行政院國家科學委員會專題研究計畫　期中進度報告

探討以人類胎盤間質幹細胞治療神經退化性疾病動物模式之可行性研究(1/3)

計畫類別： 個別型計畫
計畫編號： NSC94-2314-B-303-008-
執行期間： 94 年 08 月 01 日至 95 年 07 月 31 日
執行單位： 財團法人佛教慈濟綜合醫院神經科

計畫主持人： 林欣榮
共同主持人： 徐偉成

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（一）中文摘要：

關鍵詞：人類胎盤間質幹細胞 (pMSCs)，神經再生，神經細胞及血管分化，腦中風，巴金森病，神經再生因子

目前針對治療神經系統退化性疾病還是沒有非常有效之方法，例如急性腦中風目前是十大死亡原中之第二位，然而腦中風之主要原因為腦中不同部位之血管栓塞或硬化阻塞造成進而產生突發性肢體偏癱，失語，手腳麻木等不同程度之神經功能喪失，目前之治療方式在急性期施以抗凝血劑，抗血小板凝集劑，甚至血栓溶解劑之藥物治療，其他之慢性神經系統退化性疾病如巴金森病及杭丁頓舞蹈症，則以復健及其他預防性之保守療法為主，疾病之預後不佳，多半為行動不便須家人照料，造成社會及國家資源之耗損。腦中風造成神經細胞快速死亡，而死亡之神經細胞及血管肉皮細胞無法再生及修補因此神經功能便喪失，因此，近年來有許多學者研究在中風之動物身上植入外來之幹細胞（Stem cell），例如有骨髓中胚層幹細胞，臍帶血幹細胞甚至是胚胎幹細胞等，每位學者都發現不僅能讓中風鼠之神經功能部份回復；且植入之幹細胞可分化成神經細胞及血管內皮細胞，此種治療方式對未來腦中風及神經退化性疾病帶來一線曙光。

在許多文獻中都證實在幹細胞可在體內或體外分化為成熟之細胞例如肌肉細胞、肝臟細胞及神經細胞等，因此在骨髓幹細胞及臍帶血幹細胞慢慢被大多數人注意且被大量的運用於治療相關疾病之際，慢慢發現因老年人之骨髓較不易培養出幹細胞，而臍帶血之幹細胞數量過少，故極需要尋找另一間質幹細胞之來源，然而胎盤組織可培養出很多代之間質幹細胞，因此可想而知的是對於治療神經系統退化性疾病應可提共一使神經細胞復原及再生的好方法之一。本研究將分三年進行。

第一年將建立人類胎盤間質幹細胞體外培養模式，所須之工作包括收集胎盤組織分離血管並剪碎處理後置入細胞培養皿中加以培養，在進行 Proliferation stage 時培養基中加入含 EGF 及 FBS 等物質，待胎盤間質幹細胞之數量到達一定程度時，進行 FACS 分析以確定為間質幹細胞，同時作分盤及進行 Differentiation stage，此時培養基中加入含 retinoic acid 及 β-ME 等物質，胎盤間質幹細胞經分化處理後，我們首先觀查其細胞外形之變化，之後再利用免疫細胞染色之方法來觀查這些經分化過後之幹細胞是否表現一些

