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On the Track of Mesothelial Progenitor Cells from the Peritoneal Cavity Transplanted to Immunodefficient Mice

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Abstract: Mesothelial progenitor cells have been reported to reside in either the monolayer of mesothelium, submesothelium or within the peritoneal cavity as free floating cells. As a putative plasticity has been reported for the mesothelial progenitor cells and considering the potential implications of the establishment of a novel resource of stem /progenitor cells in gene and cell therapeutics and tissue engineering, we conducted an *in vivo* tracking of transplanted mesothelial cells. In order to induce immunodeficiency, the recipient mice were treated with 32 mg kg^{-1} of daily Cyclosporine. On days 14, 30 and 60 post transplantation, brain, heart, skeletal muscle and lung tissues were screened by a modified FISH method directed to the Y chromosome of donor cells. Fluorescence harboring cells were analyzed by flow cytometry and fluorescent microscopy. The data confirmed by PCR, demonstrated the existence morphology alteration of the donor cells in various organs of the recipient mice, notably in the skeletal muscle and lung and less in the heart and brain. Immunostaining of recovered cells from the nervous recipient tissues suggests differentiation of mesothelial cells in the new microenvironment.

Key words: Peritoneum, progenitor cell transplantation, mice, stem cells, mesothelial cells

INTRODUCTION

The serosal surface of the peritoneal and pleural cavities is lined by a monolayer of flattened mesothelial cell (Bermudez *et al*., 1998). The monolayer is supported by subserosal connective tissue consisting of fibroblasts, collagen fiber, adipocytes, leukocytes and an abundant supply of lymphocytes and microvessels (Witz *et al*., 1998). The presence of tight junctions and desmosomes has been reported (Di Poalo and Sacchi, 1990). It has been suggested that the peritoneum mesothelial cells are strongly involved in the process of inflammation in the abdominal cavity because of the expression of adhesion molecules especially ICAM-1 and VCAM-1 (Liang and Sasaki, 2000). The mesothelial cells covering the serous membranes are unique in the sense that they are of mesodermal origin (Lucas *et al*., 1996). Hemangioblasts are the common progenitors of epithelial and hematopoetic cells which have been reported to differentiate from embryonic mesothelial cells of the splancnic mesothelial (Munoz-Chapuli *et al*., 1999). The mesothelial cells share the characteristics of both epithelial and mesenchymal cell types and range from flattened to cuboidal (Wilkosz *et al*., 2005). Mesothelium in quiescent state represents a nonthrombotic, nonadhesive substrate for the serosal fluid and peritoneal leukocytes, as the leukocytes adhere and accumulate on the mesothelial surface and migrate across mesothelial lining (Muller and Yoshida, 1995). Various studies report mesothelial-mesenchymal transition of mesothelial cells which is characterized by loss of epithelial phenotype and the expression fibroblast associated markers in various benign disorders as well as fibrosis following CAPD (continuous ambulatory peritoneal dialysis) (Dobbie, 1992). EGF has been reported to induce the reversible change to fibroblast phenotype that is accompanied by an increase of integrins and enhanced adhesion on collagen type 1 (Yanez-Mo *et al*., 2003). It was first suggested that mature mesothelial cells could transform into fibroblast-like cells *in vivo* and invade the underlying subserosal connective tissue, quite unusual for mature epithelial cells. Evidence supporting the existence of mesothelial stem cells is still immature, but recent studies suggest differentiation along specific mesenchymal cell lineages as well as epithelial-mesenchymal transdifferentiation, for example, transforming growth factor-β1 (TGF-β1) induces human omental mesothelial cells to transdifferentiate to myofibroblasts *in vitro* (Yanez-Mo *et al*., 2003; Dobbie, 1992). The exact location of the reservoir of mesothelial progenitor cells is highly

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controversial, either within the monolayer itself, sub mesothelium or free floating in the serosal liquid (Yanez-Mo *et al*., 2003).

