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HOMING OF IN VITRO EXPANDED STRO-1$^-$ OR STRO-1$^+$ HUMAN MESENCHYMAL STEM CELLS INTO THE NOD/SCID MOUSE. THEIR ROLE IN SUPPORTING HUMAN CD34 CELL ENGRAFTMENT.

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Hematopoietic support and biodistribution of Stro-1$^+$ and Stro-1$^-$ Mesenchymal Stem Cells.

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SUMMARY

The Stro-1 antigen potentially defines a mesenchymal stem cell (MSC) progenitor subset. We here report on the role of human ex vivo expanded selected-Stro-1^+ or -Stro-1^- MSC subsets on the engrafment of human $CD34^+$ cord blood cells in the NOD/SCID mouse model. The data show that cotransplantation of expanded Stro-1^- cells with $CD34^+$ cells resulted in a significant increase of human CD45, CD34, CD19 and CD11b cells detected in blood or in bone marrow (BM) and spleen as compared to the infusion of $CD34^+$ cells alone.

Infusion into mice of expanded Stro-1^+ and Stro-1^- cells (without $CD34^+$ cells) showed that the numbers of Stro-1^+-derived (as assessed by DNA analysis of human β GLOBIN with quantitative PCR) were higher than Stro-1^--derived cells in spleen, muscles, BM and kidneys, while more Stro-1^--derived than Stro-1^+-derived cells were found in lungs. The transduction of expanded Stro-1^+ cells with an eGFP gene did not modify their cytokine release and their homing in NOD/SCID mouse tissues.

The difference between the hemopoeitic support and the homing capabilities of expanded Stro-1^+ and Stro-1^- cells may be of importance for clinical therapeutic applications: Stro-1^+ cells may rather be used for gene delivery in tissues while Stro-1^- cells may rather be used to support hematopoietic engraftment.

INTRODUCTION

Recent results have shown that cotransplantation of human *ex vivo* expanded mesenchymal stem cells (MSCs) together with hematopoietic stem cells hastens hematopoietic recovery following a bone marrow (BM) transplantation in animal models [1-4] and in humans [5-7]. Human BM contains two cell compartments: the hematopoietic cell compartment and the stromal cell compartment that comprises MSCs [8,9]. MSCs are able to give rise to multiple mesodermal tissue types, including bone, cartilage, tendon, muscles, cardiomyocytes, fat and brain [10-16] and a marrow stromal connective tissue which supports the differentiation of hematopoietic stem cells (HSCs) [17,18]. However, MSCs are heterogeneous and little is known on the role of MSC subsets in the hematopoietic engraftment support and in their homing in various tissues [19]. Stro-1 antigen is present on CFU-F cells in adult human BM and potentially defines a MSC precursor sub-population [20-22]. The aim of the present study was to evaluate the role of ex vivo-expanded Stro-1^+ and Stro-1^- MSCs on engraftment of human $CD34^+$ cord blood cells in non obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Our data showed that the levels of human hematopoietic engraftment (as assessed by the presence of CD45, CD34, CD19 and CD11b cells) in the blood, spleen and mouse BM were higher when Stro-1^--derived cells were co-infused with $CD34^+$ cells than when Stro-1^+-derived cells were used.

In a second step, we investigated the homing of expanded Stro-1^+ and Stro-1^- cells (infused without $CD34^+$ cells) in BM, spleen, liver, brain, heart, lungs, kidneys and muscles of NOD/SCID mice. 8 week-old NOD/SCID mice received 3.5 Gy irradiation and 24 hours later the cells were infused. We analysed the homing of cells by PCR quantitation of DNA of human β GLOBIN. Results showed that DNA amount from expanded Stro-1^+ cells was higher than that of expanded Stro-1^- cells in spleen (8 times), muscles (x6), BM (x2), liver (x1.5) and kidneys (x1.5). No significant difference was observed in brain, while more Stro-1^- than Stro-1^+ cell DNA was found in lungs (x3.5).

In conclusion, expanded Stro-1^+ cells better migrated than expanded Stro-1^- cells in a majority of mouse tissues. This indicated that Stro-1^+ cells would be potentially a good vector to bring specific therapeutic genes into tissues. In order to test this hypothesis, we infused into NOD/SCID mice expanded Stro-1^+ cells transfected with an eGFP gene. The specific eGFP DNA was found in every investigated tissue, namely BM, liver, brain, heart, spleen, kidneys, muscles and lungs.

The difference between the hemopoeitic support and the homing capacities of Stro-1^+ and Stro-1^- cells may be of importance for clinical application of MSCs [12]: Stro-1^+ cells would be rather used for gene delivery in tissues and Stro-1^- cells (or unseparated MSCs since they contained around 90% Stro-1^- cells) for hematopoietic engraftment support.

MATERIALS AND METHODS

Collection and isolation of $CD34^+$ cells from human umbilical cord blood (hUCB)

Human UCB samples were obtained from full-term deliveries after informed consent of the mother and were used in accordance with the procedures approved by the human experimentation and ethic committees of Hopital St Antoine in Paris. hUCB were collected in bags (Macopharma, Tourcoing, France) containing heparin and processed within 24 hours. Samples were diluted 1:2 in phosphate buffered saline (PBS) without Mg^{2+}/Ca^{2+} (B Braun Medical, 92-Boulogne). Low density mononuclear cells (MNCs) were collected after centrifugation on Ficoll-Paque density gradient (1.077g/l Biochrom, Berlin, Germany) and washed in PBS. CD34 cells were isolated using the MACS cell isolation kit and MidiMACS columns (Miltenyi Biotec Gmbh, Bergish Gladbach, Germany) according to the manufacturer's instruction. A purity of more than 95% was currently obtained. Selected CD34 cells will be referred as "$CD34^+$ cells" in the following. They were frozen in fetal calf serum supplemented with 10% dimethylsulfoxide (B/Braun Medical SA, Boulogne, France) until use.

