Magnetic carbon nanotubes: a new tool for shepherding mesenchymal stem cells by magnetic fields

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Abstract

Aims. In this paper, we investigated the interaction between magnetic carbon nanotubes (CNTs) and mesenchymal stem cells (MSCs) and their ability to guide these cells injected *in vivo* by using an external magnetic field. This could pay the way for the development of new strategies for manipulation/guidance of MSCs in regenerative medicine and cell transplantation. *Materials and methods*. MSCs were cultured in a CNT-containing medium. We investigated if CNTs and magnetic field can alter cell viability, proliferation rate and the ability of cells to differentiate in osteocytes and adipocytes. We also studied the effect of interaction with CNTs in terms of cell phenotype and cytoskeletal conformation. Finally we investigated the ability of MSCs labelled with CNTs to move towards the magnetic source *in vitro* and the possibility to be guided into target organ *in vivo. Conclusion*: We have demonstrated that CNTs interact with the MSCs without compromising their viability, proliferation rate and their ability to differentiate. We showed that CNTs, when exposed to magnetic fields, are able to shepherd MSCs towards the magnetic source both *in vitro* and *in vivo*.

Key words: mesenchymal stem cell, magnetic nanoparticle, MSCs homing.

Introduction

Carbon nanotubes (CNTs) [1] are molecular-scale tubes of graphite carbon with a high aspect ratio: lengths varying from several hundred nanometres to several micrometers with diameters of 0.4–2 nm for single-walled carbon nanotubes (SWCNTs) and 2–100 nm for coaxial multi-wall (MWCNTs) types. CNTs exhibit unique properties [2] which account for their substantial scientific and industrial interest with thousands of original publications reported annually. In particular, biomedical applications for CNTs are currently under intense investigation because of their molecular and unique physicochemical properties [3-4]. However the insolubility of CNTs in aqueous media has been a major technical barrier to their biomedical applications. The recent progress in chemical modification and functionalization of nanotubes has largely overcome this problem such that it is now possible to solubilize and disperse CNTs in water, thus enabling their use and application in biophysiological environments [5]. Equally important is the recent demonstration that bioactive species, e.g., proteins, carbohydrates, and nucleic acids can be conjugated with CNTs [6]. Material therapeutic advances are likely to be realized by the exploitation of the unique physical properties of CNTs, e.g., hyperthermic cell ablation based on the strong near- infrared light absorbance by SWCNTs [7], bacterial electroporation by the field emission properties of MWCNTs [8], cell permeabilisation by application of static electric field [9] and thermal release from SWCNTs following application of a radiofrequency current for tumour ablation [10]. CNTs also possess intriguing magnetic properties which derive from the metal catalyst impurities entrapped at CNT extremities during their manufacture, enabling them to react to external magnetic fields. This property has been utilised by Cai *et al.* to develop a physical technique for *in vitro* and *ex vivo* gene transfer known as 'nanotube spearing', capable of effective cell transfection with plasmid DNA [11]. Similarly, we have recently demonstrated that MWCNTs are able to interact with cells and, when exposed to a magnetic field, induce their migration towards the magnetic source [12-13].

The aim of the present paper was to investigate the use of this magnetic force exerted by CNTs in the manipulation and homing of mesenchymal stem cells (MSCs). They are bone marrow-derived cells with the capacity of replication and differentiation *in vitro* into several tissues including bone, cartilage, stroma, fat, muscle and tendon [14]. MSCs can be isolated by relatively simple procedures and then expanded without loss of their ability to differentiate into multiple lineages. As such, these cells have several clinical potential in regenerative medicine for repair or replacement of damaged tissues. MSCs do not express the haematopoietic markers CD34, CD45 or CD14 and can be identified by positive staining for a number of non-specific surface adhesion molecules such as CD29, CD44, CD73, CD105, CD 90 [14]. Additionally, MSCs do not express co-stimulatory and MHC class II molecules; therefore they escape the immune detection *in vivo* and for this reason, are considered good candidates for cellular therapy in an allogeneic setting [15]. Numerous *in-vitro* studies have demonstrated that MSCs from various species can suppress T-cell proliferation in both autologous and alloreactive states. MSCs interact and suppress cell-mediated immune responses (dendritic cells, CD4+, CD8+ T cells, NK cells) directly and through soluble factors, evading each aspect of the cellular rejection process and acting as universal suppressors of immune reactivity [16]. The interactions between allogeneic MSCs and immune cells may play a role in the induction of tolerance and offers a new perspective for the reduction of acute rejection of transplants and modulation of inflammation. [17-18].

