoxicity of the

Dex-DOTA-Gd^{3+} was evaluated in vitro. Dex-DOTA-Gd^{3+} labelled EPCs were transplanted in a rat model of ischemic limb and MR images were acquired.

Dex-DOTA-Gd^{3+} efficiently labeled EPCs for a long period of time without any cytotoxicity enabling an MR signal sufficient to track the EPCs intramuscularly injected to the limb.

Introduction

Recent progress in stem cell isolation from different sources such as blood, bone marrow and adipose tissue, coupled with a better understanding of their functions, has spread the applications of stem cell transplantation therapies in cardiac regeneration[1-2], hindlimb ischemia[3-5], stroke recovery [6], etc. When implanting stem cells in ischemic tissue, cells likely will differentiate into functional cells or release growth factors that act in a paracrine manner[7-9]. A system to track stem cells *in vivo* would provide insights into many basic and practical questions related to stem cell therapy such as the mechanism implicated in cell migration, recruitment, mobilization, and incorporation into tissue[7]. MRI is one non-invasive method that has the potential to reach levels of sensitivity and resolution necessary for detection of small numbers of cells and of their location within the ischemic tissues.

MRI tracking of cells labeled with magnetic contrast agents based on iron oxide has been used for visualization of many aspects of the angiogenesis process in different cell types such as hematopoietic and neural progenitor cells[10-12]. One of the advantages of labeling cells by superparamagnetic iron oxide particles (SPIO) is the high sensitivity in comparison with other contrast agents[13]. Wilhem et al. reported that cells labeled with SPIO can be detected in a small numbers (30-60 cell in agarose gel) by high resolution MRI [14]. Other studies reported stem cell homing to the organs and bone marrow,[15] differentiation and migration of oligodendrocyte progenitors into brain parenchyma, migration and incorporation of labeled cells to sites of tumor angiogenesis,[16] and magnetically guided EPCs to a target site.[14] However, despite the great advantages of labeling with SPOI, some articles reported some disadvantages. MR signals in cells labelled with iron oxide transplanted into a mouse hind limb did not show significant differences from the first days to day 28 post transplantation, but the cells evidenced a progressive decrease when monitored by the genetically introduced bioluminescent signals. Histological analysis confirmed that macrophages loaded with iron oxide particles were located between muscle bundles, but no transplanted cells were detected near the macrophages[15]. Therefore, one of the problems when labeling with iron oxide is its instability to remain in the cells *"in vivo"*, probably having been leaked and subseqently the free SPIO are likely endocytosed by macrophages. The same situation was described in iron oxide labeled mesenchymal stem cells transplanted into a rat model of infarcted myocardium.[16] In addition, iron oxide induces negative contrast in MRI which in some cases can be difficult to attribute to the signal loss of the labelled

cells, due to many other sources of negative contrast of MRI into the body.

Our group recently reported a polymeric MRI contrast agent based on a gadolinium chelated poly(vinyl alcohol) . In this study NIH-3T3 cells were labeled with the contrast agent via electroporation. Cell viability and proliferation were not affected by the polymer labeling and MR measurements showed that labeled cells could be clearly tracked "*in vivo*"[17]. In this study we selected endothelial progenitor cells (EPCs) as a stem cell tracking model. EPCs isolated from peripheral blood and bone marrow have been used in therapeutic angiogenesis of ischemic limbs, stroke, and myocardial infarction[18-25]. The advancement in techniques capable of visualizing the EPCs, which are activated by cytokines at the site of injury, is imperative for understanding the mechanism implicated in the proliferation, recruitment, mobilization and incorporation of EPCs into the foci of vasculogenesis. Although the detection sensitivity of gadolinium chelates is generally lower than iron oxide, their positive contrast is easy to detect in a determinant tissue.[26-27] Therefore, we synthesized a water soluble gadolinium chelate as an alternative MRI contrast agent, called Dex-DOTA-Gd^{3+}, for labeling EPCs and delivered it into the EPCs by electroporation method to investigate its feasibility for cellular imaging and its capability to track the fate of the cells *in vivo* for a long period of time. In addition we defined a procedure to properly visualize labeled EPCs transplanted into a rat model of ischemic limb.

Materials and methods

H-NMR measurements

H-NMR spectra were recorded in a 300 MHz 7.1T NMR spectrometer (Gemini 2000/300; Varian Inc., CA., U.S.). Paramagnetic species Gd(III) concentration was measured by an inductively coupled plasma atomic emission spectroscopy (ICP-AES, Model 7510, Shimadzu Co., Kyoto, Japan).

