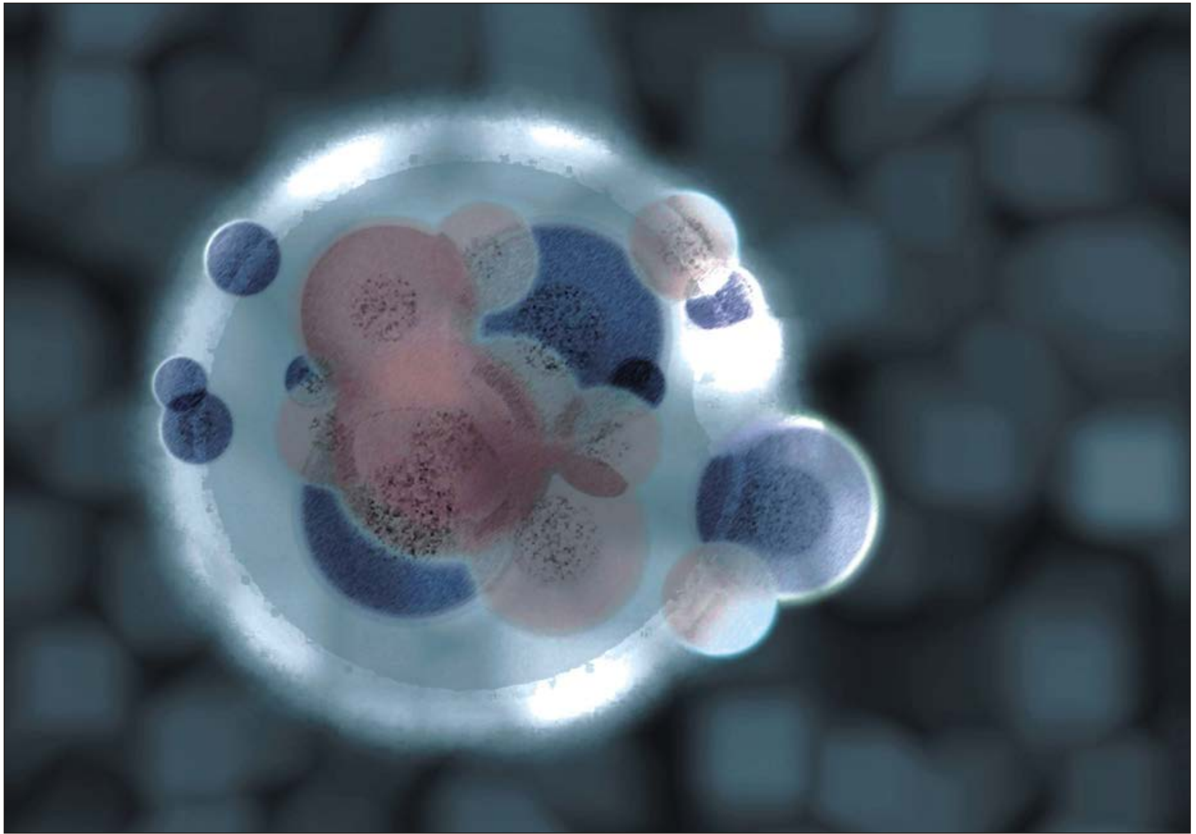

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Characterization and Transdifferentiation of Human Mesothelial Progenitor/Stem Cells of the Peritoneum Cavity

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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. A putative plasticity has been suggested for these cells as an epithelial to mesenchymal transition and transformation into myofibroblasts and smooth muscle have been suggested. In order to investigate the plasticity and nature of mesothelial cells, cell populations from peritoneal dialysis fluid of early stage non-peritonitis patients were first screened for dominant marker determination by RT-PCR and immunofluorescence. Then, cell colonies were isolated by culture and FACS using HBME-1 and CD34 markers. Efficacy of cell colony isolation by the mentioned methods was validated by flow cytometry. Later, specific media for the differentiation of the mesothelial colonies were defined. The culture of mesothelial cell colonies in knockout serum cultures containing specific growth factors showed a surprising but a relatively low yield of differentiation capacity along extra mesodermal lineage directed to neurons. This was evident by morphological characteristics of neurons and expression of neuronal specific cell markers consisting of the immature neuron markers Tubulin III and Nestin and also the structural neuronal marker Neurofilament 200 as revealed by Western blot. This study could completely violate the previously assumed plasticity of mesothelial progenitor cells and lead us to the definition of a new source of adult stem cells.