具神經細胞之物質，包括 MAP-2, Neu-N, Nestin 及 GFAP 等，所以藉此體外培養模式的確可以證實胎盤間質幹細胞經處理後會分化成神經細胞。

(二)研究計畫之背景及目的：

Stem cells are clonogenic cells that have the capacity for self-renewal and multilineage differentiation. During embryogenesis, totipotent embryonic stem cells that are derived from the blastocyst give rise to ectoderm, mesoderm and endoderm lineage cell populations (Weissman, 2000). It has become evident that stem cells persist in adult tissues, although they represent a rare population localized in small niches (Woodbury et al., 2002). Adult stem cells are not totipotent, but they are capable of self-renewal and differentiation into multiple specialized cell types (Kopen et al., 1999). In tissues from postnatal animals, stem cells have been successfully isolated from liver, intestine, bone marrow and brain (Wei et al., 2000). Neural precursors that differentiate into neurons, astrocytes and oligodendrocytes may hold significant therapeutic potential for the replacement of damaged or diseased neural tissue resulting from congenital neuropathological conditions, brain injuries and neurodegenerative disorders. Although regional neurogenesis continues throughout the lifespan of rodents and humans, the number and availability of neural stem cells (NSCs) is limited in the postnatal central nervous system (CNS) (Barami et al., 2001; Shetty and Turner, 1996). The transplantation of NSCs has been shown to provide functional improvement in vivo (Barami et al., 2001; Borlongan et al., 1997; Masada et al., 1997; Shetty and Turner, 1996).

Recent evidence indicates that bone marrow stem cell transplantation effectively prevented the progression of neurological disease signs in some functional studies, if it is performed at an early stage in the disease (Jin et al., 2002). Subpopulations of bone marrow cells may serve as an alternative source of stem cells for the treatment of CNS disease, whereby mesenchymal stem cells differentiate into various lineages of brain cells. It has been shown that cells isolated from both bone marrow and umbilical cord blood (CB) can give rise to neural cells in vitro (Black and Woodbury, 2001; Kohyama et al., 2001; Reyes and Verfaillie, 2001; Deng et al., 2001; Sanchez-Ramos et al., 2000; Sanchez-Ramos et al., 2001; Woodbury et al., 2000; Colter et al., 2000; Colter et al., 2001) and in vivo (Azizi et al., 1998; Kopen et al., 1999; Mezey et al., 2000; Brazelton et al., 2000). Many studies have shown that bone marrow-derived cells can give rise to neural cells as well as many tissue-specific cell phenotypes, including hematopoietic, skeletal muscle, hepatic, heart and vascular endothelial cells (Terskikh et al., 2001; Gussoni et al., 1999; Petersen et al., 1999). The results of these studies have shown that host tissue-specific microenvironment conditions may be essential for the multilineage transdifferentiation of bone marrow-derived stem cells (BMSCs). BMSCs also have been used as vehicles for gene delivery to various tissues including the brain (Ding et al., 1999; Jin et al., 2002; Park et al., 2001; Suzuki et al., 2000; Kang et al., 2003). These findings suggest that bone marrow cells are a potential source of brain progenitor cells and have clinical importance in applications for tissue engineering and also as vehicles for gene therapy.

At present, cells with mesenchymal progeintor characterization were isolated from several mesenchymal tissues such as muscle, bone, cartilage and tendon (Deans et al., 2000; Ralf et al., 2000), and these tissues originated from mesoderm during embryogenesis. Placenta consisted of vessel, mesenchyma and trophocyte derived from extraembryonic mesoderm (Steinborn et al., 1998), which developed into fetal appertaining including placenta, fetal membrane and umbilical cord. Since fetal

appertaining contains a lot of mesenchyma, we have suggested that pluripotent MSCs derived from extraembryonic mesoderm, but not recruited to differentiate, may reside in a quiescent state within the placenta throughout gestation. Our study aimed at isolating and characterizing MSCs in human placenta, which would possibly open a new and rich source of MSCs for experimental and clinical needs.

Because little is known about the biologic, differentiation or engraftment properties of mesenchymal stem cells in higher order animals, we have begun to isolate and characterize the biological properties of these cells from the placenta of human. Macaques share greater biological similarities with humans than most other species with which stem cell research is being conducted, and therefore provide an unmatched opportunity to research diseases that afflict humans.

In this plan, we would develop to isolate and characterize the growth and mesodermal differentiation capabilities of placenta derived-stem cells. Then we attempted to investigate the differentiation potential of these cells along neural lineages in vitro. Finally, we used the cultured placenta-derived MSCs to treat the disease model such as stroke, and Parkinson disease.

(二)研究方法、進行步驟及執行進度 (第一年)

The major purpose of this year was to isolate, culture and characterize the human placenta derived stromal cells (PMSCs).