Isolation and growth of human bone marrow (BM) MSCs

BM cells were obtained from iliac crest aspirates from healthy donors giving their BM for allogeneic transplantation purposes, after informed consent and were used in accordance with the procedures approved by the human experimentation and ethic committees of Hopital St Antoine. For the study of Stro-1^+ cell proportions in normal BM, aliquots of 2 ml were taken from ten different BM collections. For cotransplantation and homing studies, 50 ml of BM were taken from two different donors. BM samples were diluted 1:3 in PBS without Mg^{2+}/Ca^{2+}. MNCs were separated on Ficoll-Paque density gradient (1.077g/ml) and washed in PBS. They were plated at a concentration of $1x\ 10^6$ to $10x\ 10^6$ MNC/ml in T-75 cm^2 tissue culture flasks in Dexter medium [McCoy's 5A medium supplemented with 12.5% heat-inactivated fetal calf serum, 12.5% heat-inactivated horse serum, 1% sodium bicarbonate, 1% sodium pyruvate, 0.4% MEM non essential amino acids, 0.8% MEM essential amino acids, 1% MEM vitamin solution, 1% L-glutamine (200 mM), 1% penicillin-streptomycine solution (all from Invitrogen, Groningen, The Netherlands), 10^{-6} M hydrocortisone (Stem Cell Technologies), 2 ng/ml human basic recombinant fibroblast growth factor (FGFb, R&D System, Abington, UK)] and incubated at 37°C and 5% CO_2 in humidified atmosphere. After 1 week, nonadherent cells were removed, and the complete medium (except hydrocortisone)

was used. When 50% confluence was obtained, cells were harvested after a 2 min-incubation with 0.25% trypsin and 1 mM EDTA (Stem Cell Technologies) at 37°C. The collected adherent cells were incubated with anti-Stro-1 antibody, and the Stro-1^+ cells were separated using immunomagnetic beads (as described below). Stro-1^+ and Stro-1^- cells were harvested and cultured in complete medium without hydrocortisone. When 90% confluency was observed, cells were detached by trypsin incubation and replated at 1:3 dilution in T-75 cm^2 flasks. These expanded cells will be referred as "expanded Stro-1^+" or "expanded Stro-1^-" MSCs.

Immunomagnetic selection with an anti-Stro-1 antibody

Stro-1^+ cells were isolated by magnetic immunobeads (Dynabead M450, Dynal Asa, Oslo, Norway) linked to Stro-1 antibody (produced by one of us, P Charbord and purified by Biocytex, Marseille, France) as previously described [22-23]. Briefly, anti-Stro-1-coated beads were added to the stromal cell suspension and positive cells were recovered with a magnetic particle concentrator (MPC-1) and washed 3 times before use.

FACS analysis

PE-labelled monoclonal antibodies used in this study were : anti-CD34, anti-CD45, anti-CD19, anti-CD11b, anti-CD105 (SH2), anti-CD73 (SH3) (Becton-Dickinson). Cells were incubated with antibodies in PBS supplemented with 0.5% bovine serum albumin (BSA, Sigma Chemicals Co, St Louis, MO, USA), for 20 min at 4°C. Cells were washed, resuspended in 200µl of PBS 0.5% BSA, and analysed with a FACScalibur® (BD Pharmingen), with the acquisition of at least 10,000 events per test.

NOD/SCID mouse model

All experiments and procedures were performed in compliance with the French Ministry of Agriculture regulations for animal experimentation (Act n°87-847 October 19th, 1987, modified May, 2001). NOD-LtSz-*scid/scid*(NOD -SCID) mice, from breeding pairs originally purchased from Jackson Laboratory (Bar Harbor, ME), were bred in our pathogen-free unit and maintained in sterile microisolator cages. Eight-week old mice were sublethally irradiated with 3.5 Gray from a ^{137}Cs source (2.115 Gy/min).

a) Cotransplantation of CD34$^+$ cells plus expanded Stro-1$^+$ or Stro-1$^-$ MSCs.

12 to 24 hours after irradiation, $1x10^6$ expanded Stro-1^+ or Stro-1^- MSCs resuspended in PBS were injected into the retro-orbital plexus of mice together with or without $1x10^5$

$CD34^+$ cells. Control groups included mice receiving only selected $CD34^+$ cells, or receiving no cells. Therefore, four groups of mice (eight mice per group) were defined: "$CD34^+$ + Stro-1^+", "$CD34^+$ + Stro-1^-", "$CD34^+$ alone", "no cells". At 3, 6, 9 and 12 weeks post transplantation, 50 µl of peripheral blood was collected from the retro-orbital plexus, and the percentage of human hematopoietic CD45 cells in murine blood cells was counted by FACS analysis. Differences in the human CD45 cell engraftment was made by calculating the areas under the curves (AUC) at different time points (3, 6, 9 and 12 weeks).

12 weeks after injection, mice were sacrificed and blood, spleen and BM were collected. Cells were isolated, resuspended in PBS and the percentages of human CD34, CD45, CD19 and CD11b were measured by FACS.

b) Infusion of expanded Stro-1^+ or Stro-1^- MSCs for homing studies.