Key words: Stem cells, peritoneum, transdifferentiation, $CD34^+$, HBME-1

INTRODUCTION

Stem cells are self regenerating, multipotential cells that are capable of producing the full complement of cellular diversity within a region, while progenitor cells are dividing cells that will give rise to a more restricted progeny of a stem cell (Wagers and Weissman, 2004). Many adult tissues contain populations of stem cells that have the capacity for self renewal after trauma, disease, or aging. These cells may be found within the tissue or in other tissues which serve as stem cell reservoir (Pittenger *et al*., 1999), for example, adult bone marrow consists of both mesenchymal and hematopoietic cell lineages that can be induced to differentiate *in vitro* and *in vivo* (Liechty *et al*., 2000).

The lateral mesoderm divides into somatic (parietal) and visceral (splanchnic) layers, which consequently gives rise to the monolayer of mesothelial between the body wall (somatopleure) and the gut wall (splanchnopleure) (Herrick and Mutsaers, 2004). Blood islands appear in the yolk sac in the third week of gestation and shortly afterwards appears in the lateral mesoderm (Anzai *et al*., 1999). Hemangioblasts are the common progenitors of endothelial and hematopoietic cell lineages which seem to originate from the splanchnic mesoderm and more precisely from the embryonic splanchnic mesoderm (Munoz-Chapuli *et al*., 1999).

The monolayer of the mesothelium is supported by subserosal connective tissue consisting of fibroblasts, collagen fibers, 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 Poala and Sacchi, 1990). Mesothelial cells from normal serosal tissue or fluid demonstrate cobblestone epithelial morphology in culture, however they can change through passages or

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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 (Herrick and Mutsaers, 2004; Witz *et al*., 1998; Yanez-Mo *et al*., 2003; Zeimet *et al*., 1997; Demir Weusten *et al*., 2000; Segers *et al*., 2006; Afify *et al*., 2002; Comin *et al*., 2006).

Mesothelial cells express cytokeratin 8 and 18, vimentin, calretinin and desmin (Herrick and Mutsaers, 2004), WT-I (Wilm's tumour marker) (Memssen *et al*., 2006; Gulyas and Hjerpe, 2003), HBME-1 (a marker of cell tumor of tyroid) (Volante *et al*., 2004; Sun *et al*., 2001; Frierson *et al*., 2003), CD31 (epithelial tumor marker) (Darai *et al*., 1998; Newton *et al*., 1999; Sapino *et al*., 2001) and CD34 (Doyonnas *et al*., 2001; Flint and Weiss, 1995; Lee *et al*., 2006), but it is not rather clear in what state of cell transition *in vivo* or *in vitro* do mesothelial cells express these markers (Herrick and Mutsaers, 2004; Yanez-Mo, 2003). CD34 which was initially characterized as a hematopoietic progenitor cell antigen has been detected in endothelial cells, mesothelial cells, solitary fibrous tumor and vascular neoplasms as well as certain connective tissue (Steidl *et al*., 2004; Leis *et al*., 2004; Venable *et al*., 2005; Thiemann *et al*., 1999; Knapp *et al*., 1995; Shojaei *et al*., 2004; Zeigler *et al*., 1999).

Evidence supporting the existence of mesothelial stem cells is still immature, but recent studies suggest differentiation along specific mesenchymal cell lineages as Transforming Growth Factor-β1 (TGF-β1) induces human omental mesothelial cells to transdifferentiate to myofibroblasts *in vitro*; smooth muscle differentiation from mesothelial cells has also been reported (Herrick and Mutsaers, 2004; Yanez-Mo *et al*., 2003).

In order to clarify the uncertainties regarding the specific markers of mesothelial progenitor cells, origin of cells and their differentiation capacity, we first screened cell populations in the peritoneal dialysis fluid from non peritonitis patients. CD34, CD38, CD90, CD31 and HBME-1 markers were analyzed by immunofluorescence and reverse transcriptase polymerase chain reaction. Afterwards, mesothelial cell colonies were isolated by culture and FACS. Specific isolation of mesothelial cells by the aforementioned methods was verified by flow cytometry. Specific differentiation of mesothelial cell colonies was induced through the addition of growth factors in specific media.