As it was first suggested that mature mesothelial cells could transform into fibroblast-like cells *in vivo* and invade the underlying subserosal connective tissue, quite unusual for mature epithelial cells. Mesothelial cells from normal serosal tissue or fluid demonstrate cobblestone epithelial morphology in culture, however they can change through passages or continuous peritoneal dialysis to fibroblastic phenotype and undergo epithelial mesenchymal transition, which is characterized by a reduction in cytokeratin and E-Cadherin and on the other hand with an increase in α-integrin and vimentin expression (Comin *et al*., 2006; Segers *et al*., 2006; Weusten *et al*., 2000). Mesothelial cell line express cytokeratin 8 and 18, vimentin, calretinin and desmin (Herrick and Mutsaers, 2004). Mesothelial cells also have been reported to express tumor and hematopoietic cell lineage markers, for example: WT-I (Wilm's tumour marker) (Gulyas and Hjerpe, 2003; Menssen *et al*., 2000), HBME-1 (a marker of cell tumor of the tyroid) (Frierson *et al*., 2003; Sun *et al*., 2001; Volante *et al*., 2004), CD31 (epithelial tumour marker) (Darai *et al*., 1998; Newton *et al*., 1999; Sapino *et al*., 2001), CD34 (Doyonnas *et al*., 2001; Flint and Weiss, 1995; Steidl *et al*., 2004), but it is not clear in what state of cell transition *in vivo* or *in vitro* do they express these markers (Herrick and Mutsaers, 2004). Mesothelial cells are presumed to be the progenitor cells of malignant mesothelioma, a cancer strongly correlated with asbestos exposure (Broaddus *et al*., 1997; Reddel *et al*., 1989). Although the existence of mesothelial and germ line stem cells in the postnatal adult mammalian ovary have been implied, their isolation and characterization has not been achieved as yet (Bukovsky *et al*., 2004; Johnson *et al*., 2004).

Due to the ethical concerns limiting manipulation of embryonic stem cells, adult stem cells have shown applicability in use in tissue engraftments, cell and gene therapy, repair and tissue regeneration mostly concerning the nervous system, heart, skeletal muscle and liver. Thus, the possibility of defining a novel resource of adult stem cells could be promising. In order to clarify the capacity of mesothelial progenitor cells for tissue engraftment and their possible differentiation potential, we conducted an *in vivo* tracking of transplanted mesothelial cells from the peritoneal cavity (Kamminga *et al*., 2005). In order to exclude the presence of myeloid derived cells in the system and the free floating cells from the peritoneal cavity, we transplanted mesothelial cells comprising of separated mesothelial monolayer, submesothelium after eliminating vessels and adipose tissue (Muller and Yoshida, 1995). Furthermore nonadhesive cells were excluded by culture. Cells were characterized by immunostaining prior to transplantation. A practical way to detect these cells post transplantation was Y chromosome detection (Kim *et al*., 2005; Niyibizi *et al*., 2004; Wang *et al*., 2002; Zhang *et al*., 2002).

Male Balb/C mice were chosen as donors while the females were treated with Cyclosporine to induce immunodeficiency. We accomplished FISH (Fluorescent *in situ* Hybridization) method for Y chromosome hybridization. The presence of fluorescent-labeled cells in interval of 14, 30 and 60 days was analyzed by flow cytometry and fluorescent microscopy. The data were confirmed by PCR for Y chromosome testis-specific pseudogene (TSRY) (Wang *et al*., 2002). Recovered cells form the recipient cells were isolated by FACS and studied for marker alterations.

MATERIALS AND METHODS

Cell isolation: Murine mesothelial cells were isolated by surgical equipment by simply separating the mesothelial monolayer under a stereomicroscope and then the removed cells were made single cell suspension by 1% Collagenase/PBS from Clostridium difficile (Sigma-Aldrich) by incubating at 37°C for 10 min (Muller and Yoshida, 1995). Prior to dissection of mice, 2 mL PBS was injected directly into the peritoneal cavity to attain the free-floating cells. The resultant suspension of mesothelial cells were placed in T-25 (Orange) flasks containing DMEM (Himedia) supplemented with 20% FBS (Biosera), 100 U mL^{-1} Penicillin and 100 μg mL^{-1} Streptomycin (Merck). Cells were subcultured after reaching confluence with Trypsin-EDTA (Merck) treatment. Cells were washed with PBS in order to eliminate FBS immunogenecity. All animal experiments protocols have been approved by the University Animal Care Committee.