One of the unresolved problems in the use of MSCs therapy relates to site specific homing of MSC homing to the organ of interest as several studies in animal models have reported that both allogeneic or autologous MSCs [19-20-21] migrate and settle in many tissue and organs, e.g., kidney, skin, thymus, liver, spleen and bone marrow, especially after injury. These findings suggest that MSCs may participate in ongoing cellular turnover and replacement in a wide variety of tissues, especially following injury. In a seminal study, Francois et al reported the fate and potential therapeutic effect of the re-infusion of hMSCs after total body irradiation in the mouse model. Whereas in non-irradiated animals, hMSCs homed in low levels in various tissues (lung, bone

marrow and muscles) and no significant colonisation was found in other organs. In contrast, total body irradiation induced an increase in hMSCs colonization levels in the brain, heart, bone marrow and muscles [22]. This observation suggests the potential use of MSCs to repair damaged normal tissues after accidental irradiation or in patients submitted to chemo-radiotherapy. Others researchers have demonstrated that MSCs are preferentially attracted by injured tissues because these express specific receptors or ligands to facilitate trafficking, adhesion, and infiltration of MSCs to the injury site [22-23]. Moreover other reports have shown preferential migration of MSCs to the lungs following their injection in healthy animals [20-21]. These cells become entrapped in lungs presumably as a consequence of significant differences in the diameter of MSC and lumen of the pulmonary capillary bed (>15μm vs. 10 μm, respectively). Allers et al reported that lungs are the long-term destination site for human MSC in recipient mice [20]. The bio-distribution in the body of MSCs is the same irrespective of administration (intravenous or intra-arterial injection). Moreover, is time dependent and the level of cell colonization is relatively low in unconditioned recipient [20-21]. Thus on the available evidence, MSCs distribute to a wide variety of organs and tissues following systemic administration. This dispersion of the injected cells reduces the intended local effect on the target organ. As several clinical applications of MSCs require the administration of cells by the intravenous route [24-25-26-27], many researchers are exploring novel technologies for stem cell guidance. Recently Kobayashi et al used magnetically labelled mesenchymal stem cells with superparamagnetic iron oxide nanoparticles (SPIONs) to guide them to the desired osteochondral defects of the patellae after intra-articular injection [28]. The aim of this work is to provide a new method for in-vivo stem cell guidance which assures 1) minimal manipulation of MSCs, 2) long magnetization 3) negligible citotossicity, and 4) enhanced engraftment of MSCs without compromise their therapeutic potency. We demonstrate that MSCs cultured with magnetic carbon nanotubes maintain their biological activity in terms of viability and differentiation. Moreover they can be shepherded *in vitro* and guided towards the target organ *in vivo* by using an external magnetic field.

Materials and methods

MWCNT samples

MWCNTs (provided by Nanothinx S.A., Greece) were produced by catalytic chemical vapour deposition (CCVD or CVD) of hydrocarbon sources on substrates of metal oxides (Al_2O_3) impregnated with metal catalysts (Fe). The MWCNTs produced in this way have been characterised previously [29]. Briefly, the carbon content of the sample is 97.06% (i.e., metal particle content 2.94%), the metal particles being Al and Fe in a ratio Fe/Al of 7±1 (w/w); the amount of amorphous carbon in the carbon content is <1% and the nanotube diameter range from 20 to 40 nm.

Pluronic F127 (PF127, polyoxyethylene-polyoxypropylene block copolymer (Sigma, St. Louis, MO) surfactant was dispersed in Dulbecco modified culture medium at a concentration of 0.1%. MWCNTs were added to this solution (0.4 mg/ml) which was heated over a hot plate (70°C) with magnetic stirring for two hours; the resulting mixture was sonicated with a Branson sonicator 2510 (Bransonic, CT, USA) at 20 W. The mixture was then centrifuged at 900g for 10 minutes to remove residual non-suspended nanotubes and impurities. The concentration of the PF127-CNT solution, measured by spectrophotometric analysis [30] was 100 μg/ml. After preparation, a small drop (10 μl) of dispersion was placed onto a plasma-cleaned silicon wafer and allowed to dry under flux. Samples were imaged by Focused Ion Beam (FIB) microscopy which revealed an average length of the nanotubes after dispersion of 1 μm. The FIB system used in the present work was FEI 200 (Focused Ion Beam localized milling and deposition) delivering a 30 keV beam of gallium ions (Ga+).