Synthesis of Dex-DOTA-Gd^{3+}

Amination of dextran (MW 40kD) was conducted as follows: dextran (10 mmol sugar unit) was dissolved in 60 ml anhydrous dimethylsulfoxide (ADMSO) and 1,1'-carbonylbis-1H-imidazole (CDI, 7.5 mmol) was added. Reaction proceeded under nitrogen conditions at room temperature for 4 hours. 1,3 propanediamine (75 mmol) was then added to the resulting reaction mixture and stirred overnight at room temperature. The reaction product was subsequently purified by dialysis (Spectra/Pore membrane, MW cut-off = 10 kDa) in distilled water. The remaining solution was lyophilized and dextran-diamine was obtained. About 1% of the total amino groups were reacted with flourescein-5-EX, succinimidyl ester (FITC, Invitrogen, Molecular Probes. Eugene, Oregon, USA).

^1H NMR (D_2O) : δ 4.99(br,CHO_2), 3.605(br,CHOH), 3.769(br,CHOH), 5.2 (br,CHO), 3.253(br,C(=O)$NHCH_2$), 2.88 (br,CH_2NH_2), 1.792(br,$CH_2CH_2CH_2$), 3.53 (br,CHOH), 3.922-3.983 (br,$CHCH_2$).

Dextran-diamine was diluted in 60 ml of ADMSO and reacted with mono-N-succinimidyl 1,4,7,10 tetraazacyclodecane-1,4,7,10 tetraacetate (DOTA, 1 mmol of NH_2 in the dextran-diamine: 1.2 mmol of DOTA) under nitrogen conditions at room temperature for a day. The reaction mixture was purified by using a dialysis membrane (Spectra/Pore membrane, MW cut-off = 10 kDa) in distilled water. The final solution was lyophilized and dextran-diamine-DOTA was obtained.

^1H NMR (D_2O) : δ 5.02 (br,CHO_2), 3.63 (br,CHOH), 3.77 (br,CHOH), 5.2 (br,CHO), 3.3 (br,C(=O)CH_2N), 3.51 (br,NCH2C(=O)OH), 3.326 (br,C(=O)NHCH_2),br,CH_2CH_2N), 1.91 (br,$CH_2CH_2CH_2$), 3.92 (br,CHOH).

Dextran-diamine-DOTA was diluted in 50 ml of distilled water and was treated with 2.2 molar excess of gadolinium chloride solution which added drop wise during stirring. After the stabilization of the solution with 1M NaOH to obtain a final pH of 6.6-7.0, the reaction product was stirred for a day at room temperature. After this procedure, the reaction mixture was purified by using a dialysis membrane (Spectra/Pore membrane, MW cut-off = 10 kDa) in distilled water. The final solution was lyophilized and dextran-DOTA-Gd^{3+} was obtained (Dex-DOTA-Gd^{3+}).

MRI measurements

T_1-weighted images were obtained in a 1.5-T compact MR imaging system (MRmini, Dainippon Sumitomo Pharma, Osaka, Japan) with a TR of 2000 ms and TE of 9 ms (FOV, 4 x 8 cm; matrix, 126 x 256; slice thickness, 1 mm; Slice gap, 0 mm; number of slices, 35).

Isolation of EPCs

Bone marrow (BM) was flushed from femurs and tibias of F344 rats (4 weeks old, male) after previous cytokine inducted mobilization of bone marrow-derived endothelial progenitor cells by using granulocyte colony-stimulating factor (G-CSF, Kirin Pharma, Japan. 200 μg/kg/day, 5 days subcutaneously injected)[28-29]. CD34 and FLK-1 positive bone marrow cells were isolated by means of magnetic beads (Streptavidin Microbeads, Miltenyi Biotec GmbH, Gladbach, Germany) coated with antibody CD34 and FLK-1 (sc-6251 and sc-7324, Santa Cruz Biotechnology,INC.,CA.,USA) by using Biotin Labeling kit-NH_2 (Dojindo Molecular Technologies, INC, Japan). Cells were placed in fibronectin coated dishes and cultured with an endothelial cell basal medium (EBM-2) supplemented with EGM-2 SingleQuots (Clonetics Lonza, walkerville, MD., USA)[18].

Histological Identification of EPCs

One week after isolation, cells were detached by using ReagentPAck^{TM} (Clonetics Lonza, walkerville, MD., USA) and $1x10^5$ were placed in fibronectin coated dishes (3.5 mm glass botton dish. Matsunami Glass IND., LTD., Japan) and fluorescent staining of adherent cells was used to confirm the EPC phenotype. EPC were incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate-labeled acetylated LDL (DiI-acLDL; 10 μg/ml, Biomedical Technologies, n=3) at 37°C for 4 h.[19,21,24] EPCs were further

