行政院及所屬各機關出國報告
（出國類別：進　修）

人類幹細胞體外培養及合併基因療法之應用

服務機關：台北榮民總醫
出 國 人：職　　稱：主 治 醫 師
姓　　名：邱　士　華
出國地區：美國加州
出國日期：91.9.1至91.11.30

報告日期：91年12月31日

系統識別號:C09103385

公 務 出 國 報 告 提 要

頁數: 12　含附件: 否

報告名稱:

人類幹細胞體外培養及合併基因療法之應用

主辦機關:

行政院輔導會臺北榮民總醫院

聯絡人／電話:

／

出國人員:

邱士華　行政院輔導會臺北榮民總醫院　眼科部　主治醫師

出國類別: 進修

出國地區: 美國

出國期間: 民國 91 年 09 月 01 日 -民國 91 年 11 月 30 日

報告日期: 民國 91 年 12 月 31 日

分類號/目: J3／醫療　J3／醫療

關鍵詞:　人類幹細胞體外培養及合併基因療法之應用

內容摘要: 本次三個月短期計劃，主要是前往美國加州希望之城（City of Hope）醫院 / 貝克曼醫學研究中心，史竹支教授實驗室學習人體幹細胞體外培養及組織工程再造之技術。體外幹細胞培養之技術包涵人體血液及骨髓分離出CD34+Lin- 之Hematopoietic stem cell以及 Bone marrow mesechymal stem cell（CD34-CD45R+ Lin-爲主）之純化技術，同時利用流產胚兒胎腦部組織之下腦室周圍（Subventricular Zone; SVZ）培養出人體胎兒腦神經幹細胞（Human fetal brain stem cell）。 同時史教授實驗近年已利用Severe combined immunodeficiency (SCID) mice做爲人體胚胎組織培育之動物模型—SDIC-hu mice model，特別已成功將人類胰島組織可經由此種（動物體內無排斥反應）SCID-hu體內組織再生工程（Tissue engineering），而使得在三個月內可組織增生至30-40倍，同時增生之組織經由免疫單株抗體染色，確定其有成熟分化之胰島小體，並且可分泌出胰島素（Insulin）之功能性蛋白，以及昇糖激素（Glucagon）和Somatostatin之功能性蛋白等。 此外，我們也利用經由人類胰臟胰島管（Pancreatic duct）周圍組織分離出具多型分化之胰島幹細胞，由於全世界科學仍無法確定此類胰島幹細胞（Pancreatic stem cell; PSC）所帶有之特定生物標的（Biomarker），因此我們也利用小鼠反轉錄幹細胞病毒（Murine Stem cell vector）來攜帶Green fluorescence gene（GFP）進入PSC細胞中，並經細胞流速儀純化95%以上PSC-GFP（+）細胞之步驟，再進入SCID mice中以觀察此種PSC-GFP（+）細胞是否可成功分化成人類胰島小體（Pancrease Islet），並具有分泌Insulin、glucagon、somatostatin等蛋白質功能。

本文電子檔已上傳至出國報告資訊網

摘 要

本次三個月短期計劃，主要是前往美國加州希望之城（City of Hope）醫院 / 貝克曼醫學研究中心，史竹支教授實驗室學習人體幹細胞體外培養及組織工程再造之技術。體外幹細胞培養之技術包涵人體血液及骨髓分離出 $CD34^{+}Lin^{-}$ 之 Hematopoietic stem cell 以及 Bone marrow mesechymal stem cell（$CD34^{-}CD45R^{+}$ Lin^{-}為主）之純化技術，同時利用流產胚兒胎腦部組織之下腦室周圍（Subventricular Zone; SVZ）培養出人體胎兒腦神經幹細胞（Human fetal brain stem cell）。 同時史教授實驗近年已利用 Severe combined immunodeficiency (SCID) mice 做為人體胚胎組織培育之動物模型— SDIC-hu mice model，特別已成功將人類胰島組織可經由此種（動物體內無排斥反應）SCID-hu 體內組織再生工程（Tissue engineering），而使得在三個月內可組織增生至 30-40 倍，同時增生之組織經由免疫單株抗體染色，確定其有成熟分化之胰島小體，並且可分泌出胰島素（Insulin）之功能性蛋白，以及昇糖激素（Glucagon）和 Somatostatin 之功能性蛋白等。