Isolation and culture of placenta-derived stromal cells (PMSCs)

After informed consent, human placentae were obtained by vaginal delivery or caesarean sections from women with uncomplicated full-term pregnancies. The placenta had the loose chorionic, amniotic sacs and decidua removed, and was extensively washed with phosphate buffered saline (PBS), and then placentae were flushed with perfusate, Iscove's modified Dulbecco's medium (IMDM) supplemented with 12.5 U/ml heparin, 50 U/ml penicillin, 50 mg/ml streptomycin, through the arterial-venous circuit to eliminate tissue residual blood. After that, the placenta was cultivated for 12-24 hours at 20℃, at that time the organ was perfused and soaked with a volume 250 ml of perfusate. Mononucleated cells were isolated from the perfusates by density gradient fractionation (below 1.073 g/mL, Pharmacia, USA), washed and resuspended in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) supplemented with 10% fetal bovine serum (FBS) (StemCell, Canada), and cultured at 37℃ and 5% CO2. Ten days later, individual colonies were collected, isolated, cultured and expanded. When the cultures reached 90% of confluence, cells were recovered by 0.25% trypsin-1 mM EDTA and followed by passages. Furthermore, bone marrow derived MSCs were isolated and cultured according to a previously reported method by Pittenger et al (Pittenger MF, 1999;284:143-147)

Confirmation of mesodermal lineage differentiation of PMSCs

To verify the multipotential differentiation of mesenchymal characteristics of PMSCs, cells were subjected to differentiation in conditions known to induce adipogenic, osteogenic and chondrogenic lineages in human cells. Before culture in the induction medium, cultures were grown to at least 80% confluence. For adipogenic differentiation, PMSCs were induced by passaging cells at a 1:10 dilution in control medium and supplemented 10 ng/ml insulin and 10–9 M dexamethasone. Adipogenic differentiation was visualized by the presence of highly refractive intracellular lipid droplets in phase contrast microscopy or staining by Oil-Red O. To induce osteogenic differentiation, the cultures were fed daily with control medium to which was added 10 mM β-glycerophosphate, 50 ng/ml ascorbic acid and 10–9 M dexamethasone for 3 weeks. Mineralization of the extracellular matrix was visualized by stainingof the cultures with Alizarin Red S (2% w/v Alizarin Red S adjusted to pH 4 using ammonium hydroxide) for 5 minutes at room temperature followed by a wash with water. Chondroblast differentiation was induced by differentiation medium supplemented with 6.25 μg/ml insulin, 10 ng/ml transofrming growth factor β1 (TGF β1) and 50 ng ascorbate-2-phosphate in control medium for 3-4 weeks. After differentiation, the cultures were washed and fixed in 4% paraformaldehyde and stained for glycosaminoglycans using 0.1% Safranin O.

Generation of neurospheres from PMSCs

Undifferentiated PMSCs cultured at high densities spontaneously formed spherical clumps of cells that were isolated in 0.25% trypsin (Invitrogen). We also collected free floating neurospheres that were released from the cell culture surface into the culture media. The spheres of cells were transferred to a Petri dish and cultured in Neurobasal medium (NB) (Invitrogen) supplemented with B27 (Invitrogen), 20 ng/ml bFGF, and 20 ng/ml EGF (Sigma) for 4-7 days. The culture density of the spheroid bodies was maintained at 10-20 cells/cm2 to prevent self aggregation.

In vitro differentiation of PMSCs to neural cells

For neural lineage differentiation, neurospheres derived from PMSCs were layered on PDL-laminin double-coated chamber slide (Lab Tek, Nalge/Nunc). Spheres were cultured and maintained for 10 days in NB media containing only the B27 supplement. During differentiation, 70% of the media was replaced every 4 days. The cells were examined at 10 days after differentiation by immunocytochemistry, western blot and reverse transcription polymerase chain reaction (RT-PCR). All data to be shown are representative of at least three different experiments.