Characterization of mesothelial cells prior to transplantation: cells were fixed on fluorescent microscopy slides with ice cold 100% methanol for 3 min at 20°C. Slides were washed with PBS to exclude the excess fixative. Cells were blocked with 5% goat serum for 30 min at room temperature. Excess blocker was washed with PBS. Cells were incubated with HBME-1 anti mouse monoclonal primary antibody (Abcam) (1/200) and CD44 (Abcam) (1/200) for 1 h at room temperature. Slides were washed with PBS and exposed to FITC-conjugated polyclonal secondary antibody (Abcam) (1/1000), for 30 min at room temperature. Slides were visualized with fluorescent microscopy (Axioskop2 plus, Zeiss). Also

primary anti mouse monoclonal antibodies of CD34 (Abcam) 1/200 was evaluated using the mentioned protocol. Cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

Preparation of immunodeficient mice: Immunodeficiency was induced by daily dose of 32 mg kg^{-1} of cyclosporine (Cycloral®) to a group of 15 Balb/C mice. The mice were kept isolated and the environment was regularly disinfected by UV irradiation. The assessment of immunodeficiency was accomplished by Elisa Cytokine assay measuring IFN-γ, as a significant decrease in the amount of cytokines could be reminiscent of a relative decrease in immunity. After 3 weeks, 1×10^6 cells were injected via tail vein. Cyclosporine treatment was continued to the end of the experiment. Two hours prior to transplantation, the mice received a single dose of 100 mg kg^{-1} Cyclosporine (Takashima and Collins, 1987). At intervals of 14, 30 and 60 days, mice were sacrificed by cervical dislocation. Heart, skeletal muscle, lung and brain tissues were removed and were digested mechanically and by Collagenase treatment as mentioned. The enzymatic reaction was brought to an end by adding an equal amount of DMEM media.

Cytokine assay: Five groups of mice were chosen for the assay, which received Normal Saline (NS), 8, 16, 32 or 64 mg kg^{-1} of Cyclosporine. After 3 weeks of receiving the immunosuppressive agent, the spleens were removed and enzymatically digested by Collagenase. Red blood cells were eliminated by ACK lysis buffer (NH_4Cl 8.29 g, $KHCO_3$ 1 g, Na_2 EDTA 37.2 mg, 800 mL H_2O, pH 7.2). The suspension was gently shaken for 5 min at room temperature and then the tube was centrifuged for 10 min at 200 g. The spleenocytes were cultured in 12 well-plastic dishes containing RPMI 1640 media. After reaching confluence, 5 μg mL^{-1} of Concavalin A (Sigma-Aldrich) was introduced to the medium. The amount of IFN-γ in the supernatant was measured pre (1 h prior) and post (24 h after) transplantation by ELISA using the Mouse Module Set (IFN-γ, Bendermed Sciences, Germany). Cyclosporine has been reported to decrease cytokines such as IFN-γ, IL-2, IL4 and IL-13. The amount of IFN-γ is demonstrative of Th-1 maturation, NK and macrophage activity and cellular immunity (Dietert *et al*., 2003).

Tracking of grafted mesothelial cells: Y chromosome was detected in the recipient tissue by means of FISH (Fluorescent *in situ* Hybridization) (Crouch *et al*., 1997; Parrilla *et al*., 2003; Pennline *et al*., 1992; Rens *et al*., 1996, 2001). Single cell suspension on Lysine-coated slides was treated with acetone for fixation. Cells were denatured with 70% formamide for 10 min, afterwards washed with SSC hybridization buffer and incubated at 60°C for 10 min. A Murine TSRY gene specific probe (5'-TCC TGG ATC AGA GTG GCT TAC CCA GG-3') (Wang *et al*., 2002), which was labeled with biotin on 3 and 5 terminals, was purchased from the Medical Smith Laboratory (University of British Columbia, Ca). 3 μg mL^{-1} probe, 3 μg mL^{-1} of Herring sperm DNA (Invitrogen) and 10 μg mL^{-1} of Mouse Cot-1 DNA (Invitrogen) (added to eliminate nonspecific bonding), 10 μL of absolute ethanol and 11 μL of acetate buffer, pH 5.6 were frozen at −70°C and precipitated, the supernatant was discarded.