MATERIALS AND METHODS

Screening of cell populations within the peritoneal fluid by flow cytometry, immunohistochemistry and reverse transcriptase polymerase chain reaction: Peritoneal dialysis fluid was attained from early stage male patients undergoing early stages of peritoneal dialysis from Imam Reza Hospital of Mashhad University of Medical Sciences. Patients showed no clinical symptoms of peritonitis. Throughout peritoneal dialysis a catheter is inserted between visceral and parietal membranes of the peritoneum. Cells were centrifuged at 9000 rpm for 10 min and then cultured in DMEM, 15% FCS (Biosera), 100 U mL^{-1} of Penicillin and 100 μ mL^{-1} of Streptomycin (Gibco).

Having discarded the non adherent blood cells after one subculture, adherent cells were trypsinized and centrifuged. Cells were washed in PBS and blocked with 5% goat serum for 30 min at room temperature. Excess blocker was washed with PBS. Mouse anti human HBME-1 monoclonal primary antibody (ab2383; Abcam, UK), CD34 (ab6330; Abcam, UK.), CD31 (ab218; Abcam, UK.), WT-1 (ab3236; Abcam, UK.), CD90 (ab11155, Abcam, UK.) were used in separate experiments diluted to 1:200 and incubated for 1 h at room temperature. FITC-conjugated goat anti mouse polyclonal secondary antibody (1: 1000) (ab6785; Abcam, UK.) was incubated for 30 min at room temperature. Cells were analyzed by fluorescent microscopy (Axioskop 2 plus, Zeis) and flow cytometry (Becton Dickinson, FACSCalibur).

Total RNA was extracted by TRIzol kit (Invitrogen, Basel, Switzerland). cDNA was synthesized from total RNA by usimg RT universal reverse transcriptase with random hexamer primers. Cells were screened for the expression of CD34 and CD38 markers. Five microliter of randomly amplified cDNA was used as template for each reaction. CD34 expression was analyzed by the amplification of 5 μL template plus 5 μL of PCR buffer 10X, 2.5 μL $MgCl^2$ 50 mM, 1.5 μL dNTP 10 Mm, 1 μL Taq polymerase, 1 μL (10 pmol) of each primer 5'-ATGGCTTCCTCCTCCCTCCT-3'; 5' ATCCCTGCTCAACCCCTCTG-3'. Thirty microliter of d H_2O was added to make a final volume of 50 μL. Thirty two cycles of 30 sec at 95°C, 30 sec at 64°C and 45 sec at 72°C were sufficient to amplify a 190 bp segment. DNA was visualized by Ethidium Bromide. CD38 was amplified by using 5 μL template, 1 μL Taq polymerase, 5 μL PCR buffer 10X, 1 μL dNTP, 3 μL $MgCl^2$ and 1 μL of each primer 5'-ACCCCGCCTGGAGCCCTATG-3'; 5'-GCTAAAACAACCACAGCGACTGG-3'. With 30 sec at 95°C, 30 sec at 65.5°C and 45 sec at 72°C for 31 cycles, a 150 bp segment was amplified (Cheng *et al*., 1996).

Isolation and characterization of mesothelial cell colonies: Standard clonogenicity assay procedures were followed (Zhang *et al*., 2006). Briefly, for colony isolation, subconfluent cultures were harvested and seeded in 96 well plates at density ranging from 10-200 cells. Adherent colonies formed were trysinized and stained for CD34 and HBME-1 markers analyzed by flow cytometry. Having in hand the putative cell surface marker of human mesothelial colony marker we made an attempt to isolate

mesothelial colonies by fluorescent activated cell sorter starting with colonies formed in the abovementioned manner. Preparation of cells for isolation by FACS was preformed as mentioned previously. Flow cytometry analysis was also preformed to validate the purity of isolated cells. Clonal cell viability was determined by Trypan Blue staining.

Differentiation of mesothelial cells colonies: Isolated colonies were transferred to differentiation media consisting of DMEM-F12, LGlutamine medium (Gibco), 15% human ES certified knockout serum (Gibco), 1% nonessential amino acids (Gibco), 1% Glutamine 1X (Gibco) human recombinant nerve growth factor (hr NGF) 3 ng mL^{-1} (Sigma-Aldrich) and 100 ng mL^{-1} of retinoic acid (Sigma-Aldrich) were used for differentiation towards neurons.