Animals

Inbred male Wistar-Furth (WF) and Lewis rats, weighing 275-300g, were purchased from Charles River Laboratories (Italy). The animals were fed on standard rodent chow (Rieper, Italy) and water

ad lib, and were kept under a 12 h light/dark cycle. All the experimental procedures were carried out with the approval of the Ethical Committee for Animal Experimentation of the University of Pisa.

MSC isolation and culture

Rat bone marrow cells were collected from tibia and femur of Wistar Furth animals following the Dobson procedure [31]. Total nucleated cells were cultured in Dulbecco modified culture medium (Sigma-Aldrich, Italy) supplemented with 10% fetal bovine serum (Eurobio, Italy), 1% L-glutamine (Sigma-Aldrich, Italy), penicillin (Eurobio, 50U/mL), streptomycin (Eurobio, 50µg/mL), amphoterycin B (Sigma-Aldrich 0,2µg/mL) and incubated at 37°C in fully humidified atmosphere containing 95% air and 5% carbon dioxide. After 7 days half of the culture medium was changed. On reaching confluence, the adherent cells were detached by 0.05% trypsin and 0.02% EDTA for 5–10 min at 37°C, harvested and washed with HBSS and 10% FBS and finally re-suspended in complete medium (primary culture, P0). Cells were re-seeded at 10^4 cells/cm^2 in 100-mm dishes (P1) for both in vitro differentiation assessment and further cellular expansion which was achieved by successive cycles of trypsinization and re-seeding. The frequency of Colony Forming Units-Fibroblasts (CFU-F) was measured using the method of Castro-Malaspina. Visible colonies with 50 or more cells (the conventional value for defining a colony) were counted and referred to 10^6 plated cells (no. of CFU-F/10^6 TNC).

MSC-CNT samples preparation

MSC cells were seeded in a T 25 flask at concentration of $2*10^5$ with DMEM culture medium after six hours of incubation the medium was changed with modified medium added with PF127-CNTs. After three days the modified medium was changed again and at the 5^{th} day the cells were ready to be used for the *in vitro* and *in vivo* assays.

Immunophenotyping and differentiation of MSC and MSC-CNT sample

The morphologically homogeneous population of untreated MSC and of MSC cultured respectively with PF127-CNTs and PF127 alone for 5 days, were analysed for the expression of cell surface molecules using flow cytometry procedures: MSC were recovered from flasks by trypsin–EDTA treatment and washed in HBSS and 10% FBS. Aliquots (1.5×10^5 cells/100µl) were incubated with the following conjugated monoclonal antibodies: CD34, CD45, CD11b (in order to quantify hemopoietic–monocytic contamination), CD90, CD106, CD73. Non-specific fluorescence and morphologic parameters of the cells were determined by incubation of the same cell aliquot with isotype-matched mouse monoclonal antibodies. Incubations were performed for 20 min, and thereafter the cells were washed and 7-AAD was added in order to exclude dead cells from the analysis. Flow cytometric acquisition was performed by collecting 10^4 events on a FACScan (argon laser equipped; Becton Dickinson) instrument, and data were analysed on DOT-PLOT bi-parametric diagrams using CELL QUEST software (Becton Dickinson, Franklin Lakes, NJ, USA).

The ability of untreated MSC and of MSC cultured for 5 days respectively with PF127-CNTs and PF127 alone, to differentiate along osteogenic and adipogenic lineages was assayed, as described previously by Pittenger et al [14]. Osteogenic and adipogenic differentiation were evaluated by cytochemical analysis. Petri dishes were stained to assess extracellular matrix mineral bound by Alizarin Red-S. Adipogenic differentiation was evaluated by Oil Red O stain for lipid-rich vescicles.

Cell migration assay

MSCs were seeded in two 30 mm Petri dish at concentration of 20000 cells/ well with DMEM culture medium and incubated for six hours to allow cell attachment, subsequently the medium was changed and the two samples were cultured for 5 days in media added with PF127-CNTs and PF127 respectively (test and control cultures). 3 hours after the treatment a magnet was then placed

close to the wells. The magnet used was a Neodymium cubic magnet (N48, Residual Induction 1.41 T, cube late 12 mm). Cell migration dynamics were followed for 72 h after magnet placement. Test and control were cultured in PF127-CNT and PF127-modified media respectively for the duration of the assay.

In order to obtain instantaneous cell pictures and to enumerate the cell concentration close and far from the magnet, cells were treated with SYTO 82 orange fluorescent nucleic acid stain (Invitrogen, Milan, Italy): cells were washed twice in 2 ml phosphate buffered solution (PBS), incubated for 30 min with Syto 82 (5 μM in PBS) and then fixed in paraformaldeide 4 %. Static image acquisition was carried out with a Nikon TE2000U fluorescent microscope equipped with NIKON DS-5MC USB2 cooled CCD camera. Image analysis was performed with NIS Elements imaging software.