stained with fluorescein griffonia simplicifolia lection I, isolectin B4 (10 µg/ml, Vector Laboratories, INC., Burlingame, CA., USA, *n*=3) for 2 h[30,31]. Additional staining with endothelial nitric oxide synthase was performed (eNOS; Santa Cruz Biotechnology, INC., CA., USA, *n*=3).[31] After staining the samples were viewed with a confocal microscope (Nikon Eclipse TE 2000-E, Nikon Corporation Tokyo, Japan). Cells demonstrating positive fluorescence were identified as EPCs [19,21,30,31]. Six days after implantation of double labeled EPCs by using Dex-DOTA-Gd^{3+} and Qtracker® cell 655 labeling kit (molecular Probes®, invitrogen detection technologies, Eugene, Oregon, USA. Cells were labeled as indicated by the supplier), a rat was sacrificed to demonstrate that MR images of the Dex-DOTA-Gd^{3+} contrast agent inside the cells actually corresponded to the EPCs transplanted in the ischemic limb. Adductor muscle was dissected and subsequently embedded in Tissue-Tek for freezing (Sakura Finetechenical Co. Ltd., Tokyo, Japan). Samples were then observed in a confocal microscope (Nikon Eclipse TE 2000-E, Nikon Corporation Tokyo, Japan) (*n*=20, 8 µm thickness). Paraffin sections of the excised tissue were then stained for macrophages as follows: frozen sections were thawed and the tissue was fixed in acetone. Sections were then washed in TBS buffer (50 mM Tris-HCl buffer containing 0.01 % tween-20 and 0.15 M NaCl), and treated with 0.6 % H_2O_2 in 80 % methanol at room temperature for 20 min. After washing in TBS buffer, Samples were incubated with mouse anti-rat CD 68 (AbD Serotec, Kidlinton, Oxford, UK) as a primary antibody at 4 °C overnight. Sections were washed in TBST and stained with Histofine® Simple Stain MAX PO (Nichirei Biosciences INC. Tokyo, Japan) a second antibody at room temperature for 30 min. After washing in TBST, the tissue was incubated in 3,3′-diaminobenzidine tetrahydrochloride solution (DAB) until a brown reaction product appeared.

Determination of Capillary Density

To quantify the effect of labeled cells transplantation on neovascularization, an assessment was performed by measuring the number of capillaries in the AP staining within 36 randomly chosen fields in light-microscopic (Nikon Coolscope II) sections taken from the ischemic hindlimb (12 measurements/rat) at day 35. Tissue specimens were taken from the adductor and semimembranous muscles. Capillary density was compared with the non-ischemic limb. Frozen sections of tissue (8 µm) were stained with alkaline phosphatase (AP) substrate kit III (Vector laboratories, Inc.Burlingame, USA) to detect capillary endothelial cells.[32,33] Additional sections were stained for von Willebrand factor (vWF, polyclonal Rabbit anti-human) (Dako LSAB System-HRP for use on rat specimens, DakoCytomation, Denmark) to further confirm the endothelial cells phenotype.

Cell Labeling by Electroporation

After isolation EPCs were cultured for 2 months (cells were used in the 4th passage for all the experiments) in fibronectin coated dishes and cultured with endothelial cell basal medium (EBM-2) supplemented with EGM-2 SingleQuots (Clonetics Lonza, walkerville, MD., USA) at 37°

C in 5% CO_2. Afterward, $5x10^5$ cells were counted and placed in 60 mm dishes and cultured for a day. Cells were then washed with phosphate buffered saline solution (PBS, Invitrogen, NY, USA) and cultured in 3 ml endothelial basal medium (phenol red free, Clonetics Lonza, Walkerville, MD., USA) for 30 min. 10 mM of Dex-DOTA-Gd^{3+} was subsequently added to the medium and electrical pulses were applied to the cells using a CUY-21 electroporator (NEPPA GENE, Tokyo, Japan) under the following conditions: field strength 300 V/cm, number of pulses 10, pulse duration 5 ms. Cells were then cultured for one hour and washed several times with PBS.

Dex-DOTA-Gd^{3+} labeled EPCs were divided and placed into fibronectin coated dishes for further microscopy analysis (35 mm dishes, 27 mm quartz bottom, $1.2x10^5$ per dish n=30). To verify if the Dex-DOTA-Gd^{3+} leakage from the cells occurred after electroporation, cells were washed with PBS and treated with 1 ml of lysis buffer (25 mM tris; pH7.8, 2 mM 1,2-diamino-cyclohexan-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100). After one hour incubation at 37□C, 100 µl of the resulting solution was placed in a 96 well plate and the fluorescence intensity was measured in a fluorometer (n=10, excitation 430 nm, emission 540 nm, Wallac 1420 ARVO SX, Perkin-Elmer Life Sciences, Boston, MA., USA). Knowing the quantity of Gd in the polymer, the Gd inside the cells could be determined by a linear regression of the fluorescent intensity of the cell lysate in a determined number versus the fluorescent intensity of the Dex-DOTA-Gd^{3+} in different concentrations.

Cytotoxicity was analyzed by an LDH cytotoxic test following the (Wako Pure Chemical Industries, Ltd, Japan, assay was performed as indicated by the supplier) co-culturing 10^4 EPCs with different concentration of Dex-DOTA-Gd^{3+} for 24 h. Additionally, the tetrazolium salt (WST-1) was used to measured cell proliferation and viability (Premix WST-1 cell proliferation assay system, Takara BIO INC, Japan). Dex-DOTA-Gd^{3+} labeled and non-labeled EPCs were put in 12 well plate fibronectin coated dishes ($4x10^4$ cell/well). The Premix WST-1 ready to use solution was added to wells (100 µl/ 1 ml medium) and cells were then incubated at 37°C in 5% CO_2 for 24 hours. Absorbance was measured and cell quantity was calculated by a linear regression of the fluorescent intensity of non-labeled EPCs in a determined number . Samples were collected each two during 10 days (n=6).