Differentiated cells were stained for lineage specific markers using antibodies (1: 200) of tubulin III (ab6330; Abcam, UK), neurofilament (ab11155; Abcam, UK), or nestin (ab22035; Abcam, UK) following the abovementioned protocol.

Total protein was extracted using protein extracted buffer (600 mM KCl, 20 mM Tris-HCl and 1% triton X-100). Cyrotubes were dropped in nitrogen tank and consequently thawed (2X). Sampled were run for 3 h (200 V, 30 mA) in a 10% polyacrilamide gel. Gel was cut and transferred onto a strip of PVDF paper by blotting for 15 min at 300 V, 50 mA using blotting buffer. Blotted paper was blocked overnight in 5% BSA and stained with primary anti human α-Tubulin III antibody (1: 500) for 1 h, secondary-Biotin anti mouse poly valent (1: 1000) for 2 h and avidin-HRP (1: 1000) for 1 h at room temperature. Mouse brain neurons were used as positive control.

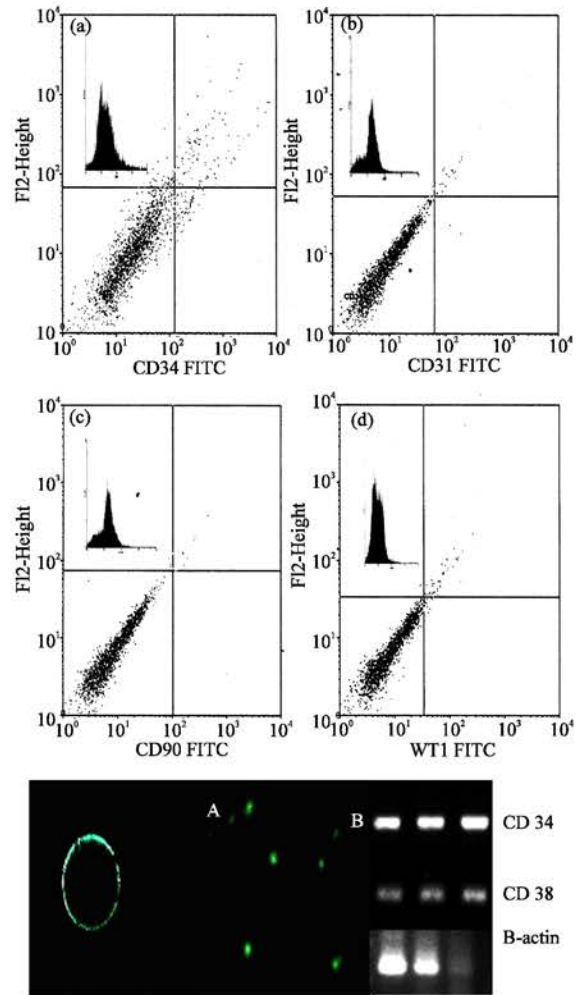

Fig. 1: Screening for cell populations of the peritoneum fluid: flow cytometry of cell populations show a CD34 (a) positive population, CD31, CD90 and WT-1 represent minor populations (b-d). HBME-1 is also positive which is a dominant marker of mesothelial cell line (A). Fluorescent probe was directed to Y chromosome to create a counterstain (B). RT-PCR bands for CD34 (200 bp) and CD38 (150 bp) RNA expression, respectively. B-actin was used as an internal control

RESULTS

Immunocytochemistry and flow cytometry analysis of mesothelial cell populations: Membrane HBME-1, a marker of thyroid tumor cells is expressed in mesothelial cell line. The majority of mesothelial cells express this membrane marker. CD34 positive cells represent 24.3% and CD31 and WT-1 represent only 1-3% of the mesothelial cell population. CD90 positive cells were insignificant. Fibroblast cells were used as negative controls. Dot plot and histogram curves were produced from gated cells from side scatter versus forward scatter curves. FITC and height-FL2 were selected as X and Y axes for dot plots and Evens versus FITC were selected for histograms (Fig. 1).

The expression of CD38 and CD34 was detected by semi quantitative RT-PCR. The amplification of CD34 of 90 bp reveals the expression of the mutual mesothelial hematopoietic marker by mesothelial cell population. CD38 the marker of late, committed hematopoietic progenitor cells was confirmed by the amplification of CD38 cDNA of 150 bp.