12 to 24 hours after irradiation, $1x10^6$ expanded Stro-1^+ or Stro-1^- MSCs resuspended in PBS were injected into the retro-orbital plexus of mice. 12 weeks after, mice were killed and blood, spleen, BM, lungs, heart, brain, liver, kidneys and leg muscles were collected for DNA extraction and PCR analysis to detect the presence of human MSCs DNA in these tissues.

DNA extraction and PCR analysis.

Genomic DNA for PCR analysis was prepared from tissues using phenol chloroform extraction after overnight incubation at 65°C in lysis buffer as previously described [24]. The DNA concentration and purity was estimated by optical density (OD) measurement. DNA analyses were performed by Real-time quantitative PCRs (Taq Man technology and ABI PRISM 7700). Amplification was performed using manufacturer-provided reagents following the standard recommended amplification conditions (Applied Biosytems, Foster City, CA) as previously described [25, 26]. One hundred nanogram of purified DNA from various tissues were amplified using TaqMan universal PCR master mix 4304437 (Applied Biosytems). The primers and probes were designed with Primer Express software (Applied Biosytems). The primers and probe for β GLOBIN were forward primer 5'GTGCACCTGACTCCTGAGGAGA3' and reverse primer 5'CCTTGATACCAACCTGCCCAGG3', the probe labeled with fluorescent reporter and quencher was: 5'FAM-AAGGTGAACGTGGATGAAGTTGGTGG-TAMRA-3'. FAM (6-carboxy-fluorescein) was used as a reporter fluorochrome and TAMRA (6-carboxy-tetramethyl-rodamine) was used as quencher. As internal control, endogenous mouse RAPSYN gene (Receptor-Associated Protein at the Synapse) was also amplified. The primers

and probe for RAPSYN gene were forward primer 5 ACCCACCCATCCTGCAAAT'3' and reverse primer 5'ACCTGTCCGTGCTGCAGAA3' [3]. Probe was chosen in order to hybridize to an internal sequence of the PCR target sequence. At each PCR cycle, the fluorescence intensity of additional reporter dye molecules was monitored. Threshold cycles (Ct) were selected in the line in which all samples were in logarithmic phase. The quantity of PCR product was calculated by Ct value. In order to determine the efficiency of amplification and the assay precision, calibration curves for β GLOBIN and RAPSYN were constructed with a 0,99 correlation (r^2) and an efficiency superior to 98%. The reagent control consisted of all constituents of the PCR reaction mixture except template DNA. The negative control DNA was isolated from the same tissues from non-transplanted NOD/SCID mice. The positive control DNA was isolated from human MSCs. Evaluation of human specificity of β GLOBIN amplification was demonstrated using tenfold dilution for 100 ng to 0 ng of human MSC DNA per assay in PCR grade water and in 0 to 100 ng of murine DNA per assay. No cross reactivity between human and murine genomic DNA was observed when amplification of human β GLOBIN or murine RAPSYN was performed with our primer-probe set. All samples were also amplified to detect the RAPSYN as an internal control for the presence of amplifiable DNA in the real time PCR chimerism assay. In order to quantify the number of human cells in mouse tissue, the numbers of copies of β GLOBIN and RAPSYN were normalized. Amplifiable DNA input in each sample was assayed by means of an active reference system, and a pair of primers and Taq Man probe specific for constant gene were used as the active reference in the real time PCR chimerism assay. Ratio between human and murine DNA copy numbers was expressed in percentage of human β GLOBIN DNA copies in RAPSYN DNA copies. Human cells contained 2 copies of β GLOBIN and murine cell contained 2 copies of RAPSYN. Therefore, we can expressed the ratio of human/murine DNA copy numbers in numbers of human cells per murine cells.

Retroviral gene transduction

Expanded Stro-1^+ MSCs were transduced with an eGFP gene in order to check if a gene transduction would modify the ex vivo cytokine release of the tranduced cells and their homing after their injection into NOD/SCID mice, as compared to non-transduced cells. The eGFP (enhanced green fluorescent protein) gene was introduced into expanded Stro-1^+ MSCs after the first culture passage using the pG13 packaging cell line producing recombinant pSF

vector carrying the eGFP gene. The pSF-eGFP vector is based on the Friend mink cell focus-forming/murine embryonic stem cell virus (FMEV). It contains the eGFP gene under the transcriptional control of the spleen focus-forming virus long term repeat (LTR), which has been combinated with a permissive leader sequence of the murine embryonic stem-cell virus (MESV) to overcome transcriptional repression of U3-mediated gene expression. Viral supernatants were passed through a 0.45µm filter to remove cells and cellular debris before use. MSCs at 20 % of confluence were exposed to viral supernatant in the presence of 1 µg/ml polybrene (Sigma) for 5 hours and this was repeated until stromal cells reached confluence. Transduction efficiency, evaluated 2 to 14 days after the last infection was determined by FACS and microscopic analysis. eGFP detection in tissues was assessed by PCR; positive controls consisted in human eGFP expanded Stro-1^+ cells and negative control consisted of murine genomic DNA extracted concurrently with each set of test samples and a reagent control. The primers for eGFP were forward primer 5'ctcgtgaccaccctgacctac3' and reverse primer 5'aagaagatggtgcgctccg3'.