Relaxivity

T_1 relaxation was estimated in each gadolinium complex. Longitudinal relaxation times (T_1) were measured by a combination of a NMR tube (650 µl distilled water diluting the polymer in different concentrations) and a small tube (containning 50 µl of benzene –d6). To obtain the signal of water protons an attenuator was used. Samples were measured in a 300 MHz (7.1 T) NMR spectrometer (Gemini 2000/300; Varian Inc., CA., USA) using an inversion recovery technique with 19 inversions times (TI) ranging from 1 to 5000 ms at room temperature (25 □C).

A typical 180□ pulse was 19 μs. T_1 values were calculated by a least-squares fitting of the signal intensities measured at 19 TI values in an exponential fashion. Gadolinium complex relaxivity was determined by a linear regression of $1/T_1$ versus gadolinium complex concentration.

Rat Ischemic Limb Model

Male F344 rats (8 weeks old) were anesthetized with isoflurane (1.5% in air). The left femoral artery and vein and their branches were ligated and totally excised through a skin incision. The femoral artery and vein were excised from their proximal origin as a branch of the external iliac artery to the distal point where it bifurcates into the saphenous and popliteal arteries. [34] Rats (*n*=8) were injected in three places with a total of 150 μl of Bolheal ® containing Dex-DOTA-Gd^{+3} labeled EPCs inside the muscle at the inguinal region where the femoral artery and vein were excised. Injections were applied as follows: to allow a normal movement of the cell through the muscle, $6.3x10^6$ labeled cells in 50 μl Bolheal ® component A (thrombin, 250 unit/ml) were intramuscularly injected in 3 different places in the abductor and quadriceps (total $1.8\text{-}2x10^7$ cells) and 50 μl Bolheal ® component B (fibrinogen,80 mg/ml) was then injected in the same sites to temporarily immobilize the cells (gelation occurred in the muscle). Ischemic limb controls (*n*=8) were injected with 150 μl Bolheal ® not containing cells.

Statistical Analysis

All data are expressed as means ±SD. Statistical significance was evaluated by an unpaired two tailed Student's *t test* for two variables. Differences were considered significant when *P* values were less than 0.05.

Results

MRI contrast agent

To develop a suitable MRI contrast agent for EPCs, we selected dextran (Mw 40 KDa) for its biocompatiblility, rapid clearance in the body[35], biodegradability [36] and approval by the U.S Food and Drug Administration (Fig. 1). Inductively coupled plasma atomic emission spectroscopy (ICP-AES) revealed 19.5% of OH groups reacted with DOTA-Gd in the contrast agent structure which corresponds to m=48 and n=199.

Figure 2a shows the MR images of Dex-DOTA-Gd^{+3} aqueous solutions at different concentrations. MR contrast agents shorten the longitudinal relaxation time T1 value, which is defined as the time constant of the exponential recovery of proton spins to their equilibrium along an applied field after disturbance. The relaxivity (R1) for Dex-DOTA-Gd^{3+}, which represents the reciprocal of the relaxation time per unit of Gd concentration in s^{-1}/mM, was determined by measuring the longitudinal relaxation time T1 values of several aqueous solutions under Nuclear Magnetic Resonance (NMR) and plotting 1/T1 versus polymer concentration. Dex-DOTA-Gd^{3+} showed a relaxivity value of 5.8 s^{-1}/mM (Fig. 2c), which, in comparison with approximate values of about 4.2 for DOTA-Gd and 3.8 s^{-1}/mM for gadolinium-diethylenetriaminepentaacetic acid

DTPA-Gd,[37,38] represents an increase of 38% and 52.6% over the relaxation value. Therefore, an enhancement of visualization of the resolution in MR images with Dex-DOTA-Gd^{3+} as a contrast agent is possible in comparison with the standard Gd^{3+} and Magnevits®. MR images of Dex-DOTA-Gd^{3+} aqueous solutions show an increase in the signal intensity with respect to an increase in the concentration of the polymer (Fig. 2a). This increase is due to a reduction of the longitudinal relaxation time. Moreover, images show the capacity of the Gd complex to return to its equilibrium after radio frequency excitation. It can be seen that Dex-DOTA-Gd^{3+} MR images from a concentration of 0.2 mM are visually different in comparison with distilled water.

Labeling of EPCs

EPCs phenotype was then confirmed by immunostaining with DiI-acLDL, staining with lectin and eNOS (Fig.3). All cells were confirmed as the EPC phenotype owing to their capacity to incorporate acetylated low density lipoprotein, to bind lectin, and endothelial nitric oxide synthase througt observation images (Fig. 3) [30,31]. Both FLK-1 and CD34 are expressed by all hematopoietic stem cells (HSCs) and EPCs but cease to be expressed during hematopoietic differentiation[18,19].