Moreover real-time image acquisition was performed with a Zeiss Axiovert 200 microscope equipped with an Axiocam MRm. Cell culture dish was placed in a temperature-controlled CO_2 incubator camera (Okulab). Cell migration dynamics was monitored for 72 hours and digital images acquired by microscopy using a 20 x Ph2 objective and stored for subsequently analysis. Images were acquired at intervals of 30 minutes in a fixed position.

Cell viability assay

WST-1 (tetrazolium salt 2-(4-iodophenyl)-3-(4-nitophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazoilium) cell proliferation assay was performed on MSC cultured with CNTs for 5 days. $25x10^3$ cells were seeded into a 96-well plate and then incubated with the culture media. After cell adhesion (six hours), 10μl of WST-1 solution (as described in quick cell proliferation assay kit, BioVision, USA) were added to the culture media and the plate was incubated for 1 h in standard conditions. The absorbance was measured on a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 450 nm with background subtracted at 650 nm. The results are given relative to the untreated control.

Cell staining and evaluation of apoptotic cell death

MSC cell death was evaluated by Hoechest 33258 on samples exposed for 5 days to MWCNT-modified culture media and compared with control and cells incubated in PF-127 0.01% modified culture medium without MWCNTs respectively. $25x10^3$ MSCs were cultured in a 24 well plate, equipped with cover slips. Hoechst 33258 (Invitrogen, Milan, Italy) 0.5ug/ml staining of cell nuclei (excitation 346 nm; emission 460 nm) enables determination of the presence of DNA condensates indicative of apoptosis [32]. After washing with PBS, cells were fixed with 40 g/l para-formaldehyde in PBS, incubated for 30 min at 4°C and then washed three times with PBS before further incubation for 20 minutes at 37°C with 0.5 µg/ml Hoechst 33258 in PBS. The cells were washed again with PBS and dried, subsequently each cover slip was inverted onto a slide containing 10 µl of mounting media (VECTASHIELD mounting media, Vector Laboratories, Burlingame, CA, U.S.A.); the excess mounting medium was removed with fiber-free paper and each cover slip was sealed with regular transparent nail polish and allowed to dry for 3 min.

The pyknotic cells were examined with a Zeiss epifluorescence microscope (Carl Zeiss, Oberkochen, Germany). The number of apoptotic nuclei was counted on five randomly selected areas from three cover slips from each experimental group. Results are expressed as number of apoptotic cells as a percentage of the total number of cells and compared to the control sample.

Monitoring cell growth rate

To evaluate the effects of the CNTs and/or the magnetic field on the MSCs growth rate, we performed four experimental groups in which MSCs were cultured in different conditions:

1. MSC: cells cultured in cell culture medium;
2. MSC-B: cells cultured in cell culture medium placed under the effect of the magnetic field;
3. MSC-CNT: cells cultured in PF127-CNT modified medium;

4. MSC-CNT-B: cells cultured in PF127-CNT modified medium under the effect of the magnetic field.

$1,5*10^5$ MSCs were seeded in a 6 well-plate and 2,5 ml of medium were added in each well; cells were removed by trypsinization at 24, 48, 72 and 96 hours and counted in a Thoma's camera. We calculated the doubling time (T_d), assuming a constant growth rate, by using the following formula: $T_d = (t_2-t_0)*\ln(2)/\ln(q_2/q_0)$, where q_0 represent the cell number at time t_0 and q_2 the cell number at time t_2.

Staining of cell cytoskeletal actin

The cytoskeletal rearrangement of MSC-CNT cells in the presence of a magnetic source was studied by means of actin staining. MSCs were seeded in a 12 well plate, equipped with cover slips, at concentration of $5*10^4$ cells/ well and incubated for 6 h in DMEM culture medium to allow cell attachment. Thus the medium was changed and the samples were cultured for 5 days in media added with PF127-CNTs. We used as comparison cells in complete DMEM and cells incubated in PF-127 0.01% modified culture medium. 6 hours after the addition of CNTs to the cells a magnet was placed close to the wells. The actin staining was performed 5 days after incubation of cells with the MWCNT and exposed to the magnetic field. The culture medium was removed and the cells were washed with PBS 1x at 4C° and then fixed/permeabilized with methanol at -20° C for 30 min. At the end of incubation cells were washed 4 times with PBS 1x at 4°C and incubated overnight at 4°C with the primary Actin antibody (mouse monoclonal, C-2:sc-8432 Santa Cruz Biotechnologies, Germany) .Subsequently the sample was washed again 4 times with PBS at 4°C and incubated 1 h at room temperature with the secondary antibody (goat anti-mouse IgG-TR, sc-2781 Santa Cruz Biotechnologies, Germany). After incubation the cells were washed with PBS (4 times) and dried; each cover slip was removed from each well and was inverted onto a slide containing 10 µl of mounting media (VECTASHIELD mounting media, Vector Laboratories, Burlingame, CA, U.S.A.); the excess mounting medium was removed with fiber-free paper and each cover slip was