Sufficient probe solution was added to each slide. Hybridization buffer consisting of 3 mL formamide, 3 mL SSPE buffer and 1 g Dextran Sulfate was also added to the slides and incubated. Different incubation times were tested ranging form 2-18 h; the optimum time was 3 h. Non-bonded DNA was washed off with SSC buffer. Avidin-FITC (Fluorescein Isothiocyanate) (Sigma-Aldrich) was added in order to bond with biotin terminals, then it was incubated at room temperature for 20 min and non-bonded dye was washed away with SSC buffer (Crouch *et al*., 1997). Cells were recovered from the slide by Trypsin-EDTA treatment and analyzed by flow cytometry (FACSCalibur, Becton Dickinson). Some slides were analyzed by fluorescent microscopy (Axioskop2 plus, Zeiss).

PCR: To confirm the existence of Y chromosome, cells were analyzed by PCR (Biometra T gradient). DNA was extracted by incubating cells in 250 mM NaCl, 1 mM EDTA, 25 mM Tris-HCl, 5% TritonX-100 and 5 U of Proteinase-K (Fermentas) for 2 h. Cells were heated at 100°C for 10 min. The resultant supernatant was precipitated with 300 μL of Isopropanol. Precipitated DNA was dissolved in TE buffer and 5 μL of the solution was used for PCR. Y-chromosome TSRY specific primers were purchased from Microsynth (Switzerland). One microliter of $MgCl_2$ 25 mM (Fermentas), 2.5 μL of 10X PCR buffer (Fermentas), 5 U Taq Polymerase (Fermentas), 3 μL dNTP 10 mM (Fermentas), 15 μL of distilled water and 1 μL of each primer (5 pMol) were used. Fourty cycles of 30S at 95°C, 30S at 60°C and 1 min at 72°C were performed. The following primers were used: Y-chromosome rev5'-GAG AAC CAC GTT GGT TTG AGA TG-3', for5'-TCC TTG GGC TCT TCA TTA TTC TTA AC-3', β-Actin rev5'-CAA GAA GGA AGG CTG GAA AAG A-3' and ß-Actin for 5'-ACG GCC AGG TCA TCA CTA TTG-3' (Wang *et al*., 2002). A final 10 min at 72°C was added to terminate the incomplete synthesis. Amplified samples were run on 1.5% Agarose gel (Biometra, Agagel and Midiwide) and visualized by SYBR Green (Roche) under UV irradiation. As the amount of Y chromosome in each PCR experiment

was unpredictable and in order to avoid under amplification of Y chromosome, β-Actin internal control samples were run in parallel lanes.

Immunofluorescence staining visualization of incorporated cells isolated by flow cytometry: Cells recovered form the recipient tissue were analyzed by FACS as outlined in section Tracking of grafted mesothelial cells. Positive cells were immunosained to prove differentiation of the incorporated donor cells in the new microenvironment. Cells were blocked with goat serum for 30 min at room temperature. Excess blocker was washed with PBS. Primary anti mouse monoclonal antibody of neurofilament (Abcam) was added (1/200) and treated as mentioned earlier.

RESULTS

Morphology: The mesothelial cells reached confluence within over a week. Colony formation was also observed 24-72 h after beginning culture. Figure 1 shows the presence of epithelial and mesenchymal shaped cells. The presence of both morphologies supports the epithelial-mesenchymal transition theory, which could occur in the non-inflammatory, non-fibrosing peritoneum (Herrick and Mutsaers, 2004). The isolation of mesothelial cells in order to observe transdifferentiation has been performed similarly (Dobbie, 1992; Muller and Yoshida, 1995; Yanez-Mo *et al*., 2003). Having eliminated nonadherent cells after a few passages, mesothelial cells became

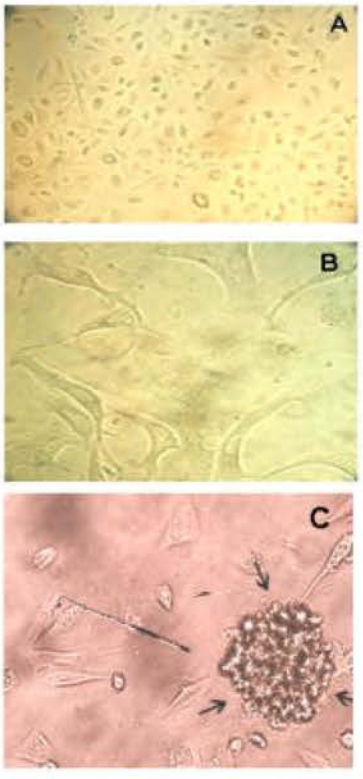

Fig. 1: Mesothelial cell culture. Cells show epithelial shaped morphology early in culture (A). Mesenchymal phenotype becomes: dominant in 3-4 weeks of culture (B). Colony formation in 24-72 h of culture (C)