EPC cells were labeled with Dex-DOTA-Gd^{3+} by means of electroporation and placed into agarose hydrogel. As can be seen in Fig. 2b, MR images of EPCs containing Dex-DOTA-Gd^{3+} in 100 µl agarose hydrogel, the amount of labeled cells necessary to obtain differences in contrast with respect to water was $1x10^6$ ($1.11x10^{-10}$ Gd mmol) and the quantity of gadolinium incorporated into a cell was 0.12 pg of Gd. Cytotoxicity was analyzed by an LDH cytotoxic test. Percentages of cell viability labeled with Dex-DOTA-Gd^{3+} at different concentrations are shown in Fig. 4a. This clearly shows, that in concentrations lower than 15 mM, high cell viability is up to 90%, whereas higher concentrations were found to slightly toxic, but in a range between 70-80% cell viability.

Therefore, we selected 10mM polymer concentration to label the cell for its low toxicity and an adequate quantity of gadolinium to achieve the required T_1 shortening and high resolution for tracking labeled cells. **Consistently with the previous results, WST-1 assay performed with 10mM polymer concentration revealed that EPCs were not affected by the contrast agent at least within 10 days after electroporation when cells were achieved maximum confluence. No statistical differences were found between the proliferation of non-labeled EPCs and Dex-DOTA-Gd^{3+} labeled EPCs.**

To analyze the distribution and stability of the contrast agent inside the cells , cells were cultured for 25 days after labeling with Dex-DOTA-Gd^{3+}. As can be noted in Fig 4b, EPCs showed a remarkably high degree of intracellular labeling with the cytoplasm containing large amounts of contrast agent; interestingly no further transport in the nuclei was observed 25 days postlabeling. Fig. 4c shows the Dex-DOTA-Gd^{3+} concentration inside cells with respect to the culture days and cell proliferation rate. The quantity of polymer remained stable within 25 days

after electroporation suggesting that Dex-DOTA-Gd^{3+} did not leak out of cells during culture days and cells grew well. The differences detected from d1 to d25 were not statistically significant (P> 0.05), demonstrating stable labeling of Dex-DOTA-Gd^{3+} over time.

***In vivo* Tracking of EPCs**

Having demonstrated successful cellular MR imaging of EPCs by means of Dex-DOTA-Gd^{3+}, we wanted to determine the efficacy of our MRI contrast agent in detecting and tracking EPCs in an *in vivo* rat model of ischemic hindlimb. Immediately after inducing ischemia, we injected rats with Dex-DOTA-Gd^{3+} labeled EPCs in the adductor and quadriceps muscle in the inguinal region where the femoral artery and vein were excised (Bolheal® was used to temporarily immobilize cells as indicated in Methods). Animals were imaged three times per week to determine the fate of the transplanted cells in the tissue. Figure 5 shows MR images obtained *in vivo* using a 1.5T animal MR imaging within 20 days postransplantation. Cells could be clearly detected in the muscles, as well as in migration for at least 16 days, until the cells totally vanished at day 19. A rat was then sacrificed six days after transplantation and the zone that appeared in the MR image was excised. As is observed in the MR images in Fig. 6a, cells are located in the adductor muscle. Frozen section slides revealed the presence of Qtracker® 655 labeled EPCs in the ischemic area. During the development of the method for tracking EPCs in vivo, we observed that between 5 to $6.3x10^6$ labeled cells diluted in 50 µl of Bolheal were necessary to achieve cell imaging for at least 14 day postransplantation.

Immunohistochemical staining for vWF and (AP) of the removed muscles at 35 d revealed the presence of numerous capillary endothelial cells in implanted Dex-DOTA-Gd^{3+} labeled EPCs rats (Fig. 7a); capillary/muscle fiber ratios for this rat group markedly increased (2-fold, P<0.001,Fig. 7b) compared with the ratio for the ischemic control group. There was not any significant difference in capillary density between the ischemic control and the normal limb (Fig. 7b, P<0.05). This blood flow recovery by the EPC transplantation suggests that our cell labeling system based on the Dex-DOTA-Gd3+ and electroporation neither affect the cell viability and cell growth nor the stem cell functions.