sealed with regular transparent nail polish and allowed to dry for 3 min. The images were analyzed by confocal microscopy.

MSC-CNT cells transplantation

Inbred male Lewis (230-250 g) rats were used as recipient of MSC-CNT cells. Briefly rats were anaesthetized by intra-peritoneal injection of Zoletil 100 (tiletamin 90 mg/Kg and zolazepam 10 mg/Kg, Virbac s.r.l., i.p.) and the portal vein was exposed through a midline incision under the spine. 10^6 cells suspended in 0.5 mL of PBS solution were injected and re-flushed 2-3 times, into the portal vein.

The following groups of transplant were performed: animals receiving untreated MSCs (control group); a); animals receiving MSC-CNT cells (group b) and animals receiving MSC-CNT cells in presence of a magnetic source (group c).

In group c after the MSC-CNTs injection into the portal vein a Neodymium cubic magnet (N48, Residual Induction 1.41 T, cube late 12 mm) was placed for ten minutes on rat liver to control the homing of the cells. After suture closure of the laparotomy, a belt equipped with a bigger magnet was placed on the rat's abdomen for 24 hours.

Histological procedure

A total of 5 animals were used for this part of the study. The liver, both lungs and both kidneys were harvested from all animals. The organs were fixed in buffered 10% formalin, immediately after their removal, for 24 hours. A total of 248 blocks in paraffin-embedded tissues were obtained after standard procedure for histological preparation. We prepared 2 slides (thickness 2µm) for each block to prepare Hematoxylin/Eosin and Perls staining. The Perls staining was performed using a kit following the manufacture's instructions (Perls Microstain, DiaPath, Martinengo, Italy). The valuation of positive cells for Perls staining was done by software for morphological analysis (AnalySIS b, Olympus). The pathologist selected the positive cells using different magnification

(10X, 20X and 40X). The total output data were obtained automatically at the end of each observation in electronic sheet format. All data were uploaded in statistical analysis program (GraphPad Prism5.0, GraphPad software).

Statistics

Values are reported as mean ± standard error of the mean (S.E.M.). Statistical significance was assessed by one way analysis of variance (ANOVA) followed by post-hoc comparison test (Tukey) at P<0, 05.

Results

In-vitro studies

Aqueous solutions of CNTs were made with PF127, a surfactant with MW 12,600 soluble in water [33]. PF127 surfactant wraps the surface of the nanotubes (PF127-CNTs), thus avoiding their aggregation by a steric mechanism. The PF127-CNT solution was analysed after its preparation and before use by FIB imaging which confirmed that the nanotubes were dispersed singly and were of uniform length (about 2±0.5 μm). PF127-CNT modified medium, prepared by mixing PF127-CNT solution and cell culture medium at ratio 1:10 (v/v), contained 10 μg/ml of MWCNTs and 0.01% of PF127. PF127-CNT-modified medium was stable over a month from preparation with no evidence of nanotube bundling or precipitation. PF127 modified medium, prepared by mixing PF127 solution and cell culture medium at ratio 1:10 (v/v), contained 0.01% of PF127.

When MSCs are cultured in a CNT-containing medium, the nanotubes interact with the cells resulting in “magnetisation” of the cells as confirmed by atomic force microscopy (AFM) analysis (see Fig-1 supplementary materials). To establish the ability of CNTs to induce the migration of MSCs in the presence of an external magnetic field, test and control MSC cultures, grown for 5 days in PF127-CNT medium and PF127 medium respectively, were exposed to the magnetic field

generated by a permanent magnet. In a previous publication we showed how the strength of the magnetic field generated by the magnet is a function of the distance from the magnetic pole [13]. After 72 h of exposure to the magnetic field, MSCs were stained with a fluorescent probe for cell imaging and counting. The images captured at different distances from the magnet revealed that the magnetic field induced migration of the MSCs cultured in PF127-CNT modified medium towards the magnetic source but no such migration was observed with the control culture (Fig. 1A). Data analysis (Fig. 1B) provided the cell density for test and control culture MSCs in the Petri dish.