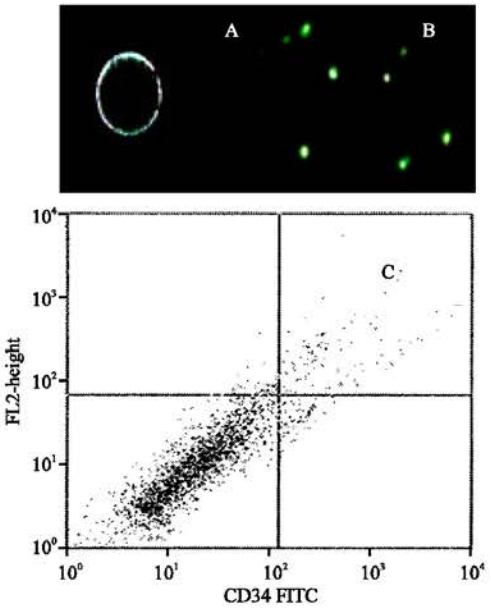

Fig. 2: Marker determination of mesothelial cell prior to transplantation. Mesothelial cells expressed HBME-1 membrane marker (A). CD44 was also expressed by these cells (B). Dot plot curve demonstrates the expression of CD34 by a number of mesothelial cells (C). Forward scatter and side scatter were selected FITC and FL-2, respectively

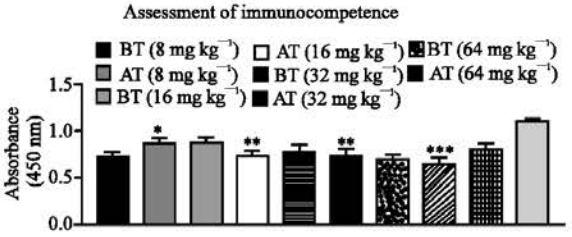

Fig. 3: IFN-γ levels upon cyclosporine treatment. Absorbance read at 450 nm (620 nm reference) for each group pre (BT) and post transplantation (AT), 1 h prior and 24 h after transplantation, respectively. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. The last 2 bars represent NS group before and after transplantation, respectively