Immunohistology

The presence of GFP protein on eGFP-tranduced cells in mouse tissue sections was assessed by a rabbit anti-GFP antibody. The human origin of these cells on mouse tissues was assessed by an antibody directed against human β-2 microglobulin. Tissues were fixed in 4% neutral buffered formaldehyde for 16 hours at room temperature and were then embedded in paraffin. Sections (5 µm) were deparaffinised and rehydrated, then permeabilised for 5 min in 0.1% Triton/PBS at room temperature. For eGFP detection, the immunocytochemistry was performed on a NexES IHC automat (Ventana, Illkirch, France) using DAB detection kit (22495, Ventana) with a 2% trypsin digestion step for 30 minutes. Slides were incubated for 30 min with rabbit anti-Green Fluorescent Protein (GFP) polyclonal antibody (Product N° AB3080, Chemicon International) diluted at 1/1000 (product N° E3432, Novocastra). A biotin-labelled goat anti-rabbit IgG antibody was used. Immunoreactivities were visualized by avidin hrpo DAB detection. Negative control consisted of rabbit IgG diluted at 1/1000 in antibody diluent (107784, Ventana). The slides were counterstained with hematoxylin and dehydrated. For β-2 microglobulin, the immunocytochemistry was also performed on a NexES IHC automat (Ventana). The monoclonal anti- β-2 microglobulin antibody (product No E.3432, Novocastra) was applied at 1/50 in antibody diluent. A secondary antibody composed of biotynyled anti-rabbit IgG was used. Immunoreactivities were visualized by

ventana kit to make alcaline phosphatase reaction with FRED substrat followed by counterstaining with hematoxylin.

Statistical analysis:

Statistical analyses were performed using table curve software (SPSS, Paris, France). The kinetics of human CD45 cell engrafment was evaluated by calculating areas under the curves (AUC) at different time points (3, 6, 9 and 12 weeks). Differences in $CD45^+$ cell AUC between mouse groups were assessed using Man-Whitney Rank Sum Test. Other statistic tests were performed using Sigmastats software (SPSS). When indicated, values are reported as mean +/- standard deviation (SD). Statistical significance was set for $p<0.05$.

RESULTS

I- Separation and flow cytometric analysis of expanded Stro-1$^+$ and Stro-1$^-$ cells.

Ten BM samples were studied. After a one week culture period, non-adherent cells containing hemopoeitic cells were discarded. The adherent cells, detached with trypsin, contained a median of 6% Stro-1$^+$ cells (mean: 7% +/- 6; range:1-25). Cells were then separated with anti-Stro-1 coated beads, and the Stro-1$^+$ and Stro-1$^-$ cells were further seeded for expansion. Confluence was usually reached after 3 days. Cells were reseeded at least twice. The total median time of culture was 15 days. Cells were then collected by trypsinization and analysed by flow cytometry. Results showed that expanded Stro-1$^+$ cells were 30% to 56% SH2$^+$ (CD105) and 87% to 97% SH3$^+$ (CD73). Expanded Stro-1$^-$ cells were 12% to 42% SH2$^+$ and 71% to 83% SH3$^+$. None of the fractions contained CD45 (hematopoietic) or CD34 cells.

In 2 independent experiments, the Stro-1$^+$ and Stro-1$^-$ cells were expanded by the same technique and further used for infusion to NOD/SCID mice (see below).

II. Cotransplantation of CD34$^+$ cells plus expanded Stro-1$^+$ or Stro-1$^-$ MSCs.

a) Human CD45 cell engraftment in blood.

Eight-week-old mice were sublethally irradiated with 3.5 Gray. 12 to 24 hours after irradiation, CD34$^+$ with expanded Stro-1$^+$ or Stro-1$^-$ cells were injected into the retro-orbital plexus of mice. Controls consisted in mice receiving only CD34$^+$ cells or no cells. The purity of hUCB CD34$^+$ cells used in these 2 series of experiments were 93% and 98%. At week 3, 6, 9 and 12 post transplantation, blood was collected from the retro-orbital plexus. The percentages of CD45 cells were determined by FACS analysis and the data from the 2 experiments were aggregated and plotted according to time (figure 1) (no CD45 cells were found in the control group of mice receiving no human cells). The data showed that the percentages of human CD45 cells in peripheral blood of NOD/SCID mice were higher at 6, 9 and 12 weeks for the group of mice receiving the combination of "CD34$^+$+ Stro-1$^-$" cells, as compared to mice receiving only CD34$^+$ cells. The differences are highly significant when comparing the areas under the curve (AUC) for each group of mice (figure 2) (p= 0.02, p=0.009 and p= 0.01 at week 6, 9 and 12 respectively). The differences were also significant when the data of "CD34$^+$ + Stro-1$^-$" group was compared to the "CD34$^+$ + Stro-1$^+$" group (p= 0.005, p=0.004 and p= 0.02 at week 6, 9 and 12 respectively). No difference was observed between the groups "CD34$^+$ + Stro-1$^+$" versus "CD34$^+$ alone". These data of human CD45

cell engraftment in peripheral blood indicated that expanded Stro-1$^-$ cells sustained human hematopoietic engraftment in NOD/SCID mice, and that no advantage was brought by coinfusion of expanded Stro-1$^+$ cells over infusion of CD34$^+$ cells alone.

b) Engraftment of human CD34, CD45, CD19 and CD11b cells in BM, spleen and blood 12 weeks after infusion.

Animals of the above groups were sacrificed at 12 weeks and cells recovered from the BM, spleen and peripheral blood were analysed for the presence of human CD34, CD45, CD19 and CD11b cells.

Cotransplantation of "CD34$^+$ + Stro-1$^-$" cells resulted in BM in an increase (as compared to the group "CD34$^+$ alone") in the percentage of human CD34 (8.7% vs 4.3%, p=0.04), CD45 (48% vs 27%, p=0.04) and CD19 (37% vs 20%, p=0.03) (Table I). No difference was found for CD11b.