此外，我們也利用經由人類胰臟胰島管（Pancreatic duct）周圍組織分離出具多型分化之胰島幹細胞，由於全世界科學仍無法確定此類胰島幹細胞（Pancreatic stem cell; PSC）所帶有之特定生物標的（Biomarker），因此我們也利用小鼠反轉錄幹細胞病毒（Murine Stem cell vector）來攜帶 Green fluorescence gene（GFP）進入 PSC 細胞中，並經細胞流速儀純化 95%以上 PSC-GFP（+）細胞之步驟，再進入 SCID mice

中以觀察此種 PSC-GFP（+）細胞是否可成功分化成人類胰島小體（Pancrease Islet），並具有分泌 Insulin、glucagon、somatostatin 等蛋白質功能。

幹細胞之簡介

幹細胞的簡單定義為能產生人體內所有組織的原始細胞，即具有所謂的"多樣化潛能幹細胞"(Pluripotent Stem Cells)，或多塑性幹細胞 (Multipotent Stem Cells)，主要來自一群未分化的細胞。由於來源的不同而稱之為不同。例如胚胎幹細胞(Embryonic Stem Cells)，神經幹細胞 (Neural stem cell) ， 淋巴幹細胞 (Lymphoid stem cells)或是骨髓幹細胞 (Bone marrow stem cells)。而其中胚胎幹細胞為最早期的幹細胞，所以其分化及可塑性潛能也最高，故也稱為"全能性幹細胞(Totipotent stem cell)"， 胚胎幹細胞可以分化成為骨骼細胞(Bone Marrow Stromal Cells)、淋巴幹細胞和骨髓幹細胞；而淋巴幹細胞可以再進一步分化成為T細胞 (T-cells)與B細胞(B-cells)；而其他的組織特異性幹細胞也可同時進一步分化成為其他組織特殊功能細胞。例如 骨髓幹細胞則可以分化成為血紅細胞 (RBC)、血小板 (Platelets)、嗜中性細胞(Neutrophil)、嗜酸性細胞 (Eosinophil)、嗜鹼性細胞(Basophil)、巨大細胞 (Mast Cells)及巨噬細胞 (Macrophages)等。

目前已可自人體腦組織成功的分離出幹細胞，並且在體外內培養，並促使幹細胞分化成為腦神經元細胞，這項成果預期將可利用幹細胞移植的策略，同時以人工分化的腦神經元細胞，除可代替病患萎縮退化的神經細胞外，也使得研究人員得知特定的藥物，有利於腦神經元細胞的分化，可望應用於新藥的發展。如果上述的計劃能達預期效果，則可望利用患者本身的幹細胞，進行人工培養分化再移植回患者，以

減少體內排斥的問題。

最近已有科學研究人員製造出人體骨骼細胞、皮膚、血管、眼角膜及肝臟，其中製造骨骼細胞已有成功的報導，相信在不久的未來徹底改變醫療的觀念及方式，除了將使無數急需組織或器官移植的病患受惠外，對於重大疾病或傷害的治療將有突破性發現。

研　究　成　果　報　告

視網膜幹細胞體外培養與臨床應用

邱士華

眼科部主治醫師

以往認為神經組織均無再生能力，一旦受傷或退化則其喪失之功能將無法恢復。幹細胞具有自我再生和無限制分裂能力，同時亦可分化成特定組織細胞。 運用幹細胞的特性，將之移植到受損的神經組織中，或許能使功能獲得部分之復原。 視網膜為精細之感光神經組織，一些導致其退化的疾病如色素性視網膜炎、老年性黃斑部退化、青光眼等將使病患的視力嚴重減退。 對於這些疾病的治療，如視網膜移植、基因治療等，至今仍無令人滿意的結果。 神經幹細胞治療能否替這些失明患者帶來一線曙光，則被受期待！