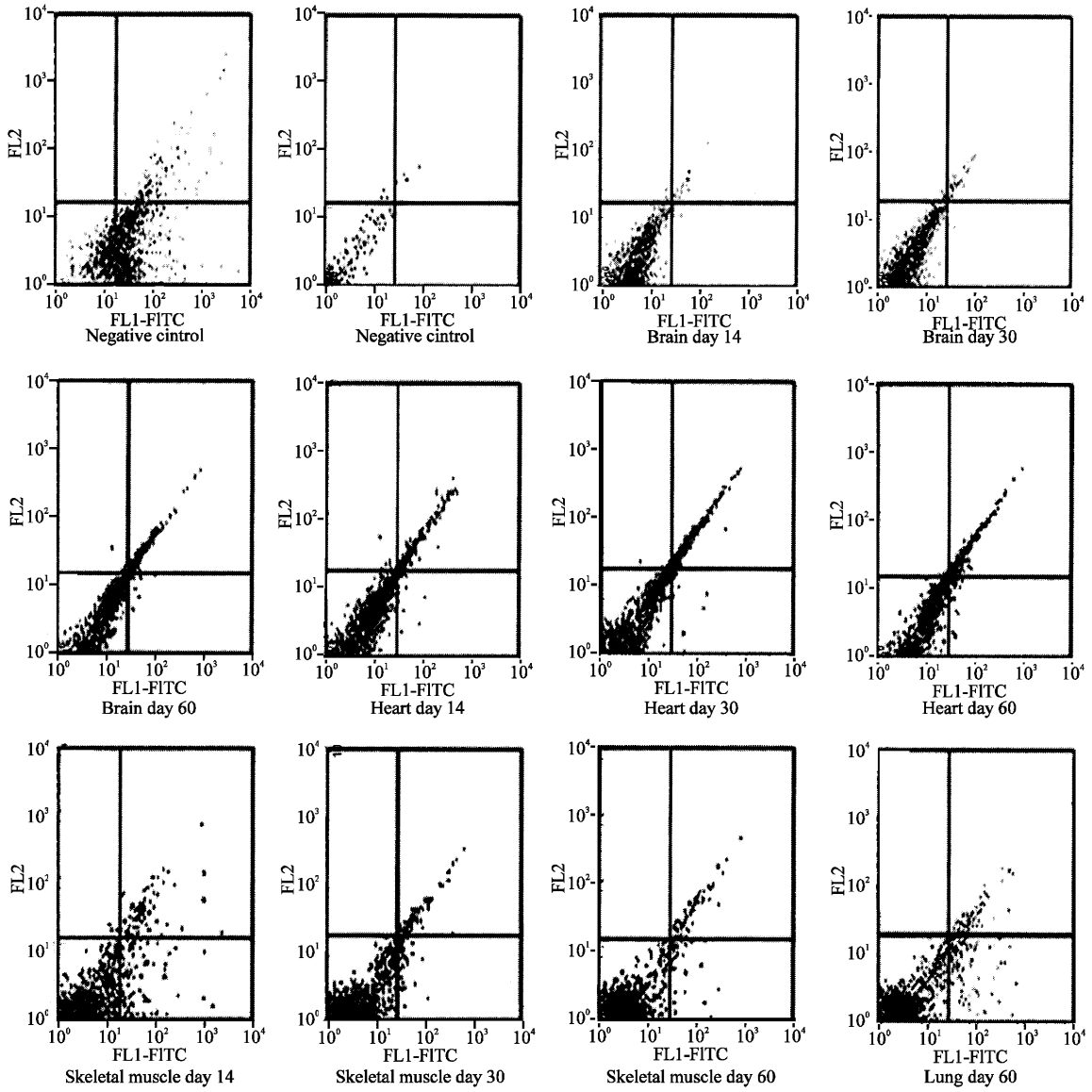

Fig. 4: Flow cytometry of cells from recipient mice tissues hybridized to a Y chromosome specific probe; curves indicate 3 intervals of screening at 14, 30 and 60 days. In each curve X and Y axes were selected as FL1-FITC and FL2, respectively

dominant in culture, which made them suitable for transplantation. Colony formation was evident 24-72 h after culture.

Characterization of mesothelial cells: In order to characterize mesothelial cells, immunostaining experiment were performed. Mesothelial cells expressed membrane HBME-1, as shown in Fig. 2A, which was stained using immunofluorescence technique. CD44 was also dominantly expressed by mesothelial cells (Fig. 2B). CD34 was expressed as a cell surface marker by a population of mesothelial cells. CD34 is a marker of hematopoietic stem cells as well as endothelial and mesothelial cell lines. Dot plot curve demonstrates a percentage of positive cells (Fig. 2C).

Cytokine assay: The amount of IFN-γ produced by spleenocytes before and 24 h after transplantation was measured by Elisa Sandwich. The protocol was advised by the manufacturer (Bendermed Sciences). An absorbance/concentration standard curve was defined to calculate the amount of IFN-γ in the supernatant of each group, which is shown in Fig. 3. The data were analyzed by one-way ANOVA and Tukey post test. p-value was