In the spleen, there was a trend for higher percentages of CD45, CD19 and CD11b in the group "CD34$^+$ + Stro-1$^-$" as compared to "CD34$^+$ alone", but differences were not significant (p= 0.08, p= 0.4 and p=0.2 for CD45, CD19 and CD11b respectively). In blood, in addition to higher levels of CD45 as reported above (figure 1), there was a trend for an increase of CD19 and CD11b cell percentages in the group receiving "CD34$^+$ + Stro-1$^-$" cells as compared to the group "CD34$^+$ alone" (p= 0.2 for both CD19 and CD11b). In contrast, no significant differences in the percentages of human hematopoietic cells were observed between "CD34$^+$ + Stro-1$^+$" and "CD34$^+$ alone" groups.

III. Homing of Stro-1$^+$ and Stro-1$^-$ MSCs in mouse tissues.

In a second series of experiments, NOD/SCID mice receiving expanded Stro-1$^+$ or Stro-1$^-$ MSCs (without CD34$^+$ cells) or no cells were sacrificed at 12 weeks and DNAs extracted from the BM, spleen, peripheral blood, brain, heart, liver, lungs, kidneys and leg muscles were analysed for the presence of specific human DNA by detection of human β-GLOBIN gene. The RAPSYN murine gene probe was used as an internal control to normalize the amount of human DNA as compared to murine DNA in quantitative PCR. The ratio of human/murine DNA copy numbers was expressed in numbers of human cells per 10000 murine cells. Human Stro-1$^+$ and Stro-1$^-$ cell DNAs were found in variable amounts in BM, spleen, brain, heart, liver, lungs, kidneys and muscles, but not in blood (table 2). Results showed that the amount of Stro-1$^+$ cell DNA was higher than that of Stro-1$^-$ DNA in spleen (8 times), muscles (x6), BM (x2), liver (x1.5) and kidneys (x1.5). No significant difference was observed in

brain, while more Stro-1$^-$ than Stro-1$^+$ DNA was found in lungs (x3.5). These data indicate that Stro-1$^+$ derived cells migrated better than Stro-1$^-$ derived cells in a majority of mouse tissues.

IV. Injection into NOD/SCID mice of expanded Stro-1$^+$ cells transduced with eGFP.

In order to check whether gene transduction might modify the cytokine release and homing functions of expanded Stro-1$^+$ cells, the cells were transduced with the gene encoding for the green fluorescent protein (eGFP). In 2 separate experiments, eGFP gene marking efficiency was 78 and 73%. eGFP expression was unchanged after 4 additional weeks of culture and the eGFP transduced Stro-1$^+$ cells showed in vitro by ELISA similar cytokine (M-CSF, GM-CSF, G-CSF, TPO, IL-6, LIF) release than non-transduced Stro-1$^+$ cells (results not shown). Then, eGFP transduced cells were infused into the irradiated NOD/SCID mouse. At 12 weeks post-injection, animals were sacrificed and blood, spleen, liver, BM, brain, heart, lungs, muscles, and kidneys were harvested and DNA extracted. In BM, eGFP cells were assessed by fluorescence in FACS analysis, and no eGFP-positive cell could be detected. In contrast, using a more sensitive technique, PCR, eGFP-positive DNA was found in BM from 5/5 tested mice (table 3). Similarly, most of the tissues tested in PCR were found eGFP-positive (table 3), indicating that the gene transfection did not alter the capacity of expanded Stro-1$^+$ cells to migrate.

In situ localization of eGFP-transduced cells was studied in tissues by microscope examination to detect GFP green fluorescence. No fluorescence was detected in any examined tissue. To improve the sensitivity of detection, the presence of GFP protein on eGFP-tranduced cells in mouse tissue sections was then assessed by a rabbit anti-GFP antibody (revealed with DAB). In parallel, the human origin of these cells on mouse tissues was assessed by an antibody directed against human β-2 microglobulin. Nevertheless, eGFP-cells remained undetectable in most tissue cross sections, except in brain where eGFP-marked cells was observed into *dentate gyrus* area (figure 3). The human origin of these cells was independently confirmed with an anti human β-2-microglobulin antibody revealed with alkaline phosphatase (figure3).

DISCUSSION

Engraftment and initiation of hematopoiesis by tranplanted HSCs depend on complex processes. Transplanted cells must home into the BM microenvironment, lodge in the

appropriate niches before they proliferate and differentiate [18, 27, 28]. Stromal cells present in this microenvironment, now commonly called MSCs, are thought to play an important role in hematopoietic stem cell engraftment. Marrow stromal cells are an heterogenous population of cells, including reticular endothelial cells, fibroblasts, adipocytes, and osteogenic precursor cells, which provide growth factors, cell-to-cell interactions, and matrix proteins.

Previous studies have demonstrated the capacity of MSCs to improve hematopoietic engraftment when they are coinfused with hematopoietic stem cells [1, 3], the first demonstration being made by Anklesaria et al [29] in a mouse model. In the humanized-sheep model of HSC transplantation, cotransplantation of BM-derived stromal cells resulted in enhancement of BM engraftment and increased levels of human cells circulating early after transplantation [30]. Recently, MSCs derived from human fetal lungs have been shown to promote engraftment of human umbilical cord blood $CD34^+$ cells in a mouse model [4]. However, MSCs are heterogeneous and little is known on the respective role of the various sub-populations of MSCs in hematopoietic homing and engraftment. Simmons et al [20, 21], and then Dennis et al [22] showed that the Stro-1 antigen is expressed in a progenitor MSC subset, that is enriched in CFU-F progenitors. Given their multipotentiality, Stro-1^+ cells appear as good candidates for cell and gene therapy. The aim of the present study was to know the functions of these cells, as compared to those lacking the Stro-1 antigen. Phenotypically, the two cell subsets did not show any significant difference in the expression of several several membrane antigens (in addition to SH2 and SH3 reported here, we tested integrin β1, integrin α1, integrin α3, integrin α5, integrin αvβ3, integrin αvβ5, CD44, endoglin, SH4, S-endo, Thy-1; unpublished results). In contrast, we found that the levels of human hematopoietic engraftment (as assessed by the presence of human CD34, CD45, CD19 and CD11b cells) achieved in the blood, spleen and BM of NOD/SCID mice were higher when expanded Stro-1^- cells were cotransplanted with cord blood $CD34^+$ cells, as compared to Stro-1^+ cells.