The migration dynamics of a single cell was followed by placing the test culture in a temperature-controlled CO_2 incubator camera (see Fig-2 supplementary materials). The lens objective was focused onto the target cell which was monitored for 72 hours. This figure shows 5 frames acquired at interval of 30 minutes in the same position (8 mm from the magnet pole).

MSCs were grown for 5 days in cell culture medium (K), in PF127-CNT modified medium and PF127 modified medium and cell proliferation was measured (Fig. 2). Cytotoxicity was expressed as percentage viability normalized to the control. We also performed the Hoechst apoptosis assay to exclude nanotubes induced apoptosis after their interaction with MSCs (see the Fig-3 supplementary materials).

We used four experimental groups for the evaluation of the effects on MSC growth rate by the CNTs and under the influence of the magnetic field (Fig.3): (I) cells cultured in cell culture medium (MSC); (II) cells in cell culture medium in presence of the magnetic field (MSC-B); (III) cells cultured in PF127-CNT modified medium (MSC-CNT) and (IV) cells cultured in PF127-CNT-modified medium in the presence of the magnetic field (MSC-CNT-B). These studies confirmed that CNTs do not induce cell toxicity or influence normal cell growth rates.

The phenotype of the cells treated with CNT was analysed by flow cytometry and the expression of markers was compared with that of untreated MSCs (table 1). We observed that after the incubation with nanotubes the cells maintain the same expression pattern as the control sample. In addition we

confirmed the ability of stem cells to differentiate in osteocytes and adipocytes when treated with magnetic nanotubes (Fig. 4).

Additionally in the *in vitro* studies we investigated the possibility that CNTs and/or magnetic field may induce modification in the cytoskeleton of cells including the orientation of the actin filaments. We stained the actin filaments in cell incubated with nanotubes and exposed to a magnetic field and did not observe any different conformation and/or orientation of the actin in comparison to the control cells (Fig.5).

In- vivo study

This was designed to test the ability of magnetic MSCs to be shepherded in *vivo* towards a target organ – the liver. The cells were with magnetic CNTs for five days. Thereafter, we injected one million cells in the hepatic portal vein of rats. Immediately after their injection, a magnet was placed on the abdominal wall overlying the liver and kept place overnight by a belt around the abdomen of the animal. In this experiment, the control animals received the same intra-portal dose of magnetic MSCs but had no magnet overlying the liver. The animals were sacrificed three weeks later with harvest of the organs for histological analysis. The distribution of the cells injected in the animals was ascertained by Prussian blue histochemical staining for intracellular detection of iron. In line with the reported literature, we performed these histochemical studies on serial sections of the liver, lungs and kidney. All the experiments were performed in triplicate. These histochemical studies revealed that magnetic stem cells were retained in the liver by the magnet with no evidence of either inflammation or neoplastic proliferation. We found significant differences by ANOVA analysis in the mean number of stained cells detected in the organs studied. As expected, the mean number of stained cells varied with the number of histological sections prepared for each organ ($p<0.0001$). The seminal finding related to the number of stained cells in the harvested livers: those derived from animals treated with a magnet overlying this organ had higher number of stained cells per histological section than in animals with livers not exposed to the magnetic field (6.284 ± 1.349 vs. 2.176 ± 0.375; $p=0.0053$). In contrast, the kidneys and lungs derived from animals whose liver

had not been exposed to a magnetic field had higher mean values of stained cells: kidneys: 10.800±3.038 vs. 6.464±1.915 (p=0.0050); lungs: 70.390±18.590 vs. 54.610±22.930 (p=0.0332). Significant differences in the number of stained cells/ tissue slice were found between liver and lung (p=0.0001), liver and kidney (p=0.0025) and kidney and lung (p=0.01309) (Fig.6 A and B**).**