Discussion

Bone marrow derived EPCs were successfully labelled with Dex-DOTA-Gd^{3+} by electroporation at 0.12 pg of Gd per cell, which is significantly low in comparison with 12 pg/cell loaded with iron oxide,[16] but is sufficient to achieve signal enhancement required in MRI for a good contrast in the body. Several investigations regarding magnetic labeling of cells for MRI tracking *in vitro* and *in vivo* have shown that iron oxide nanoparticles are suitable for imaging stem cells[10,12,39]. Nevertheless, more recent works call into question its use as a contrast agent owing to false-positive MRI signals involving inconsistencies between a persistent positive MRI signal and histologically labeled cells.[16,2, 40-42]

Commonly used Gd complex contrast agents such as DOTA- Gd^{3+} and Magnevist® exhibit a

relatively slow relaxation time in comparison with Dex-DOTA-Gd^{3+}. This is due to the fact that those contrast agents have more simple structures of low molecular weight and need higher doses of contrast agent to accomplish the same resolution. The incorporation of DOTA-Gd^{3+} in dextran probably increased the relaxation time due to a steric limitation on the rotational movement of the polymer increasing the rotational correlation coefficient.[43,44] In addition DOTA-Gd conjugated to a biocompatible dextran carrier and the free Dex-DOTA-Gd^{3+} are expected to have rapid clearance in the body and a reduced interaction with the cell membrane due to their high solubility.

Our results demonstrate that Dex-DOTA-Gd^{3+} achieved intracellular labeling through electroporation, which is an essential condition when labeling cells, due to the fact that a membrane modified with a polymer will interfere with cell-cell interactions during the recruitment of cells in the mechanism of angiogenesis. A polymer in the cell membrane can be easily detached and may be taken up and transferred to other cells *in vivo*. Fluorescent intensity of the cell lysis solution suggests that Dex-DOTA-Gd^{3+} remains stable in the EPCs for at least 25 d (Fig. 4c); a decrease in the fluorescent intensity indicates that the polymer leaked out of cells during culturing, or that cells are dying, but no significant change in the fluorescent intensity was shown in labelled EPCs. In addition, cells showed a normal proliferation rate (during 10 days)[45] after Dex-DOTA-Gd^{3+} labeling by electroporation. **An additional cell proliferation activity and viability was performed by using WST-1 assay system. Results demonstrated that EPCs were not affected after labeling by electroporation with Dex-DOTA-Gd^{3+} as an MRI contrast agent. Not statistic differences were found between the proliferation or labeled and non-labeled EPCs. At 10 day EPCs did not longer proliferate, cell number remained stable.**

MR images *in vivo* indicate that the Dex-DOTA-Gd^{3+} labeled EPCs are perfectly detectable and the fate of the cells can be followed within 16 days after transplantation in a cell density of 5-6x10^6 cell per 0.05 ml. (Fig. 5). Labeled cells totally faded after 19 days. This is likely due to cell biodistribution during cell migration which is probably caused by a cytochemical attraction of EPCs via incorporation in newly formed vessels and may also be influenced by the release of proangiogenic factors in a paracrine manner[28,46-48]. Moreover, a likely cell proliferation *in vivo* may reduce the concentration of the polymer inside the EPC cytoplasm causing a low contrast in the limb. Proliferation rate of the labeled cells *in vitro* indicated that after 10 days cells increased their quantity approximately 3 times and the fluorescent intensity remained stable. This indicates that the concentration of Dex-DOTA-Gd^{3+} remained constant , but the concentration per cell might be reduced 3 times. Analogous to the results *in vitro* in figure 2b; if the image contrast of 6x10^6 cells is reduced 3 times the signal intensity should likely be similar to the signal in 2x10^6 cells. If this intensity reduction is assumed *in vivo*, the 6.3x10^6 labeled cells transplanted in the ischemic limb 10 days after proliferation could then be tracked because the Dex-DOTA-Gd^{3+} still

has capacity to produce signal. At day 14 after implantation of labeled cells, the concentration of Dex-DOTA-Gd^{3+} probably was reduced 4 times which is the limit of signal intensity in this contrast agent .

A rat was sacrificed **6** days after implantation of labeled cells. Muscles were carefully excised taking particular attention to the place where MR images showed the location of the labeled cells. **Presence of Qtracker® 655 labeled EPC** localized in the neovascular zones of the ischemic limb which ensured that MRI signals corresponded to those generated by the labeled cells (Fig. 6a). In addition the number of macrophages found in the muscles (Fig. 6b) was insignificant but in any case was related to the obtained MR images or cells. This ensures that in contrast to cells loaded with iron oxide, Dex-DOTA-Gd^{3+} labeled EPCs have not been endocytosed by the macrophages and can be reliable when tracking labeled cells for a long period of time.

Preliminary *in vivo* data suggests that Dex-DOTA-Gd^{3+} labeled cells were incorporated into sites of neovascularization and arranged into the capillary network. Direct local transplantation of labeled cells into the ischemic limb quantitatively augmented capillary density in the ischemic limb *in vivo*.

Conclusions

MRI as a method for tracking EPCs by using our novel contrast agent in therapeutic angiogenesis of ischemic limb models would be extremely useful in anatomically localizing the transplanted cells for a long period of time. A contrast agent for imaging cells *in vivo* has to satisfy certain conditions such as: desired MRI contrast properties, *ex vivo* cell labeling before transplantation, a high degree and stabile intracellular labeling, biocompatibility without affecting the cell viability, proliferation and healing capacity, and remaining consistently detectable for long periods of time. These conditions are completely met by using Dex-DOTA-Gd^{3+} as an MRI contrast agent.

