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Research Article

Neuronal differentiation of mouse amnion membrane derived stem cells in response to neonatal brain medium.

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Abstract

Amniotic membrane derived stem cells have considerable advantages to use in regenerative medicine and their anti-inflammatory effects, growth factor secretion and differentiation potential, make them suitable candidates for stem cell therapy of nervous system. The developing and neonatal brain contains a spectrum of growth factors to direct development of endogenous and donor cells. Using an *in vitro* model, we investigated the plasticity and potential of mouse amnion membrane stem cells to differentiate into neural cells in response to neonatal mice brain medium. Mouse amniotic membrane stem cells were isolated from embryonic membrane and confirmed by flowcytometric analysis of their surface markers. Co-culture of harvested stem cells and neonatal brain derived medium was conducted for 21 days and neural differentiation of cells was explored using immunohistochemistry and flowcytometry analyses. Isolated amnion membrane stem cells showed high rate of viability and proliferation and also expressed stem cell markers such CD105 and CD90. Amnion stem cells presented neural characters such morphological changes and development of axon like appendixes as well as *Nestin* and *Map-2* expression when neighbored with brain tissue. In conclusion, the current investigation showed that amnion membrane derived stem cells are potent cells for responding to environmental signals promoting them to neural fate and could be used in regenerative medicine of neurodegenerative disorders.

***Keywords*:** fetal tissue, neuron, regeneration, stem cell

Introduction

The amniotic membrane is a fetal derived tissue, which composes of three layers: a single epithelial layer, a thick basement membrane, and an avascular mesenchymal layer (Dobreva et al., 2010). Subpopulations of stem cells exist in both the epithelial and mesenchymal layers, which term as amniotic epithelial cells (AECs) and amniotic mesenchymal cells (AMCs) (Toda et al., 2007). Amniotic membrane stem cells (AMSCs) have considerable advantages with regard to cellular therapy; they easily obtain from fetal placenta and their isolation lack any ethical problems, also millions of them can be harvested from each term amniotic membrane, which is important to prevent potential immune responses arising from cells of several unrelated donors (Pratama et al., 2011). Amniotic membrane derived stem cells don't express HLA class I and II and have low risk of rejection in transplantation procedures (Akle et al., 1981; Banas et al., 2008). They are not tumorogenic because of low telomerase activity (Miki et al., 2005) and do not form teratomas upon transplantation into the testes of SCID mice (Ilancheran et al., 2007). Amniotic membrane cells express pluripotency markers such as Oct-4, Sox-2 and Nanog (Izumi et al., 2009), also they are positively expressed mesenchymal stem cells markers, such as CD105 and CD90, and negatively expressed hematopoietic markers, such as CD45 (Kim et al., 2014). AMSCs have capacity to differentiate into three germ layer cells such as myocytes, osteocytes, adipocytes, pancreatic cells, hepatocytes, as well, as well as neural and glial cells (Broughton et al., 2012; Toda et al., 2007).

A developing and neonatal brain contains various growth factors that direct development of endogenous cells and have similar effects on donor cells in transplantation studies; in fact, neural and non-neural stem cell populations are capable of responding to this complex environment, to differentiate into neural cell types (Marcus et al.,

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2008a). This suggests that a developing brain could be considered as neuronal environment to assess the plasticity and function of stem cells.

Using an *in vitro* model, we investigated the plasticity and potential of mouse AMSCs to differentiate into neural cells in response to neonatal mice brain medium (mBM) and the aim of this investigation was to explore the effects of brain environment on the fate of amnion derived stem cells.

Materials and Methods

Cell isolation and primary culture

Mouse amniotic membrane stem cells isolation and culture were performed as described previously (Marcus et al., 2008b). Briefly, amniotic membranes were collected from 13-16 days old NMRI strain mouse embryos in sterile condition and after cutting into small pieces, were digested twice in 0.05% trypsin-EDTA (Gibco, UK) for 10 minutes at 37°C. Following trypsin inactivation with fetal bovine serum (FBS) (Gibco, UK), centrifuge was performed at 1500 rpm for 5 minutes and cell pellet was transferred to culture plates containing Modified Eagle's Medium (DMEM), (Sigma-Aldrich, USA), 10% FBS and 1% Streptomycin-penicillin (Gibco, UK) in a humidified atmosphere of 95% and 5% CO_2 at 37 ^oC.