Discussion

The experimental data obtained in this study confirm that magnetic fields can be used to control movement and location of MSCs cultured with carbon nanotubes. The cell density for control cultures MSCs in the Petri dish was homogeneous (350-400 cells/mm^2), whereas the cell density was different in the test culture. Specifically, in the test culture the MSCs density ranged from 200 cells/ mm^2 to 800 cells/mm^2, correlating respectively with distances from the magnetic pole of 9-14 mm and 5 mm. This cell streaming behaviour was closely related to the intensity of the flux density within the Petri dish which ranged from a very low value (<0.1 T) when the distance from the magnetic pole was >9 mm to about 0.5 T at a distance of 5 mm. Mathematically, an MSC interacting with CNTs is subjected to a translational force F_m in the presence of a gradient field of:

$$F_m = \frac{1}{\mu_0} \cdot \chi_{rp} \cdot V \cdot B \cdot \frac{dB}{dr} \qquad (1)$$

where μ_0 the magnetic permeability of is free space, χ_{rp} and V are respectively the magnetic susceptibility and the total volume of magnetic nanotubes attached to the cell. Eq. 1 provides thus a mathematical tool to design the manipulation of MSCs cultured with carbon nanotubes by a magnetic flux gradient. The migration dynamics of a single cell shows that the cell moves towards the magnetic source with a speed of approximating to 30 μm/h (see the dashed line crossing the cell nucleus of figure 2, supplementary materials).

Cell proliferation assays were performed to exclude any adverse effect of the nanotubes and/or the magnetic field on the viability, proliferation and functionality of MSCs. Based on the experimental data reported by Wörle-Knirsch et al [34] we also used the WST-1 cell proliferation kit. The results

showed that the PF127 surfactant marginally decreased the cell viability but the in cell viability was negligible with PF127-CNTs. The latter may be explained by the action of the surfactant which wraps the nanotubes surface and reduces the amount of free PF127 in the medium [35]. Additionally, the data on the cell growth assays showed that the MSCs doubling time was not significantly influenced by the presence of both the nanotubes and the magnetic field and no apoptotic cells were observed in all samples studied.

The in-vitro studies also confirmed that cells treated with CNTs up to 5 days maintain their ability to differentiate under specific conditions as well as the untreated cells. The lack of adverse effects on cell viability and proliferation by MWCNTs observed in the present study needs to be compared with the reported findings by Mooney et al. [36]. In this study, hMSCs were treated for 24 h with both COOH functionalised SWCNTs and OH-functionalised MWCNTs, at various concentrations. The authors demonstrated that COOH-functionalised SWCNT, used at concentration up to 32 μg/ml, did not affect cells viability, proliferation, differentiation ability and metabolic activity; but at higher concentrations, SWCNTs exerted detrimental effects on the cells. In contrast, OH-functionalised MWCNTs were found toxic at all concentrations. In the present study, the MWCNTs were not functionalised but simply dispersed in an anionic surfactant and used at concentration of 10 μg/ml. Finally, the staining of the actin filaments suggested clearly that the treatment with the nanotubes and the exposure to the magnetic field did not alter cell morphology.

The results of the in-vivo experiments demonstrate that MSCs cultured with CNTs can be shepherded by means of an external magnetic source towards a specific organ. This observation assumes importance in view of the widespread interest in recent years on MSC-based cell therapy for tissue engineering, regeneration/ repair of damaged organs and allogeneic transplantation. As MSCs also exhibit immuno-modulatory and anti-inflammatory effects they may play a role in the *in vivo* induction of tolerance. Also they reduce the incidence and severity of GVHD [17-18], as well as prolonging skin graft survival [16].

The clinical applications of MSCs require the administration of cells by the intravenous route [26]; but their subsequent dispersion in many tissue and organs, [19-20-21] reduces the number of cells which colonize the intended target organ. There is therefore a need to increase targeted stem cell localization and homing to the diseased site. The approach proposed in this paper has the potential to achieve this objective of site-specific localization of the CNT treated MSCs, by reducing their colonisation of other sites with consequent adverse effects resulting from their proliferation and differentiation in ectopic sites. In the animal model studied we were able to achieve a 3-fold increment of MSC localization in the target organ (liver) by this approach with a corresponding decrease of MSC localization in the lung.. In a recent work, Gao demonstrated that intravenous infusion of sodium nitroprusside, a vasodilator, administered prior to the cells infusion, reduced by 15% the number of cells present in the lungs increasing by 10% the cells in the liver [19]. Based on this result we intend to investigate the combined action of a vasodilator and the magnetic guidance to enhance the targeted homing of cells. Additionally, we need to optimise the protocol and design and realize a magnetic applicator device which would allow a more precise and controlled configuration of the magnetic field gradient applied to the target site.

Conclusion

In the present study we have demonstrated that when mesenchymal stem cells (MSCs) are cultured in a CNT- containing medium, the nanotubes interact with the cells without compromising their viability and proliferation rate. Moreover we confirmed that magnetically labelled cells maintained the ability to differentiate in adipocytes and osteocytes. As result of the cell interaction with CNTs, the application of a magnetic field, enables shepherding of MSCs to the desired location. In the experimental model used we were able to retain significantly more the mesenchymal stem cells within in the liver with a consequent reduction of their migration to other organs.