Mesothelial colony isolation and characterization: Mesothelial cells colonies isolated by culture and FACS using CD34 and HBME-1 markers by culture and FACS. Isolation efficacy of each system was estimated to be 99%. Figure 2 demonstrates the isolated colonies, gating of CD34 and HBME-1 positive cells and thereafter isolation and purification of them and consequently flow

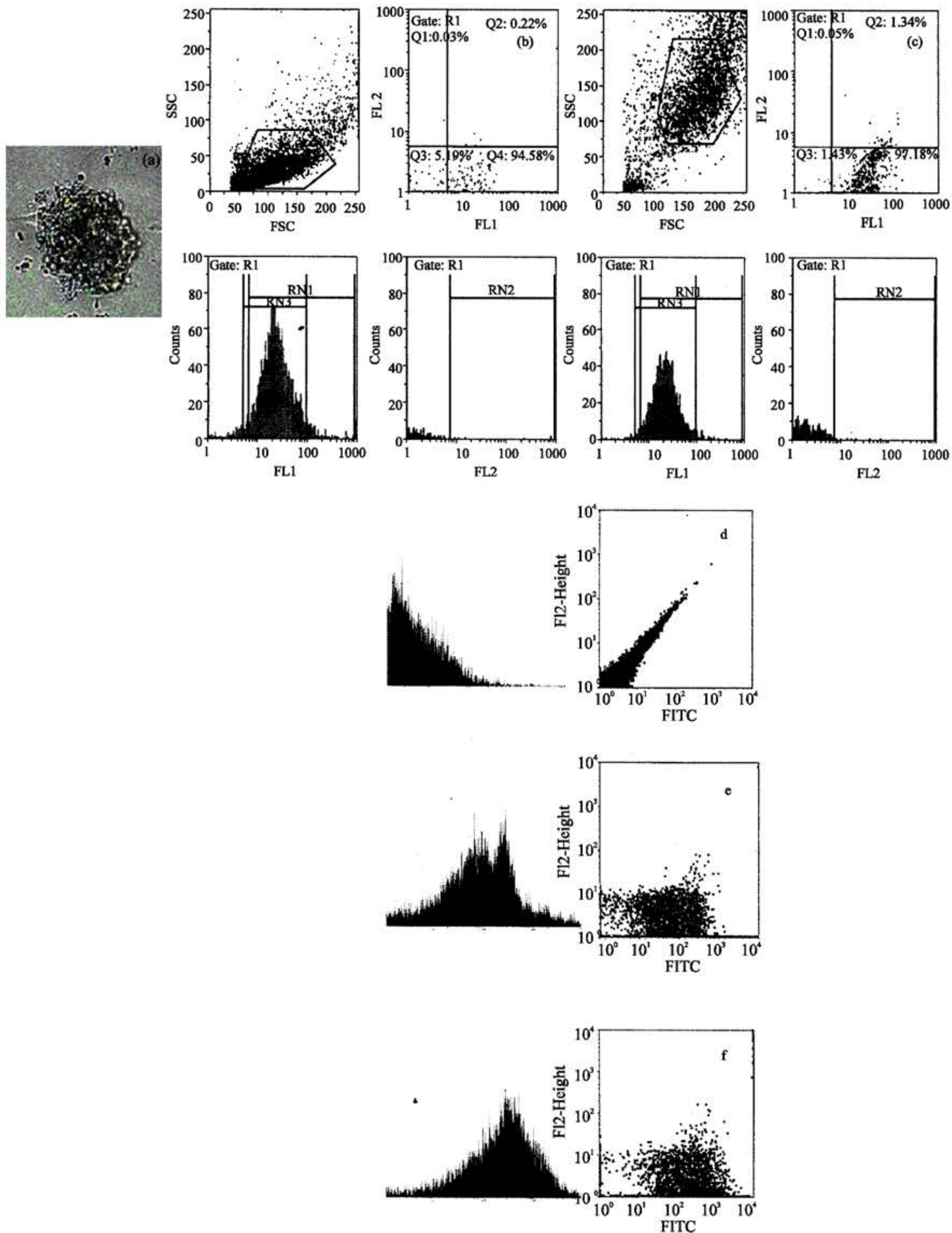

Fig. 2: Isolation and characterization of mesothelial colonies: isolation of mesothelial colonies was accomplished by culture (a), FACS mediated by HBME-1 (b), CD34 (c) directed FITC conjugated antibody. Efficacy of cell isolation by both methods was validated by flow cytometry which revealed a 99% purity of isolated cells. Histogram and dot plot of negative control (d), isolated colonies of mesothelial cells expressing HBME-1 (e) and CD34 (f)