Preparation of mouse brain medium

Mouse brain medium was prepared from neonatal mice. Briefly, 1-2 old days animals were anesthetized in CO_2 chamber under sterile condition and each brain was homogenized to obtain 1ml mBM. The brains were discarded using thin surgical forceps and then transferred to cold PBS (Gibco, UK). After 3 times washing, tissues were cut and homogenized in DMEM medium and centrifuged at 1500 rpm for 20 minutes. Supernatant was collected and was filtered using 0.2 µm filters and kept at -70 °C. All concerns of animal welfare were considered during mating and surgery.

Treatment of AMSCs with mBM and neural differentiation analysis

Amniotic membrane derived stem cells were counted using tripan blue (Gibco, UK) and 10^4 cells/ml were plated in 24 well cell culture plates. To eliminate the serum effects on neural differentiation, FBS concentration reduced to 1%. Twenty-four hours after cell seeding, the cell culture media was replaced with fresh medium containing 50-300 µl/ml of brain derived medium; experiments were lasted for 21 days and medium was changed every 3 days. Neural differentiation was investigated in 7^{th} and 21^{th} days, on the basis of morphological and immunological properties.

Immunocytochemistry analysis

The cells were fixed using 4% paraformaldehyde for 30 minutes, permeabilized with Triton X-100 (0.4%) for 10 minutes, blocked with 5% goat serum (Gibco, UK) and processed for immunocytochemistry using primary anti-mouse Map-2 antibody produced in rabbit (Abcam, USA). Goat anti-rabbit FITC conjugated antibody (Abcam, UK) was used as secondary antibody and cell nuclei were labeled with Propidium iodide (PI) (Sigma-Aldrich, USA).

Flowcytometric surface marker expression analysis

For flow cytometry analysis, the cells were stained with a specific antibody, as described previously (Park et al., 2012). In brief, cultured AMSCs were harvested using 0.25% trypsin/EDTA, and then washed with PBS and permabilized with Triton X-100 for 30 minutes. Blocking was done with goat serum for 20 minutes, and then the cells were incubated with mouse anti CD90, CD105, CD45 (all from eBioscience, USA) and Nestin primary antibodies (Abcam, USA) for overnight. After washing with PBS, cells were incubated secondary antibody for 45 minutes. Fluorescein-activated cell sorting (FACS) was performed to measure the percentage of antigen expressing cells both in control and treated samples.

Statistical analysis

All experiments were performed at least for three times and the statistical analysis was performed using SPSS.16 software.

Results

Cell harvesting from amnion membrane

Amniotic membranes, isolated from 13-16 days old embryos, were used for stem cell isolation and compared to membranes isolated from 17-19 days embryos. Cell counting showed that membrane from younger embryo was a more appropriate source for cell harvesting and the amount of isolated cells was significantly higher in comparison to the older embryos. Measurement of the viability rate of isolated cells with trypan blue staining showed that 92% of the cells were viable 3 days after enzymatic cell isolation; indicating non-damaging procedure for cell isolation.

Morphological analysis under light microscope showed the presence of 2 types of cells, which was related to the presence of both epithelial and mesenchymal stem cells in amniotic membrane (Figure 1).

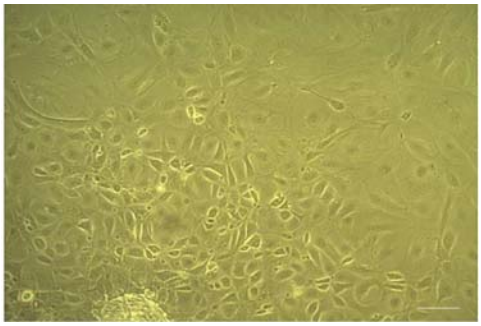

Figure 1. Stem cells isolated from mouse amniotic membrane. Both epithelial and mesenchymal like cells are present in the cell population derived from amniotic membrane. Observation under light invert microscope (200 ×).

Characterization of amnion membrane derived stem cells

To characterize the amniotic stem cells specific surface markers, flowcytometry was performed and results showed 95.71% of CD105, 88.17% of CD90 (Figures 2A and 2B) and low rate expression of the hematopoietic marker CD45 (Figure 2C).