本實驗是將新生大白鼠(Sprague-Dawley rats)眼球內的睫狀體色素邊緣(pigmented ciliary margin)與腦皮質取下，分別置於含有纖維母細胞生長因子(FGF)與基礎上皮生長因子(bEGF)的無血清培養皿中培養。 培養約七天後，來自睫狀體色素邊緣與腦皮質的細胞均形成神經球(neurospheres) 並有不同型態的細胞分化發生。 另外，我們也將成人眼球內的睫狀體色素邊緣取下做相同的培養，也發現神經球的形成；免疫螢光染色並證實有細胞分化的標記表現。我們初步的實驗結果與先前國外的報告相符：視網膜幹細胞位於睫狀體色素邊緣。 雖然咸認神經幹細胞治療極有發展潛力，目前仍有許多瓶頸待克服，例如：如何增加幹細胞培養的數量達到可用來治療之標準、如何調控幹細胞分化的方向、取得

胚胎幹細胞所引起的倫理爭議等等。

關於未來研究方向，首先希望能找出最適合的神經幹細胞培養條件，克服幹細胞數量不足的問題以便為以後的實驗奠定基礎；利用 adeno-associated virus 為載體，試圖將一些螢光標記與生長因子的基因植入神經幹細胞中。 此外，將建立一套視網膜退化的動物實驗模型，把基因改造過的神經幹細胞植入受損的視網膜內，評估其神經復原程度。

Background:

Neural degeneration is the cause of debilitating visual impairment associated with prevalent ocular diseases, such as retinitis pigmentosa (RP), age-related macular degeneration (ARMD), retinal detachment and glaucoma. Retina is a complex, highly differentiated, image-sensing and image-processing nerve structure. In human, the repair of a diseased retina was believed to be impossible because the postembryonic retinal neurogenesis was rare, if any. Transplantation of retinal pigment epithelium, iris pigment cells and even a whole layer of retina tissue have been tested to restore a functional vision in animals and human but all failed [1-4]. Attempts to repopulate the retina with grafted neurons have also been unsuccessful because donor cells prefer not to integrate with those of the host [5]. Gene therapy with growth factors has not been demonstrated to be able to effectively rescue the retina and the intraocular inflammatory reactions caused by virus vectors were concerned, because it could cause further damages of retina and deteriorate the visual defects

[6].

The recent identification and characterization of neural progenitors with stem cell properties has open new avenues that may be useful for treating functional impairments caused by the death of specific neural cell populations [7,8]. Tropepe et al. have reported that adult mammalian retinal stem cells are localized to the pigmented ciliary margin and not to the central and peripheral retinal pigmented epithelium [9]. Cultured neural stem cells may be transplanted to repopulate degenerating retina by differentiating into photoreceptors (cell-replacement therapy) [10,11]. Cultured neural stem cells can also be genetically engineered to synthesize and secrete neuroprotective factors and can be used to rescue degenerating photoreceptors and reconstruct [12,13]. In addition to promoting survival, the genetically modified cells can differentiate into photoreceptors and reconstruct the degenerating retina (Ex vivo gene therapy).

Although several studies support the therapeutic application of neural stem cells, there are some problems to the practical and successful use of neural stem cells [14]. First is the issue of availability of neural stem cells in sufficient quantity for therapeutic purposes. Second, it is not understood whether the extended exposure to the mitogens would lead to genetic changes of neural stem cells. Third, although there is evidence that transplanted cells can differentiate into site-specific cells, details regarding the proportions of grafted cells that remain undifferentiated or that differentiate into some other cell types remain incomplete. Lastly, there are ethical concerns associated with stem cells derived from the embryonic tissue.

For more practical and successful use of neural stem cells in rescuing degenerating retina, further work is necessary to identify the optimal conditions for the maintenance, storage and differentiation of neural stem cells into desirable cell types.

Methods:

(1) Newborn Sprague-Dawley rats were sacrificed and their dissociated cells obtained from brain cortex, neural retina and pigmented ciliary margin of eyes were cultured independently in serum-free media with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Neurospheres formed were not passaged.

(2) Dissociated cells from pigmented ciliary margin of adult human eyes were cultured in the same way. Immunostaining of differentiated cells was performed to demonstrate the presence of markers for different differentiated retinal cells.