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Disclosure Statement

The authors declare "No competing financial interest exist."

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Figures legends

Figure 1. MRI contrast agent synthesis. Schematic structure of Dex-DOTA-Gd^{3+} which consists in a dextran derivative modified with a gadolinium (III) chelate DOTA-Gd^{3+}.

Figure 2. MRI contrast agent properties: (a) MR images of Dex-DOTA-Gd^{3+} aqueous solutions. T1 weighted images of the samples were acquired with a 2D and 3D spin echo sequence 300 repetition times (TR) and a echo time (TE) of 12 ms, image acquisition matrix of 128×256. Fifty µl Dex-DOTA-Gd^{3+} solutions containing 0.1, 0.2, 0.3, 0.4, and 0.5 mmol polymer unit. (b) MRI images of EPCs containing Dex-DOTA-Gd^{3+} as a contrast agent (10 mM in unit, 19.5 Gd introduction ratio when labeling) in 100 µl agarose hydrogel, 1.5 T. Sequence: 2D spin echo, coronal, slice 1 mm, TR=2000 ms, TE=9 ms, image acquisition matrix of 128×256 ; 0.12 pg Gd/cell. (c) Relaxivity measurements of Dex-DOTA-Gd^{3+}. T1 was measured by a combination of a NMR tube (650 µl distilled water diluting the polymer in different concentrations, and a small tube containing 50 µl of benzene–d6). An attenuator was used to obtain the signal of water proton.

Figure 3. Phenotype identification of isolated EPCs. One week after isolation, fluorescent staining of adherent cells was used to confirm the EPC phenotype. Cells were incubated with DiI-acLDL, Isolectin B4, and eNOS. After staining the samples were visualized with a confocal microscope. Cells demonstrated positive fluorescence which ensures the EPC phenotype. Scale bar 50 µm.

Figure 4 (a) EPCs viability measured by LDH assay at different Dex-DOTA-Gd^{3+} concentrations. (b) Stability of Dex-DOTA-Gd^{3+} in EPCs after electroporation (solid mark) and cell proliferation (open mark). Fluorescent intensity mean (±sd) are shown and statistical differences were not found which respect to the first day after electroporation, (n=5).* P>0.05. (c) EPCs proliferation activity and viability was assessed by WST-1 cell proliferation assay. The cell quantity was measured by its absorbance. Cell number mean (±sd) are shown and statistical differences were not found between labeled and unlabeled EPCs (n=6)* P>0.05. (d) Confocal microscopy of Dex-DOTA-Gd^{3+} labeled EPCs after electroporation. Scale bar 40 (Left) and 20 (Right) µm.

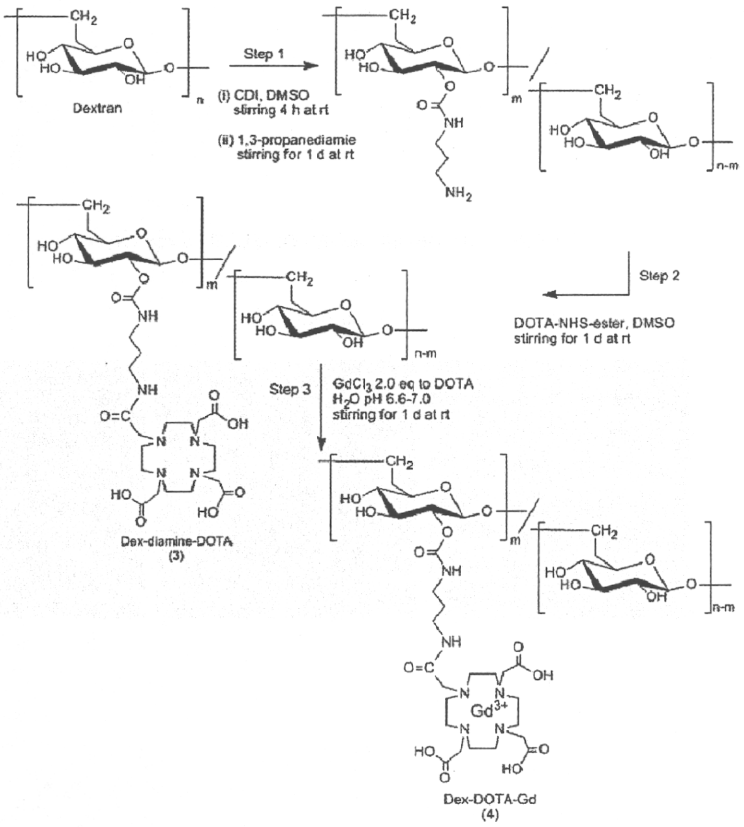
Figure 5. MR images of Dex-DOTA-Gd^{3+} labeled EPCs (2×10^7) after intramuscular injection in ischemic limb rat models. Bolheal was used as a scaffold. T_1 weighted images were acquired at 0, 1, 5, 9, 12, 14,16, and 19 days postransplantation in a 1.5 T compact MR imaging system. Sequence: spin echo, coronal, slice 1 mm, TR=2000 ms, TE=9 ms, image acquisition matrix of 128×256.