Effect of 150-300 µl/ml of BEM on AMSCs

Treatment of AMSCs with 150, 200 and 300 µl/ml of mBM showed high concentration of medium had cytotoxic effects on AMSCs. Observation of mBM treated AMSCs under an invert microscope showed their cell death after mBM addition to the cell culture medium especially at 300 and 200 µl/ml concentrations. Although the cell death rate in 150 µl/ml of mBM was lower in comparison to higher doses, any neural differentiation was not obvious through 21 days of 150 µl/ml mBM treatment (Figure 3).

Effect of 50 µl/ml of mBM on AMSCs

Investigation of AMSCs treated with 50 µl/ml of mBM showed this concentration of mBM had significant effect on neural fate of the cells. Results from flowcytometry analysis showed decline in Nestin expression in AMSCs, 88.52% and 11.5% in third and seventh days after treatment, respectively (Figure 4).

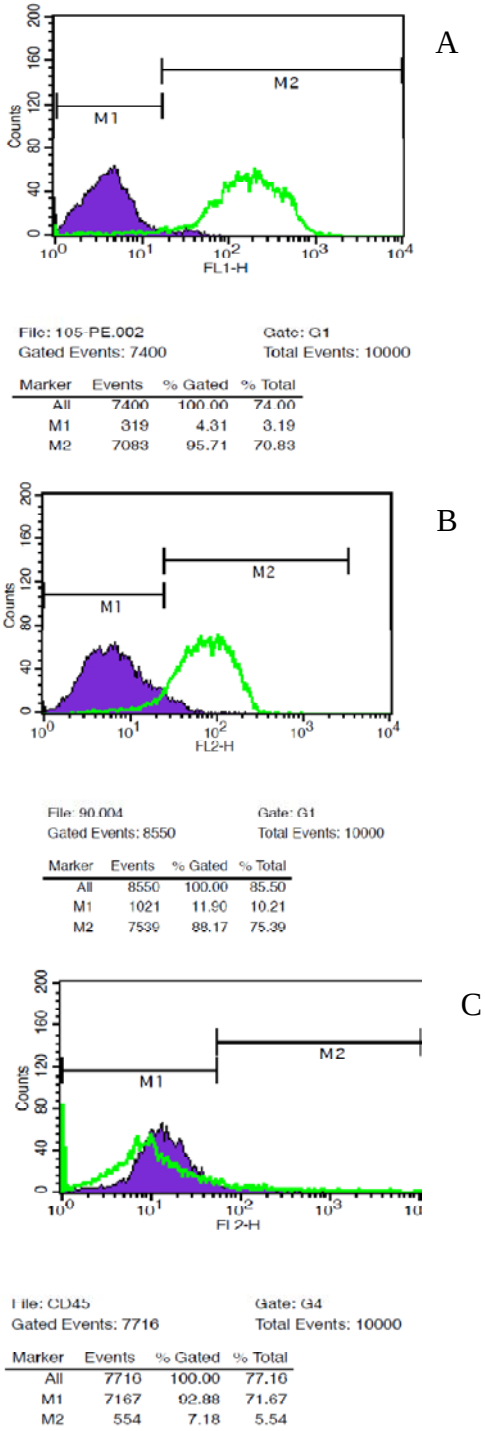

Figure 2. Flowcytometry analysis showing expression of A) CD105, B) CD90 and C) CD45 cell surface markers on AMSCs.

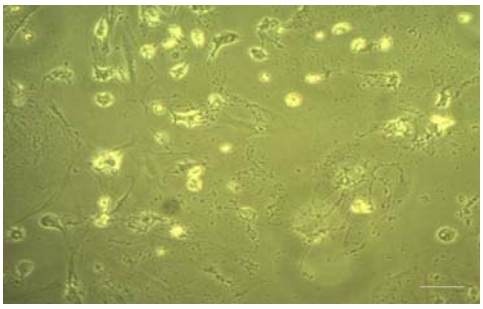

Figure 3. Cytotoxic effect of high dose of brain derived medium on amniotic stem cells. AMSCs underwent broad cell lysis under treatment with 150-300 µl/ml mBM. Observation under light invert microscope (200 ×).

