行政院國家科學委員會專題研究計畫　期中進度報告

以明膠為基底之生醫複合材料作為培養椎核細胞並誘導椎核組織再生之研究(1/2)

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中文摘要

關鍵詞(keywords)：椎核組織再生、明膠生醫材料支架、三度空間椎間盤細胞培養

椎間盤退化及其相關之脊椎病變是導致背痛及醫療支出增加的主要原因之一。儘管絕大部分導致椎間盤退化的病因仍屬未知，已有許多證據指出椎間盤退化始發自椎核部分（nucleus pulposus），椎核部分糖蛋白（proteoglycan）含量的減少使得其含水量下降，而隨著含水量的下降程度逐漸嚴重，椎核組織會變得纖維化，進而損害椎間盤正常傳遞受力的功能。目前對於退化性椎間盤病變的手術治療包括椎間盤切除術及脊椎融合手術，椎間盤切除術對於緩解神經根疾症有不錯的效果，但卻可能導致脊椎運動單元的不穩定，而脊椎融合手術雖有助於椎節受力傳導的改進並限制不穩定椎節的異常活動，但此手術方式在機械力學及臨床研究上均顯示有許多的不盡理想之處。雖然仍有許多研究致力於提高脊椎融合手術的成功率，但有最理想的解決退化性椎間盤病變的方法應爲椎間盤的重建並恢復其功能，因此許多研究則專注於促進椎核組織再生的研究，而移植椎核細胞即爲其中一個頗具潛力的方法，而經由結合生醫複合材料可以增加移植之椎核細胞的數目及活性。

在此計畫的第一年，我們首先利用 Gelatin 和 Chondroitin-6-sulfate 爲基材，運用 0.1% glutaraldehyde 作爲交連劑，研發出一種生醫材料支架，目的在提供一個適合椎間盤細胞貼附、增生並生產細胞外基質的環境。緊接著就此明膠生醫材料支架特性加以探討。由掃瞄式電子顯微鏡的照片中，可以發現此支架大型孔隙的大小平均約 100 ì m，我們預期此一較爲鬆散的結構可以使培養的細胞較易深入支架的中心並可以較爲均勻的分佈其中，而此支架之內的空間可以容納較多的新生細胞外基質堆積。此外，由於椎間盤是人體內最大的無血管組織，因此生醫支架吸收體液的能力對其中培養細胞的養分供給就十分重要。我們針對人體椎核再生所設計的支架大小爲直徑一公分、厚零點二公分的圓盤，此一大小的圓盤狀支架的平均乾重爲 28.6 ± 1.5 mg (26.6-30.8 mg)，飽含水分後的濕重爲 177.9 ± 22.0 mg (136.4-203.8 mg)，我們所設計的支架約可吸收 149.3 ± 21.8 mg (109.1-174.6 mg) 的細胞培養液，約爲本身乾重的 5.2 ± 0.8 倍(4.0-6.2 倍)，期望此含水能力足以應付在生物體內無血管環境的養分供給及代謝廢物的排除。如每一個支架與 1×10^6 個人類椎間盤細胞一起培養兩小時，可以有 $4.40 \pm 0.76 \times 10^5$ 個細胞($3.50\text{-}5.50 \times 10^5$ 個)貼附在支架上。在利用人類的椎間盤細胞與此明膠生醫材料支架三度空間培養的先期實驗中，在兩週及六週的培養後，MTT test 顯示出實驗組的生醫支架有很強的細胞活性表現，並且可在組織切片的染色中發現大量的細胞聚集，顯示此支架對細胞生存及增生有所助益。

根據在計畫第一年研究所得的成果，計畫第二年將進行更深入的實驗，包括大量的使用此明膠生醫材料支架做一系列的椎間盤細胞三度空間培養，以 MTT test 檢驗其培養細胞的活性，測量於支架中由培養的椎間盤細胞所生成的 collagens

及 glycoaminoglycans 以評估其產生細胞外基質的能力，並將進行組織切片及免疫組織化學染色。

英文摘要

關鍵詞：regeneration of nucleus pulposus, gelatin scaffold, 3-dimentional culture of disc cells

Disc degeneration and associated spinal disorders are a leading course of morbidity resulting in substantial pain and increased health cost. Although the cause and pathophysiology of intervertebral disc degeneration are largely unknown, some evidences indicate that disc degeneration can originate in the nucleus pulposus. Progressive decrease of proteoglacan content causes dehydration of the nucleus pulposus. As the degree of dehydration decreases, the tissue becomes more fibrous with a compromised ability to transmit intervertebral forces optimally. Surgical treatments for a symptomatic degenerative disc include disectomy and spinal fusion. Discectomy is effective in relieving radicular pain, but it can lead to instability of the spinal column. Spinal fusion permits load bearing and enables passive restraint to the motion segment. However, the disadvantages of disc arthrodesis are well documented in mechanical tests and clinical studies. Although there are still lots of efforts being made to improve the techniques in disc arthrodesis, the ultimate solution for anterior column support should not be overlooked. It has been long realized that the better solution for treating anterior column instability caused by degenerative disc disease is to re-establish the disc space and the function of a healthy disc. One of the potential methods is enhancement of disc regeneration via transplantation of nucleus pulposus cells. The amount and viability of the transplanted nucleus pulposus cells could be improved by combining with some bioactive substrates and growth factors.