Flow cytometric analysis of surface epitopes

For phenotypic characterization by flow cytometry, undifferentiated PMSCs, PMSCs-derived neurospheres and adherent cells were harvested by trypsinization, washed twice with PBS and suspended at a concentration of 1×106 cell/ml and incubated with antibodies to the following antigens: CD13, CD34, CD45, CD90, CD73, HLA-ABC, HLA-DR, CD14, CD29, CD44, CD105, CD117 (BD, PharMingen) for 20 minutes. For FACS analysis, we used primary antibody directly conjugated with APC, or FITC. Monoclonal antibodies to CD34, CD3, CD4 and CD8 were used to identify cells as hematopoietic. The stained cells were thoroughly washed with two volumes of PBS and fixed in neutralized 2% paraformaldehyde solution. For an isotype control, nonspecific mouse or rabbit IgG (DAKO, Chemicon or Santa Cruz) was substituted for the primary antibody. The labeled cells were

analyzed on a FACScan argon laser cytometer (Becton Dickinson, San Jose, CA).

RT-PCR analysis of total cellular RNA

Before and after neural differentiation of PMSCs, total cellular RNA was isolated with Trizol (Invitrogen) reverse transcribed into first strand cDNA using oligo-dT primer and amplified by 35 cycles (94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute) of PCR using 20 pM of specific primers. PCR amplification was performed using the primer sets. All primer sequences were determined using established human GeneBank sequences for genes indicative of neural lineages or control genes. Duplicate PCR reactions were amplified using primers for GAPDH as a control for assessing PCR efficiency and for subsequent analysis by 1.5% agarose gel electrophoresis. Primer sequences for all the aforementioned genes were described previously.

Quantitative real-time RT-PCR

To assess the efficiency of neural differentiation and compare the levels of expression of brain-derived neurotrophic factor (BDNF) and microtubule associated protein-2 (MAP2ab) expression in differentiated PMSCs, quantification was performed using real-time RT-PCR. Total cellular RNA was isolated using conventional protocol. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and probe (5′FAM and 3′TAMRA) were purchased from Applied Biosystems (Foster, CA). Quantitative real-time RT-PCR was performed using this kit according to the manufacturer and an ABI7700 Prism Sequence Detection System. Primer and probe sequences were designed using Primer Express software (PE-Applied Biosystems, Warrington, UK) using gene sequences obtained from the GeneBank database. All probes are designed with a 5′fluorogenic probe 6-FAM and a 3′quencher TAMRA. The expression of human GAPDH was used to standardize gene expression levels.

Immunocytochemistry and FACS analysis of neural differentiated cells

For analysis of neural differentiation of PMSCs neurospheres, differentiated cells were fixed with 4% paraformaldehyde, and incubated with 10% goat serum to prevent nonspecific antibody binding. The cells were incubated overnight at 4°C with antibodies. For detection of differentiated neuronal or glial cell proteins, we used several species-specific monoclonal antibodies directed against glial acidic fibrillary protein (GFAP) (1:2000, Dako, Carpinteria, CA), MAP2ab (1:250, Sigma, St Louis, MO), nestin (1:250, Sigma), Neu N (1:500, Sigma), NF160 (1:500, Sigma) and myelin basic protein (MBP) (1:250, Chemicon, Temecula, CA). After extensive washing in PBS, the cells were incubated for 30 minutes with FITC or Alexa Fluor 568 conjugated secondary antibodies (1:250, Molecular Probe, Eugene, OR). Controls in which primary antibodies were omitted or replaced with irrelevant IgG resulted in no detectable staining. Specimens were examined using a Leica TCS SP2 laser scanning microscope equipped with three lasers (Leica Microsystems, Exton, PA). Immunocytochemical studies were repeated at least three times. Western blot analysis of differentiated cells Protein extracts were prepared from undifferentiated or differentiated pATSCs by the treatment of lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin and 1 mM sodium orthovanadate. Total protein (30-40 μg/ml) was resolved on 12.5% acrylamide gel and electroblotted onto Polyvinyldiethylfluoride (PVDF) membrane (Amersham). The blot was probed with either mouse anti-nestin (1:500) or mouse anti-MAP2ab antibodies (1:500). Immunoreactive bands were detected using horseradish

peroxidaseconjugated anti-mouse IgG antibodies (Amersham) and visualized by enhanced chemiluminescence (Amersham).

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