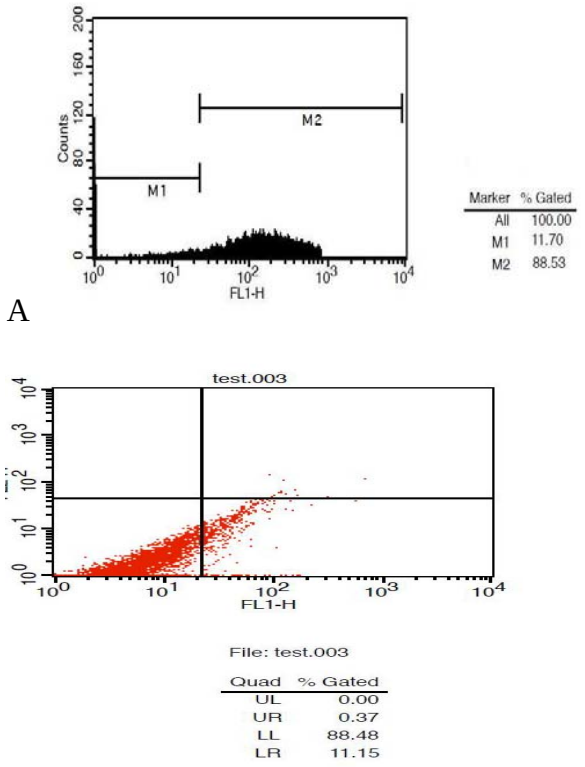

Figure 4. Flowcytometry analysis showing expression of Nestin in AMSCs. Nestin expression was measured 3 (A-M2) and 7 (B- LR) days after neural differentiation of AMSCs which represented 88.53% and 11.15% of Nestin expression in cells, respectively.

In addition to changes in Nestin expression, 7 days after mBM treatment, AMSCs started to neural related morphological changes and axon like process developed gradually. Twenty-one days after treatment, neuronal related changes of AMSCs was significant in comparison to untreated AMSCs that was accompanied with high rate of Map-2 expression on the basis of results obtained from immunohistochemistry analysis (Figiures 5 and 6).

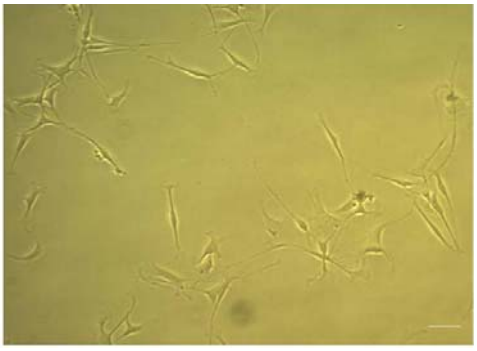

Figure 5. Differentiation of AMSCs toward neural cells in the presence of BEM. Neural related changes such long bipolar or multipolar process and some cell to cell contacts are evident in figure. Observation under light invert microscope (200 ×).

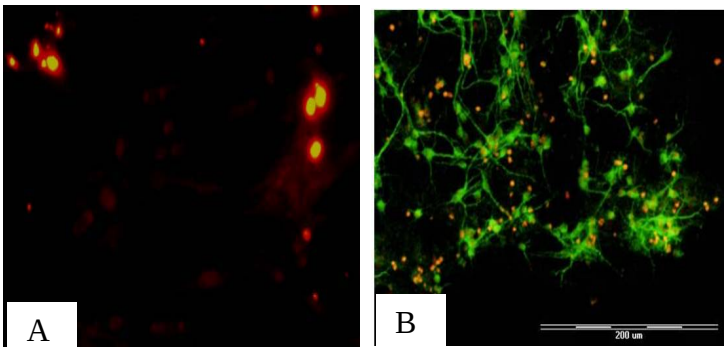

Figure 6. Immunoflurescent assay representing the negative Map-2 expression in A (undifferentiated AMSCs, in the absence of mBM), and positive Map-2 expression in B (differentiated AMSCs, in the presence of mBM). Map-2 expression is obvious in stained cells (green) and nuclei are stained with PI (red). Observation under immunofleurecent microscope.