Executive Summary

MWCNTs dispersion

• Multi-wall carbon nanotubes (MWCNTs) have been efficiently dispersed in Pluronic F127.

Cytocompatibility study on mesenchymal stem cells

• MSCs showed excellent metabolic activity following 5-days incubation with cell culture medium modified with 10 μg/ml of MWCNTs**.**

• Magnetic carbon nanotubes interact with MSCs without compromising their proliferation rate.

• No apoptotic phenomenon was detected following incubation of MSCs with MWCNTs at concentrations of 10 μg/ml.

• MSCs treated with magnetic nanotubes maintain their ability to differentiate *in vitro* into osteocytes and adipocytes.

MSC magnetization with carbon nanotubes

• Atomic force microscopy (AFM) analysis confirmed that MSCs cultured in a CNT-containing medium showed a strong interaction with the MWCNTs resulting in cell "magnetisation".

• The treatment with carbon nanotubes and magnetic fields does not alter cell phenotype.

• MSCs treated with magnetic nanotubes move towards the magnetic source *in vitro.*

• In vitro studies about the possible cytoskeleton modification induced by CNTs and/or magnetic field revealed that in cells incubated with nanotubes and exposed to a magnetic field there is no different conformation and/or orientation of the actin in comparison to the control cells.

***In vivo* studies**

- One million of magnetized cells were injected in the hepatic portal vein of rats.
- The application of a proper magnetic field changes the MSC biodistribution
- The histochemical studies revealed an increment of CNT labelled MSC accumulation in the liver of the animals exposed to the magnetic field, with no evidence of either inflammation or neoplastic proliferation.

Conclusions and perspectives

- MWCNTs hold the potentiality to be used as nano-devices to improve the protocol for transplantation and homing of stem cells *in vivo* in numerous clinical applications.

Legend to figures

Fig.1 Imaging of MSCs shepherding: (A). MSC-CNT-cultures (a) MSC-control (b). Cultures 72 h after magnet placement. Images taken at different distances from the magnet pole (1, 2 and 3 correspond respectively to 5, 9 mm and 14 mm from the magnetic pole). Cell is stained with Syto 82 fluorescent dye. **(B).** MSC density for test and control cultures after 72 h from the magnet placement at different distances from the magnet pole. Assays performed in quadruple and results are the mean±S.E.M. (vertical bars).

Fig.2 Cell proliferation assay: Cell viability assay for MSCs cultured with the control medium (K), the PF127-CNT-modified medium and PF127 modified medium. The viability was measured with the WST-1 assay and results are given in percent related to untreated controls. Results are the mean±S.E.M. (vertical bars) of four experiments each carried out in six-plicate.

Fig.3 MSCs doubling time: Cells were cultured in culture medium and in PF127-CNT-modified medium, with and without the external magnetic field B; Data expressed as mean ± S.E.M. of three experiments each carried out in quadruple.

Fig.4 Osteogenic and Adipogenic stem cell differentiation. Osteogenic differentiation**:** (A) Control stem cells; (B) Stem cell treated with magnetic nanotubes; (C) Stem cell treated with PF127. Adipogenic differentiation**:** (D) Control stem cells; (E) Stem cell treated with magnetic nanotubes; (F) Stem cell treated with PF127.

Fig.5 Staining of cell cytoskeletal actin: (A) Control MSCs; (B) MSCs incubated with nanotubes in presence of magnetic field.

Fig.6 Histochemical analysis: (A) Liver's section with Perls labelled mesenchymal stem cells with magnetic nanotubes; (B) Mean and S.E.M. values of positive cells for Perls staining in each type of organ.

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References annotations.

[11] Cai et al. developed a physical technique for in vitro and ex vivo gene transfer known as 'nanotube spearing', capable of effective cell transfection with plasmid DNA by using magnetic carbon nanotubes.

[12] This paper demonstrated that MWCNTs are able to interact with mammalian cells and, when exposed to a magnetic field, induce their migration towards the magnetic source..

[13] This paper describes the dynamics of cell movement in a magnetic field consequent on CNT attachment.

[20, 22] Two of the most important studies focused on the biodistribution of MSCs *in vivo*.

[28] The authors propose a method for magnetic guidance of MSC based on the use of superparamagnetic iron oxide nanoparticles.

[36] The first investigation on the effects of carbon nanotubes on MSC viability, proliferation and differentiation.

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