In the first year of this study, we develop a gelatin-based scaffold which aims to provide a suitable environment for attachment and proliferation of disc cells and to help the subsequent production of extra-cellular matrix. This scaffold was made of gelatin powder and chondroitin-6-sulfate and cross-linked by 0.1% glutaraldehyde. Serial studies were conducted to test its properties and effects on disc cells.

SEM study showed diameter of the macro-pores of this gelatin scaffold was 100 ì m in average. This loose structure is hypothetically good for cultured disc cells residue all over the scaffold even deep into the center. Roomy space inside the scaffold offers a rich space for deposition of newly produced matrix. Since the intervertebral disc is the largest avascular tissue inside human body and its nutrition depends on diffusion, the ability of absorbing fluid is important for the survival of cultured disc cells. For culturing human disc cells, scaffolds were designed in disc shape with diameter of 8 mm and thickness of 2 mm. Average dry weights of the gelatin scaffolds was 28.6 ± 1.5 mg (range 26.6-30.8). Average wet weight of the full-hydrated gelatin scaffolds was 177.9 ± 22.0 mg (range 136.4-203.8). Our gelatin scaffolds were able to absorb 149.3 ± 21.8 mg (109.1-174.6 mg) culture medium. It was 5.2 ± 0.8 times (range

4.0-6.2) of their initial dry weight. Hopefully this will be able to offer enough nutrient supply in the in-vivo avascular situation. The amount of cultured disc cells which could attach to the scaffolds was measured by cell affinity test. By culturing $1x10^6$ human disc cells with the gelatin scaffolds for 2 hours, $4.4 \pm 0.7 \times 10^5$ cells (range $3.5\text{-}5.5 \times 10^5$) could attach onto the scaffold. Some promising results were also achieved from pilot studies of culturing rabbit nucleus cells and human disc cells onto the scaffolds. After 2-week and 6-week culture, MTT test demonstrated evidences of strong activities of cultured human disc cells. Histology revealed that this newly designed scaffold was good for survival and even proliferation of cultured disc cells. Serial further studies will be conducted in the second year based on the promising results we have now. Larger scale of 3-dimentional disc cell cultures will be performed in this gelatin scaffold. MTT test will be used to demonstrate the activities of the cultured disc cells. The contents of collagens and glycoaminoglycans produced by cultured cells will be measured to demonstrate the ability to produce extracellualr matrix. Histological and immunohistochemical staining will be performed to show the microscopic picture of this 3-dimentional culture system for disc cells.

報告内容

前言

It has been estimated that degraded intervertebral disc is responsible for 23% of low back pain cases [1]. Disc degeneration and associated spinal disorders are a leading course of morbidity resulting in substantial pain and increased health cost [2-6].
The disc has a composite structure comprised of the nucleus pulposus core, a multi-layered lamina of annulus fibrosus, and cartilaginous end plates. The nucleus pulposus is a gel matrix consisting of a hydrophilic polymer called proteoglycans (PG), and randomly interlaced collagen fibers. Because of the hydrophilicity of PG, the nucleus pulposus contains 75 to 80% water.
Although the causes and pathophysiology of intervertebral disc degeneration are mostly unknown, some evidences indicate that disc degeneration originates in the nucleus pulposus [7-9]. The PG content in the nucleus pulposus decreases progressively and the nucleus pulposus becomes dehydrated gradually [10, 11]. The gradual transformation within nucleus pulposus becomes more and more significant with age and is leading to degeneration in intervertebral disc. As the disc degenerates, the water binding capacity decreases and the disc becomes deflated. The hydration decreases and the tissue becomes more fibrous with a compromised ability to transmit intervertebral forces optimally. As the nucleus shrinks, a more compressive load is shifted to the annulus fibrosus. The excessive compressive load will cause annular layer delamination and eventually lead to damage in the annulus, through which the nucleus is herniated. For patients with disc herniation without obvioius instability,

discectomy is effective in relieving pain. But it can lead to instability of the anterior column [12, 13]. A frequently used surgical treatment of degenerative disc disease with instability is spinal fusion. Fusion permits load bearing, and enables passive restraint to the motion segment. However, the disadvantages of disc arthrodesis are well documented in mechanical tests [14] and clinical studies [15, 16].
Although there are still lots of efforts being made to improve the techniques in disc arthrodesis, the ultimate solution for anterior column support should not be overlooked. It has been long realized that the better solution for treating anterior column instability caused by degenerative disc disease is to re-establish the disc space and function instead of disc arthrodesis [17-21].

研究目的

The very early stage of degeneration of intervertebral disc happens in the degradation of proteonglycans and loss of normal hydration of nucleus pulposus. This is the leading event of following degenerative cascade of the spinal column. The rationale of initiating regeneration of early degenerative nucleus pulposus is to halt the following degenerative process of the entire spine. These are still no well-established treatment modality for the early degeneration in the intervertebral disc, especially the nucleus pulposus.