For decades, studies on MSCs were hampered by the lack of sufficient numbers of human cells to infuse in humans. The possibility to expand ex vivo these cells has actually renewed the clinical interest for MSCs. Indeed, the spatial organization of stem cells in the marrow, mediated by the hematopoietic microenvironment and extracellular matrix, may be crucial for hematopoietic regeneration after HSC transplantation [18, 27, 28]. Based on animal data, including the results presented here, it is postulated that stromal cells infused into humans can engraft in the marrow microenvironment that have been damaged by chemotherapy or irradiation, and therefore would improve the hematopoietic recovery after HSC transplants. A

series of studies has brought evidence that these cells do play a role [5-6]. However, randomized studies in humans (that are in progress) need to be done to clearly prove this point.

In a second part of our study, we analyzed the homing of human Stro-1-selected MSCs in tissues of mice. By quantitative PCR analysis, we found that expanded Stro-1^+ cells migrated better than expanded Stro-1^- cells in BM, spleen, muscles, liver and kidneys. No difference was observed in brain, while more expanded Stro-1^- than Stro-1^+ cells were found in lungs. This latter result in lungs fitted with the data reported by Noort et al [4] although their data were obtained with fetal lung MSCs. The homing of MSCs in various tissues has been earlier reported [31, 32], but this is the first observation of a difference of migration of MSC subsets, based on Stro-1 antigen expression.

We recently demonstrated that allogeneic MSCs were able to engraft in a patient with aplastic anemia, to improve the stromal microenvironment and to contribute to donor stromal cell regeneration [33]. The fact that in this observation, allogeneic MSCs were only detectable by real time PCR in BM recipient did not mean that the cells did not repopulate host tissues. Indeed, Horwitz et al [34] found less than 2% infused MSCs in children suffering from osteogenesis imperfecta, while the biological effect of this infusion was impressive on the correction of the disease, particularly in the new dense bone formation. Similarly, Koç et al [35], despite founding less than 2% donor cells in the BM-derived MSCs of patients with Hurler's syndrome and metachromatic leukodystrophy infused with expanded MSCs, noted a reversal of disease pathophysiology in some tissues.

The high plasticity of MSCs has opened new areas of clinical applications [12, 19]. Transplantation of MSCs would attenuate or possibly correct genetic disorders of bone, cartilage and muscle, as already pointed out above [34, 35]. Here, in order to ensure that the gene transduction would not modify Stro1^+ MSC functions, the cells were transduced with the eGFP gene and analyzed for ex vivo cytokine release and homing in NOD/SCID mice: the eGFP-transduced Stro-1^+ cells had the same cytokine release and migrated to the same tissues (as assessed by PCR) in NOD/SCID mice than the non-transduced cells.

Noteworthy, we did not report on the presence of eGFP cells detected by microscope examination in tissue sections because this technique had proven to have, in preliminary testing, a very low sensitivity. Visualization of eGFP-positive cells in tissue cross sections was difficult and was only revealed by immuno-staining with a rabitt anti-GFP antibody in *gyrus dentate* brain section.

The presence of eGFP-positive cells was thus assessed by a technique more sensitive, PCR: by this technique, a majority of the tissues were found positive. In particular, the BM thatwas found eGFP -negative by direct microscope examination and by FACS analysis, was actually eGFP-positive by PCR in 5/5 tested mice. In a baboon model, Devine et al [32], also found positive homing of eGFP cells using a sensitive quantitative PCR technique. These results were also obtained by one of us [36] in irradiated macaques.

Very rare pluripotent stem cells persist into the stromal cell compartment of adult bone marrow [37, 38]. In given circumstances (lesion, new environment) they may proliferate and differentiate. Interestingly, in our non human primate model [36], infused MSCs homed preferentially to altered tissues, what is promising for specific tissue repair. However, it's remained unknown if these MSCs were engrafted or just resident in the tissues where they migrated. We are presently investigating if the expanded MSCs that home in altered tissues of NOD/SCID actually differentiate into true local MSCs and acquired the specific tissue antigen, or just remained in the tissue without changing their phenotype.

Our data may be interesting for gene therapy : Stro-1^+ cells that were not modified by gene transduction would probably be used in humans for targeting tissues. Indeed, the potential of MSCs as vehicles for gene delivery or protein production has been indicated by many authors [12-19,23]. Nolta et al [1] have shown that MSCs transfected with the IL-3 gene can improve engrafment of human HSC in immunodeficient bnx mice by producing the IL-3 protein in vivo. The same result was obtained by Brouard et al [3] in the NOD/SCID mouse model with a murine stromal cell line.

In conclusion, the present data demonstrate that the functions of MSCs depend on their Stro-1 phenotype : expanded Stro-1^+ home in higher numbers than expanded Stro-1^- cells in the majority of mouse tissues, but support less engrafment of hemopoietic progenitors. In contrast to hemopoietic support, for which selection of Stro-1^+ cells seems unnecessary (around 90% of unselected MSCs are Stro-1^-), a pre-selection before ex vivo gene tranfection for in vivo gene delivery appears useful to improve MSC homing, at least in NOD/SCID mice.