Figure 6. Identification of transplanted EPCs in the ischemic limb. Six days after Dex-DOTA-Gd^{3+} and the Qtracker® cell 655 labeled EPCs transplantation, a rat was sacrificed to demonstrate that MR images of cell inside the muscle really match to the EPCs transplanted in the ischemic limb (a). The MR image corresponds to the rat before sacrifice (1) Frozen section of tissues dissected specifically in the area were the MR image showed were stained with Qtracker® cell

655 (2) differential interference contrast DIC (3) and merge image (4). (b) Immunohistochemical analysis for identification of macrophage in the transplanted area, HE staining (A), CD68 (B), CD68 negative control, without reaction with the secondary antibody (C), CD68 positive control from rat spleen (D). Arrow indicates a positive macrophage. Scale bar 50 μm.

Figure 7. Immunohistochemical staining of non-ischemic and ischemic limbs and capillary density. Immunohistochemical staining for AP and vWF staining turns viable endothelial cells. 5 fields from 2 muscles samples of each animal (n=9) were randomly selected (a).Capillary density is shown as the capillary/muscle fiber ratios from frozen sections prepared from muscles of ischemic limbs 35 d postransplantation of Dex-DOTA-Gd^{3+} labeled EPCs and ischemic controls. *P<0.05 vs normal limb and ischemic control(b).

Agudelo. Fig. 1



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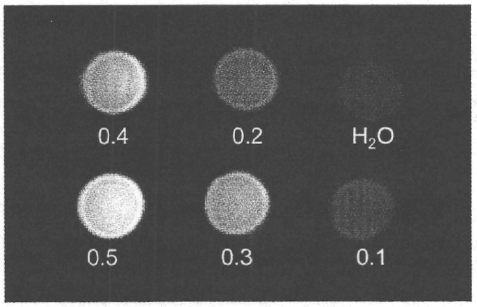

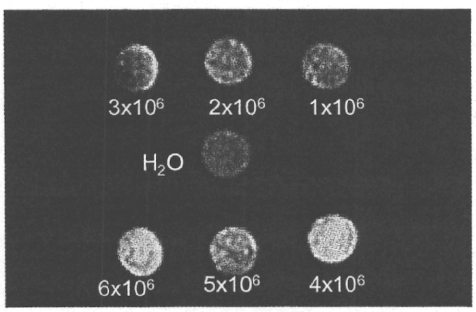

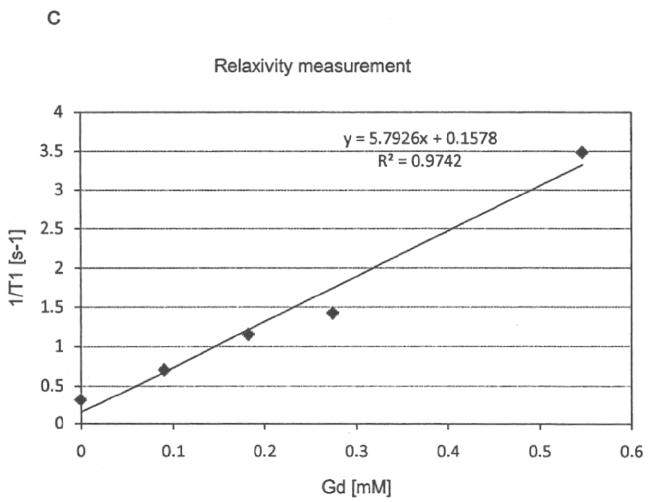

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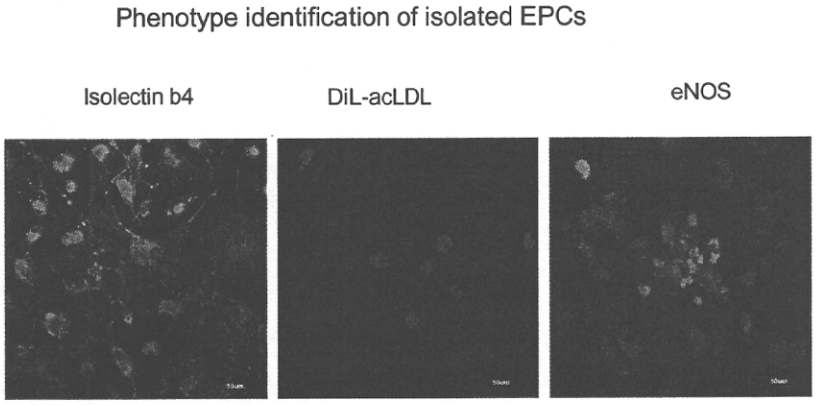

Agudelo. Fig. 4