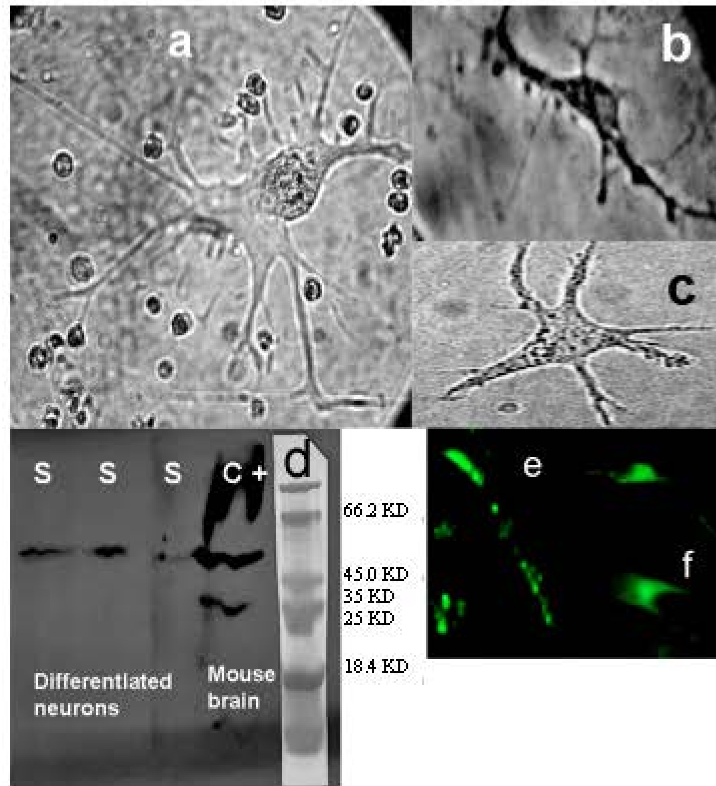

Fig. 3: Differentiation towards nonmesodermal lineages by colonies of mesothelial cells: neuron like cells resulting from neural induction of mesothelial HBME-1 positive cell clones (a-c). Tubullin-βIII (50 KD) protein expression by mentioned cells testified by Western blotting. Lanes of samples and one lane of mouse brain neurons as positive control were loaded followed by a molecular weight ladder from protein transfer stage from left to right, respectively (d). Differentiation of mesothelial cells to neurons verified by the expression of surface markers. To mention: neurofilament high molecular weight (200) (e) and Nestin (f)

cytometry curve revealing dot plot and density plot curves suggesting a high efficiency of isolation. Trypan Blue staining also suggests viability for mesothelial colonies.

Study of differentiated cells resulting from mesothelial colonies: Colonies isolated by FACS were transferred to neuron-specific media comprised of knockout-DMEM, FCS, L-Glutamine, nonessential amino acids plus human recombinant nerve growth factor and of Retinoic Acid. Neuron specific cell differentiation was apparent 24-48 h afterwards as the neutrites started to elongate form exons. Next, the other processes started to grow and develop into cell dendrites. Newly differentiated neurons became more morphologically typical after a few days in neuron specific culture in which axon and dendrite formation was evident. Differentiated neurons survived one to two weeks in culture, which consequently underwent apoptosis due to the lack of appropriate environmental stimulation or cell to cell signaling which is marked by the secretion of survival factors by astrocytes *in vivo* or in co-cultures (Tomita *et al*., 2006; Buzanska *et al*., 2006; De Hopp *et al*., 1998; Sun *et al*., 2005; Yoshida *et al*., 2006). Figure 3 demonstrates the formation of neurons from epithelial shaped mesothelial cells characterized by the formation of axons and dendrites.

Differentiated cells expressed neuron specific cell surface markers such as: Tubulin III and Nestin which are immature neuron markers. Cells also expressed late structural neuronal markers such as neurofilament 200. Therefore, in addition to morphology studies, cell surface staining also confirms neuronal differentiation. Western blotting was also positive for the expression of Tubulin III protein by the differentiated cells. Figure 3 demonstrates the positive control which in this case was mouse brain neurons in comparison with differentiated neurons which

all expressed levels of α-Tubulin III with a molecular weight of 50 kD which is obvious in the figure.