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Legends of figures

Figure 1. Percentages of human CD45 cells in the blood of NOD/SCID mice at 3, 6, 9 and 12 weeks after infusion of human cord blood $CD34^+$ cells +/- ex vivo expanded Stro-1^+ or Stro-1^- cells. Each point corresponds to the mean +/- SD of CD45 cell percentages in 8 mice.

Figure 2 : Percentages of human CD45 cells detected in peripheral blood of NOD/SCID mice. Columns represent the mean value (from 8 animals) with standard deviations of the areas under the curve (AUC) of CD45 cell percentages in the blood of NOS/SCID mice. An asterisk (*) means a statistically significant difference with a $p<0.05$ as compared to infusion of "$CD34^+$alone", ** a difference with a $p <0.01$.

Figure 3 : Distribution of human eGFP MSC derived cells in brain of NOD/SCID mice.
Pictures were taken in confocal microscopy from 5 µm-thin sagital sections of brain of NOD/SCID mice. Panels b and c were respectively x40 and x400 magnifications of brain cross sections incubated with anti-GFP antibody counterstained with hematoxylin. Panel d was a x400 magnification of brain cross section incubated with anti- β-2 microglobulin antibody counterstained with hematoxylin. Black arrows indicate positive GFP cells (in panels b and c) and human β-2 microglobulin positive cells (in panel d) in the *dentate gyrus* area. Panel a was a x40 magnification of a cross section incubated with a non relevant antibody (negative control).

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Group of mice	Human cells	$CD34^+$ $Stro\text{-}1^-$	$CD34^+$ $Stro\text{-}1^+$	$CD34^+$
BM	CD45	48 ±22	33 ±16	27±25
	CD19	38 ± 13	21 ±13	20± 17
	CD11b	3± 1	4±1	3 ±3
	CD34	9 ± 3	5±2	4± 4
Spleen	CD45	35 ± 25	14±8	15±16
	CD19	19± 23	7±6	11± 12
	CD11b	8±10	0.8±0.7	3 ± 4
Blood	CD45	14± 12	3. ±2	4 ± 4
	CD19	9 ± 7	1.6 ±1.3	3 ± 3
	CD11b	4± 6	0.25 ±0.25	1 ± 1

Table 1. Percentages of human CD45, CD19, CD11b and CD34 in BM, spleen and blood of animals, 12 weeks after cotransplantation of $1x10^5$ hUCB $CD34^+$ cells and $1x10^6$ expanded $Stro\text{-}1^+$ or $Stro\text{-}1^-$ cells in NOD/SCID mice.

tissues	Stro-1$^+$ cells	Stro-1$^-$ cells
Spleen	8 ± 5	1.5 ± 1
BM	13 ± 7	6 ± 3
Brain	4 ± 5	4 ± 0.6
Heart	1.6 ± 1.1	2 ± 2
Liver	0.6 ± 0.4	0.4 ± 0.6
Lungs	2 ± 3	7 ± 11
Kidneys	2.9 ± 1.9	1.9 ± 1.7
Muscles	2 ± 2	0.2 ± 0.3

Table 2: Homing of expanded Stro-1$^+$ or Stro-1$^-$ cells in mouse tissues. The results were expressed as numbers of equivalent human cells per 10000 murine cells (as evaluated by number of copies of human β GLOBIN DNA in mouse tissue DNA) in each analysed tissue.

tissue	BM	Lungs	Liver	Muscles	Kidneys	Spleen	Brain	Heart
Stro-1^{+} eGFP	5/5	4/5	3/5	5/5	4/4	5/5	3/5	5/5

Table 3: Detection of eGFP positive tissue by PCR in NOD/SCID mice 12 weeks after infusion. Results are presented as numbers of positive tests / number of mice analyzed