Discussion

Amniotic membrane derived stem cells have characteristics of neural cells and express some markers for neuronal and glial cells (Toda et al., 2007). Amnion membrane cells differentiate into functional neurons in spinal cord injury models (Sankar and Muthusamy, 2003). Intra-cerebral grafting of amnion derived cells in a mouse model of Parkinson' disease showed that human amniotic cells had an advantage to use in Parkinson' disease because they synthesize and release dopamine, acetylcholine, catecholamine and neurotrophic factors, such as nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor (Kakishita et al., 2000; Sakuragawa et al., 1997). Amniotic mesenchymal cells exhibited the phenotype of neuroglial progenitor cell and when subjected to a

neural cell differentiating protocol, extended long bipolar or multipolar processes (Sakuragawa et al., 2004).

Although brain assumed as impotent tissue for regeneration, it provides a neurogenic cues, influencing the fate of inherent or extrinsic cells. Several studies have showed inductive effects of developing or adult brain on neural and non-neural stem cells; for example adult bone marrow stromal cells (Kopen et al., 1999) and human umbilical cord blood cells (Zigova et al., 2002), acquired neural and glial fates subsequent to transplantation into the rodent brains. Neural cells transplanted to developing brain of rats populated large areas of the CNS and underwent region specific differentiation and appropriate for their final location (Campbell et al., 1995; Fishell, 1995).

In the current study we investigated the neural differentiation of AMSCs in response to brain tissue as well as the ability of the neonatal brain environment to promote neural fate in mouse amniotic membrane derived stem cells witch obtained results demonstrated the mouse brain medium may contain factors to promote neural fate in AMSCs. Characterization of mouse amniotic membrane derived stem cells demonstrated the expression of amniotic membrane specific markers such CD105 and CD90 and absence of hematopoietic cells specific factor CD45, in a manner conforming to previous studies(Roubelakis et al., 2012). In *in vitro* studies, neural differentiation of stem cells and also the survival of differentiated neurons, depends on the presence of inducing and growth factors (Mao and Lee, 2005) and amniotic membrane stem cells have been differentiated into neural cells in the presence of different neural inducing factors such as bFGF, RA and EGF (Niknejad et al., 2010). In current study all inducing factors were absent in the culture medium of AMSCs and they underwent treatment with pure mBM; moreover, to eliminate the inhibitory effect of serum on neural differentiation (Kawasaki et al., 2000), the serum in the medium was decreased to minimum amount. Conducted co-culture of AMSCs and brain derived medium promoted AMSCs towards morphological changes related to neural fate and developing the axon-like processes. Expression analysis showed early expression of Nestin and its reduction throughout the first days of experiment which was accordance the fact that Nestin supports survival and proliferation of stem/progenitor cells (Park et al., 2010), and upon differentiation becomes dowenregulated (Michalczyk and Ziman, 2005). As in postmitotic terminally differentiated neurons Map-2 is highly enriched in the dendritic compartments and primarily this protein is associated with neurons (Sanchez et al., 2000), we investigated its expression in neural differentiated AMSCs which confirmed last stage of neural development in AMSCs cultured in vicinity of brain medium. These results were comparable to the effects of inducing factors on the neuronal differentiation of amniotic membrane derived cells (Tamagawa et al., 2008).

Results from different studies on rodent brains have showed that early in postnatal life, the brain continues to undergo extensive development of different growth and neurotrophic factors such as fibroblast growth factors, BDNF, NT-3, and NGF which are important self-renewal or inducing factors for neural development (Friedman et al., 1991; Kopen et al., 1999; Maisonpierre et al., 1990). Neural related changes in AMSCs co-cultured with mBM may be explained with the presence of such factors which influenced on their fate and promoted them towards neural development. Though AMSCs synthesize and release some neurotrophic factors such as nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor (Kong et al., 2008), which could have self-inducing effects on them, non neural differentiation related changes observed in mBM untreated AMSCs which declines the hypothesis of differentiation of AMSCs because of their self- produced factors.

Conclusion

In conclusion, results from this study showed the amniotic membrane derived stem cells are potent to respond to environmental signals promoting them to neural fate. AMSCs are easily isolated from the amnion membrane and provide an accessible source of autologous cells for transplantation approaches, so they could be useful vehicles for treating a variety of central nervous system to replace damaged cells in response to proper intra-tissue factors. Anti- inflammatory effects, growth factor secretion and differentiation potential are characters that make them suitable candidates for stem cell therapy of the nervous system.

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Conflict of Interests

The authors declare no any conflict of interest.

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