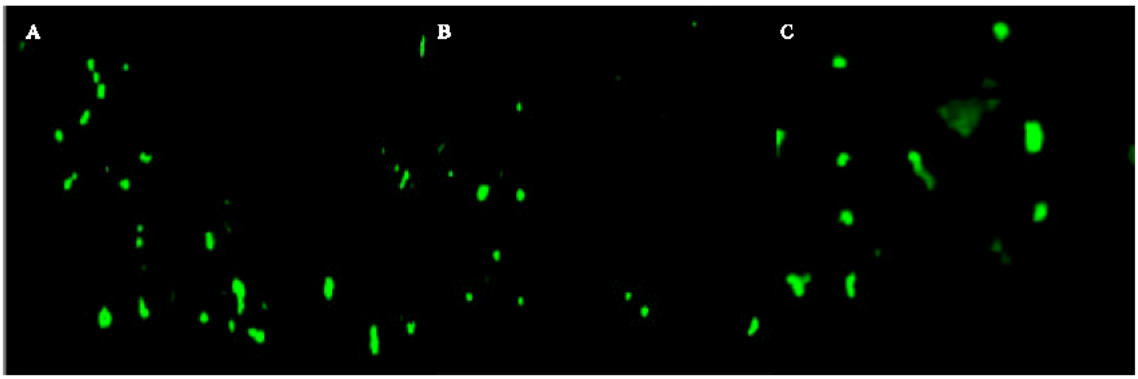

Fig. 5: Fish staining of Y chromosome pseudogene. Fluorescent harbouring cells within the recipient tissues. Donor cells recovered from the lung (A), heart (B) and skeletal muscle (C)

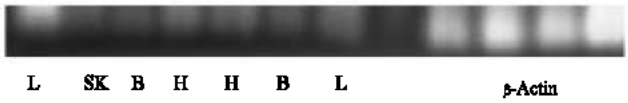

Fig. 6: PCR results after amplification of a Y chromosome-specific sequence in different tissues from the recipient mice. Lung (L), brain (B), skeletal muscles (SK) and heart muscle (H) samples are shown. β-actin was also run in parallel lanes

calculated 0.0004 with F factor of 3.995. The amounts of post transplantation IFN-γ in the supernatant of groups received 8, 16, 32 or 64 mg kg^{-1} of Cyclosporine were significantly lower in comparison to the NS group ($p<0.05$, $p<0.01$, $p<0.01$ and $p<0.001$, respectively). The data shows the potency of the drug especially in higher doses in reducing immunity pre and post transplantation. As a dose of 64 mg kg^{-1} was not tolerated in long-term treatment and led to death, we used a dose of 32 mg kg^{-1} day^{-1} for present experiments.

Flow cytometry analysis of recipient cells: In order to detect and quantify donor cells in the recipient tissue, hybridized cells were analyzed by flow cytometry. The data were analyzed by Cell Quest Software. Figure 4 shows the presence of positive cells in comparison with the positive and negative controls. Side scatter and the forward scatter axes were chosen as FL2 and FLI-FITC, respectively. The scattered cells in the scatter diagram that seemed to be a separate group were gated consequently. The right lower quadrant shows the fluorescent harboring cells, which obviously were not enough to create a separate group as the number of incorporated cells in the recipient tissues rationally could not exceed a 1-2%. Each sample was compared with positive and negative controls, which the donor cells were chosen from male and female mice, respectively.

This figure shows samples taken from brain, heart, skeletal muscles and lung in intervals of 14, 30 and 60 days. The number of fluorescent harboring cells varied from 0.15 to 1% in brain and heart tissues. Skeletal muscle and lung tissues of the recipient showed a relatively higher percentage of fluorescent cells than heart and brain tissues (1-2%). The possibility of nonspecific bonding was ruled out by the addition of Mouse Cot-1 DNA and Herring sperm DNA. Results demonstrate the presence of donor cells with a relatively high percentage in the skeletal muscle and lung tissues, with a less amount in the heart tissue and with the least amount in the brain. The experiments were repeated 15 times to ascertain the validity of the results.

Fluorescent microscopy: Figure 5 demonstrates cells from the donor 60 days after transplantation which have resided in different recipient tissue and harbor the nuclear dye due to hybridization with the Y chromosome specific probe. Donor cells were detected in lung, heart and skeletal muscle.

PCR: PCR results at this level confirm the existence of donor cells in the recipient tissue in the given intervals (Fig. 6). As mentioned, the β-Actin internal control was run in a parallel lane in case of under amplification. The expected size of the amplified segments for TSRY gene is

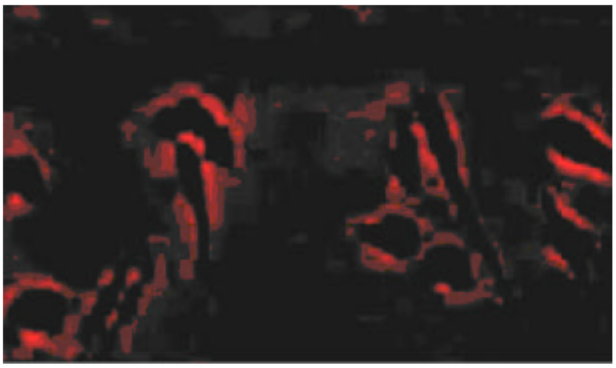

Fig. 7: Immunostaining of recovered cells from the brain. Cells recovered from the recipient tissue and isolated by FACS were positive for murine neurofilament-200, a late marker of neural cells. This data clearly proves incorporation and differentiation of donor cells within the recipient tissue (40X)

270 bp and around 200 bp for β-Actin. Y chromosome-harboring cells were found in the brain, lung, heart muscle and skeletal muscle tissues.