Figure 1

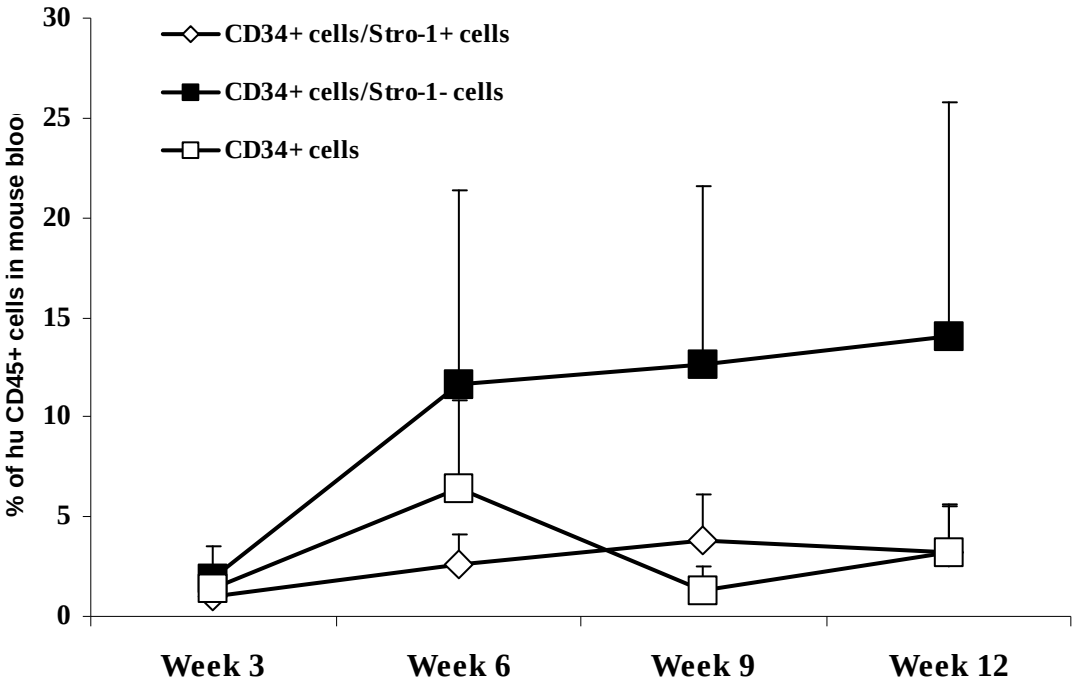


Figure 2

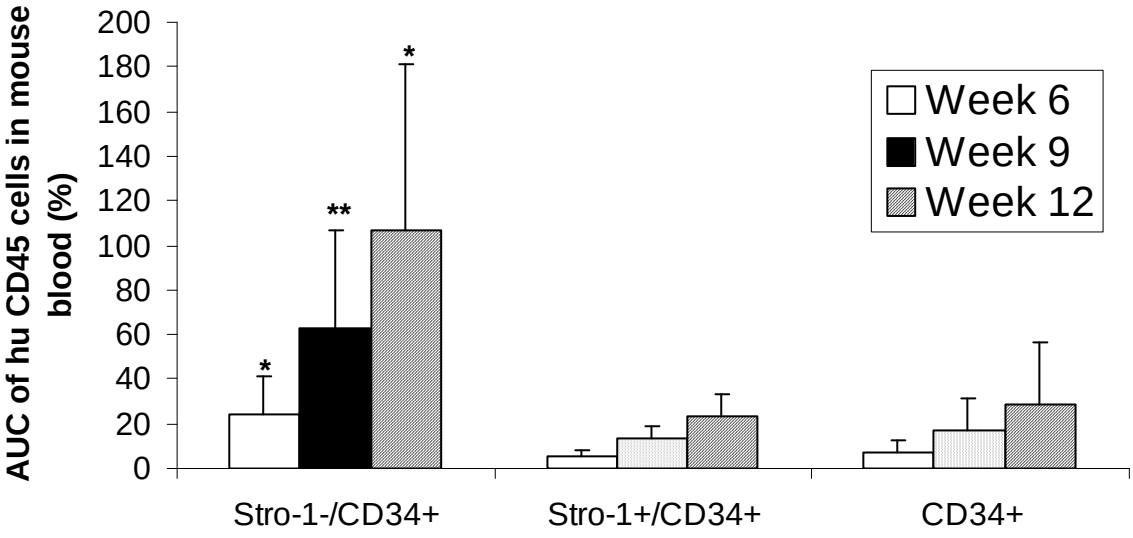
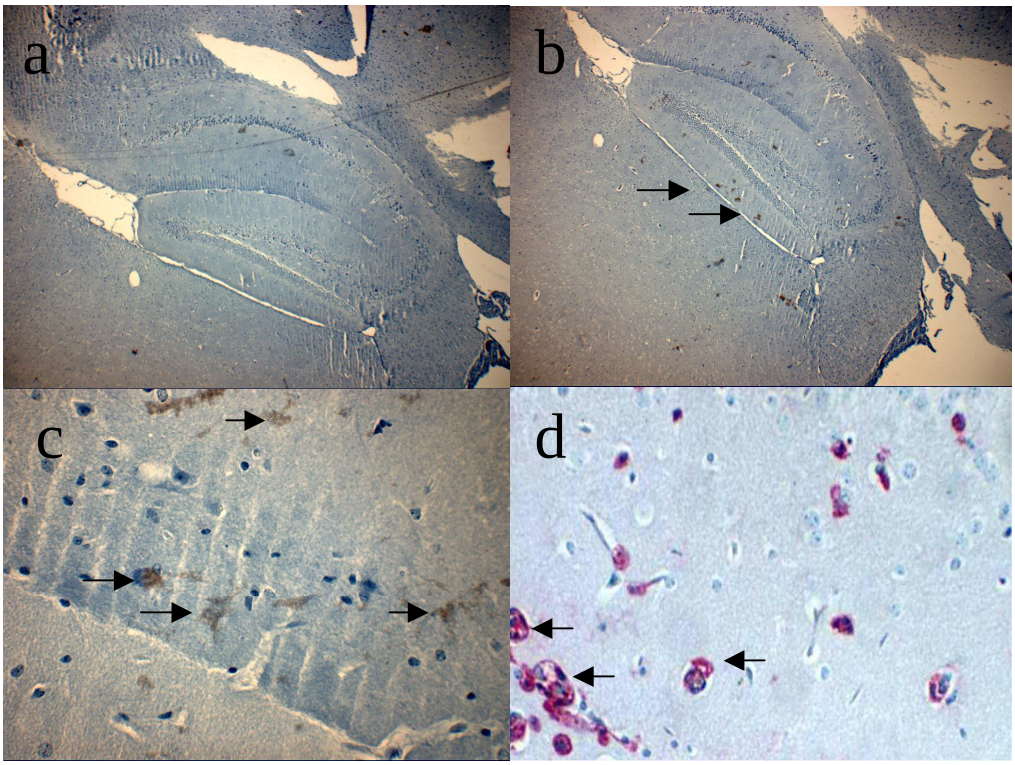


Figure 3



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Homing of in vitro expanded Stro-1$^-$ or Stro-1$^+$ human mesenchymal stem cells into the nod/scid mouse. Their role in supporting human CD34 cell engraftment

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