DISCUSSION

Adult somatic stem cells possess extensive self-renewal capacity, as their primary role is to replenish aged and functionally impaired tissues (Pettinger *et al.*, 1999). In this study, mesothelial cells from the effluent of patients undergoing early stage peritoneal dialysis were used for characterization and *in vitro* differentiation studies. Patients showed no clinical symptoms of peritonitis or fibrosis of the peritoneum. Peritoneal effluents of early stage peritoneal dialysis subjects dominantly retain mesothelial cells. Cell populations of the peritoneum dominantly expressed HBME-1 and CD34 and which the later is apparently reminiscent of cell differentiation state. An insignificant population of CD90, WT-1 and CD31 cells were also detected. We isolated colonies from the peritoneum fluid cell populations by culture. In order to verify the identity of the colonies and judging by dominant markers resulting from the aforementioned cell screening, the given colonies also isolated by FACS proved to be mesothelial cells expressing HBME-1 and CD34. The efficiency of isolation by culture and FACS were calculated to be more than 98%. A recent report suggests that mesothelial stem cells express the same markers as mesothelial cells (Yung *et al.*, 2006).

Having characterized and isolated mesothelial colonies, HBME-1 positive cells were transferred to specific media for differentiation. Specific differentiation of mesothelial colonies to neurons was dominantly induced by retinoic acid and human recombinant nerve growth factor. Evidence supporting differentiation of mesothelial progenitor cells to any of the given lineages within or beyond its embryonic developmental layer is still lacking in previous works. Considering the developmental point of view, the mesothelium is an epithelial that derives from the mesoderm rather than the ectoderm. The epithelial mesenchymal transition of these cells in states of continuous culture or peritoneal dialysis confirms the mesodermal origin of these cells. What is obvious is that hemangioblasts are the common progenitors of hematopoietic cell lineages and endothelial originates from the embryonic splanchnic mesoderm (Munoz Chapuli *et al.*, 1999). In the present study, CD34 marker which is specifically expressed by the progenitors of the endothelial and hematopoeitic cells was especially emphesized. The mutual expression of CD34 by mesothelial cells lineages confirms this theory. The CD38 population was most likely among the nonadhering population of the cells. The detection of CD38 suggests the contamination of late blood derived hematopoietic progenitor cells, as the existence of circulating CD34/38 positive progenitor cells from the bone marrow has been reported (Aiuti *et al.*, 1998; Moore *et al.*, 1997). Bone marrow mesenchymal stem cells can differentiate into several types of mesenchymal cells, including osteocytes, chondrocytes and adipocytes, but, under appropriate experimental conditions, can also differentiate into nonmesenchymal derived cells for instance, neural cells (Wislet-Gendebien *et al.*, 2005; Guo *et al.*, 2005; Kohyama *et al.*, 2001). The formation of neurons from mesothelial cells confirmed by immunostaining studies suggests a multipotent plasticity of mesothelial cells which violates the previously assumed potential of differentiation of these cells which described them as progenitor cells.

What is apparent is that populations of multipotent colony forming stem cells reside in the peritoneum from mesothelial origin that retain their undifferentiated state *in vivo* probably in an undefined microenvironment, but demonstrate plasticity in regenerating the peritoneum in states of injury and trauma, as pervious studies reveal a considerable proliferation capacity among mesothelial cells (Herrick and Mutsaers, 2004). What is obvious from this study is that mesothelial cells retain a multipotent capacity of differentiation to further than mesodermal-derived cell lines. The transdifferentiation of mesothelial cells to neurons could be testified in clinical studies and electrophysiological work, in which the functionality of resulting neurons can be evaluated. As obtaining mesothelial cells from the peritoneum is more clinically feasible and less invasive than bone marrow aspiration this source of stem cells could be an alternative source rather than bone marrow stem cells. The further capacity of mesothelial cells in differentiation to other cell lines should also be investigated. 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. Thus, the definition of a novelle resource of adult stem cells definitely renders a massive impact on regenerative biomedicine. Future work will address the electrophysiological and clinical study of these neurons. Complete insight into the neuron functionality and microenvironmental manipulation of the given cells should be performed prior to clinical implications. Furthermore, the full differentiation potential of mesothelial stem cells will also be challenged in further *in vitro* differentiation studies.

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