Immunofluorescence visualization of incorporated cells isolated by flow cytometry: Cells recovered from the recipient tissue harboring FITC were isolated by FACS and stained for lineage specific marker expression. Figure 7 demonstrates cells expressing neurofilament 200 heavy weight, which suggests a differentiation theory. This data clearly violates the previously assumed progenitor identity of these mesothelial cells.

DISCUSSION

A stem cell is succinctly defined as an undifferentiated cell with the ability to proliferate, to self-renew and to generate a large number of progeny, while the term progenitor cells refers to the offspring of the stem cell that have a more limited restricted cell fate (Laflamme and Murry, 2005). The existence of mesothelial progenitor cells has been reported by various works (Herrick and Mutsaers, 2004). Circulating endothelial progenitor cells have been isolated in humans (Laflamme and Murry, 2005). As reviewed in numerous works, bone marrow cells generate nonhematogenic cells-like muscle cells in both *in vitro* (Song *et al*., 2002) and *in vivo* models (Sato *et al*., 2005). Human bone marrow has also shown engraftment and migration in neural tissue when implanted in to rats as well as the ability to differentiate to neural cells *in vitro* (Song *et al*., 2002). Several lines of evidence also indicate that the direct injection of non-induced mesenchymal stem cells in the heart improves the ventricular function (Laflamme and Murry, 2005). Hemangioblasts are the common progenitors of epithelial cells and it has been suggested that hematopoetic cells are differentiated from embryonic mesothelial cells of the splancnic mesothelial (Munoz-Chapuli *et al*., 1999).

As the definite plasticity of mesothelial cells is not transparent, we conducted an *in vivo* tracking of these cells through a transplantation experiment, hoping to clarify the uncertainties. As mentioned, mesothelial cells dominantly consisting of mesothelial cells in epithelial-mesenchymal transition were transplanted to immunodeficient mice. Cells were characterized prior to transplantation. Cells expressed HBME-1 and CD44 dominantly. CD34 expression was significant but not dominant. After 14, 30 and 60 days post transplantation, cells were detected in brain, heart, skeletal muscle and lung tissues in different mice. The data analyzed by flow cytometry and confirmed by PCR, reveal a relatively low percentage in the brain tissue (less than 0.5%), which is expected due to the brain blood barrier. The highest percentage was detected in the skeletal muscle, lung and heart tissues (not exceeding 1-2%), as for the lung and heart tissues were obviously one of the first organs that the cells entered post transplantation. Marker determination of the recovered cells from the brain reveals definite marker alteration of donor cells which suggests the occurrence of transdiffererntiation and implies plasticity in nature for mesothelial cells.

Evidence supporting differentiation of mesothelial progenitor cells to any of the given lineages beyond its embryonic developmental layers is lacking in the previous works. Present findings suggest that 60 days after transplantation, these cells were still detected in tissues. It is rational to assume the cells were trapped for this extended amount of time and they established residence

and incorporated into the tissue because they simply differentiated to tissue specific cells. It is highly unlikely that cells of another origin would survive such a longtime in an incompatible microenvironment without a suitable trophic signal or not to undergo apoptosis and engulfment and to be eliminated soon after entering the recipient tissue (Alberts *et al*., 2002). Fusion is a possible and not necessarily rare phenomenon in transplantation of stem cells, which should be studied in further work (Rodic *et al*., 2004).

Could the common evolutionary background of hematopoetic cell lineages and mesothelial cells account for the observed differentiation? This data clearly violates the previously assumed plasticity of these cells considering them as progenitor cells (Herrick and Mutsaers, 2004). Such a presumable differentiation capacity could enormously effect cell therapeutics and tissue regeneration. Further attempts should be aimed at *in vitro* differentiation of these cells to support this observation.

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