Primary results:

(1) With our regimen, neurospheres did developed from both brain cortex and pigmented ciliary margin of newborn rats within seven days (Fig. 1). In addition, some morphological differentiation also existed (Fig. 1). However, neurosphere was not found in neural retinal culture of rats. The retinal stem cells are located in the pigmented ciliary margin, which is compatible with other studies.

(2) The growth rate of retinal stem cells was slower than that of cortical stem cell. Without passage and supplement of growth

factors, the numbers of neurospheres in cultures of retinal stem cells started to diminish gradually after about 6 weeks and neurospheres almost disappeared after 8 weeks.

(3) Neurospheres may form in adult human eyes (Fig. 2) and survived more than 6 months. Immunostaining showed the markers of different differentiated retinal cells (Fig 3).

Future work:

(1) Find out the optimal condition to maintain and expand the population of retinal stem cells. Such as co-culture with RPE cell, addition of activator of cAMP, cGMP and NO.... etc.

(2) Transfect the retinal stem cells with adeno-associated virus carrying the gene of green fluoresxein protein to label the retinal stem cells that would be transplanted into injured retina. It is easier to observe the differentiation and growth pattern of the transplanted retinal stem cells if they were labeled.

(3) Transfect the retinal stem cells with adeno-associated virus carrying the gene of survival factors such as bcl2 (an antiapoptotic factor) for ex vivo gene therapy.

(4) Set up an animal model of retinal neural degeneration by transient retinal ischemia induced by raising the intraocular pressure, which can be considered comparable to an acute glaucoma attack, a central retinal artery occlusion, or an ischemic optic neuropathy. Transplant the genetically modified stem cells into injured retina.

References:

1. Del Cerro M, Humayun MS, Sadda SR, et al. Histologic correlation of human neural retinal transplantation. Invest. Ophthalmol. Vis. Sci. 41(2000)3142-3148.
2. Gouras P, Du J, Gelanze M, et al. Transplantation of photoreceptors labeled with tritiated thymidine into RCS rats. Invest. Ophthalmol. Vis. Sci. 32(1991)1704-1707.
3. Gouras P, Du J, Kjeldbye H, et al. Long-term photoreceptor transplants in dystrophic and normal mouse retina. Invest. Ophthalmol. Vis. Sci. 35(1994)3145-3153.
4. Seiler MJ and Aramant RB. Intact sheets of fetal retina transplanted to restore damaged rat retinas. Invest. Ophthalmol. Vis. Sci. 39(1998)2121-2131.
5. Berson EL and Jakobiec FA. Neural retinal cell transplantation: ideal versus reality. Ophthalmology. 106(1999)445-446.
6. Anand V, Chirmule N, Fersh M. Additional transduction events after subretinal readministration of recombinant adeno-associated virus. Human Gene Therapy. 11(2000)449-457.
7. Gage FH. Mammalian neural stem cells. Science. 287(2000)1433-1438.
8. Anderson DJ. Stem cells and the pattern formation in the nervous system: the possible versus the actual. Neuron. 30(2001)19-35.
9. Tropepe V, Coles BL, Chiasson BJ, et al. Retinal stem cells in the adult mammalian eye. Science 287(2000)2032-2036.

10.Chacko DM, Rogers JA, Turner JE et al. Survival and differentiation of cultured retinal progenitors transplanted in the subretinal space of the rat. Biochem. Biophys. Res. Commun. 268(2000)842-846.

11.Nishida A, Takahashi M, Tanihara H, et al. Incorporation and differentiation of hippocampus-derived neural stem cells transplanted in injured adult rat retina. Invest. Ophthalmol. Vis. Sci. 41(2000)4268-4274.

12.LaVail MM, Yasumura D, Matthes MT, et al. Protection of mouse photoreceptors by survival factors in retinal degeneration. Invset. Ophthalmol. Vis. Sci. 39(1998)592-602.

13.Cayouette M, Behn D, Sendtner M, et al. Intraocular gene transfer of ciliary neurotrophic factor prevents death and increases responsiveness of rod photoreceptors in the retinal degeneration slow mouse. J Neurosci. 18(1998)9283-9293.

14. Ahmad I. Stem cells: new opportunities to treat eye diseases. Invset. Ophthalmol. Vis. Sci. 42(2001)2743-2748.