文獻探討

Although direct cell implantation had been reported successful in treating disc degeneration in limited human and animal studies [22, 23], the importance of extracellular matrix had also been emphasized [24, 25]. Therefore we plan to make a gelatin-based scaffold to enhance the survival and proliferation of the cultured disc cells and the productivity of the extracelluar matrix.

研究方法

- Fabrication of Gelatin Scaffolds

The goal of developing this bioactive scaffold is for better attachment, proliferation and matrix production of disc cells, especially nucleus pulposus cells.
1 g of gelatin powder (Sigma Co., St. Louise, USA) and 9 mg of chondroitin-6-sulphate (Sigma Co., St. Louise, USA) were mixed in 20 ml double distilled water. The solution was cross-linked by soaking in 0.1% glutaraldehyde solution then lyophilized to obtain copolymer sponge as scaffold. The SEM was performed for morphological observation of the gelatin scaffold. The scaffold was prepared in a disc shape with 8 mm diameter and 2-3 mm of thickness. This shape was designed to be able to be inserted into the nucleus space through the window

created by annulotomy during a conventional open discectomy.

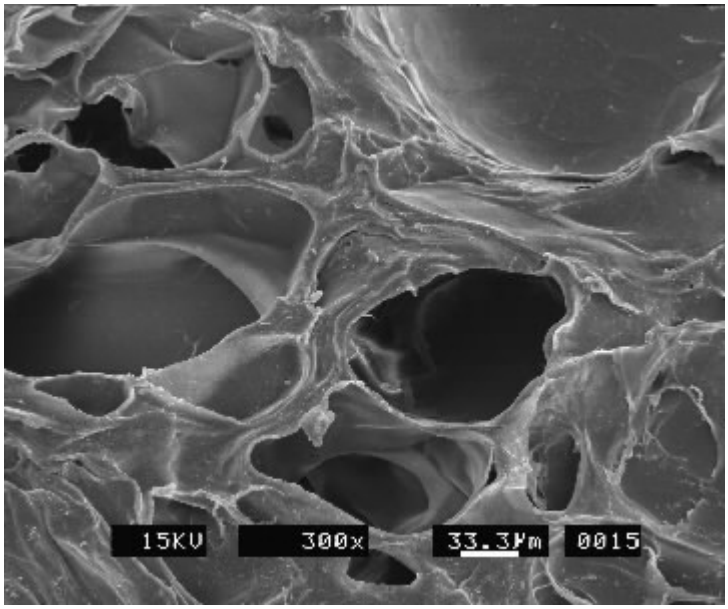

Fig. 1 SEM picture of the gelatin scaffold

- Fluid Water absorbing capacity of the gelatin scaffold

Since the intervertebral disc is the largest avascular tissue inside human body and its nutrition depends on diffusion, the ability of absorbing fluid is important for the survival of cultured disc cells. The weight of scaffolds was measured when they were freshly prepared before being soaked into any fluid. Then scaffolds were immersed into the cell culture medium for at least 2 hours to make scaffolds fully hydrated. Then the weight of scaffolds was measured again reflecting the weight in fully hydrated status. This study was aimed to estimate the ability of absorbing fluid of this gelatin scaffold and implied its ability to supply nutrition to the cultured disc cells in the *in-vivo* avascular environment.

- Number of cultured disc cells attached to the scaffold

Even though the cellularity of the nucleus pulposus is very low, the larger number of cultured disc cells initially attached on the scaffold will shorten the time needed to produce enough extracullular matrixes. Therefore the amount of cultured nucleus pulposus cells which could attach to the scaffolds was measured. Under the approval of IRB of National Taiwan University Hospital and the agreements of patients, surgical specimens of discectomy for six patients were obtained for primary culture of nucleus pulposus cells. After enough cell number was achieved, the cultured disc cells were used in the following study. Gelatin scaffolds were sterilized and pre-wetted before cultured disc cells were seeded. The number of cells remained in the culture medium was calculated and the number of cells attached on the scaffolds was obtained.

- Some pilot studies on the scaffolds cultured with human nucleus pulposus cells

Activities of the cultured cells in the scaffolds assessed by Microculture tetrozolium (MTT) assay

The MTT assay is a quantitative colorimetric assay for cell survival and proliferation. This test bases on the ability of live cells to utilize a pale yellow substrate, a tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co) , and tis subsequent modification into a dark blue formazan product [26, 27]. The tetrazolium is cleaved in mitochondria, so the reaction only occurs within living cells. The assay detects living cells and the signal generated is dependent upon the degree of activation of the cells. Histological studies including H-E stain and Alcian blue stain were also performed in some scaffolds after 6 weeks of culture.

結果與討論

- Morphological observation of the gelatin scaffold

The SEM picture of this gelatin scaffold was showed in figured 1. The size of the macro-pores was 100 ì m in average. This loose structure is hypothetically good for cultured disc cells residue all over the scaffold even deep into the center. Roomy space inside the scaffold theoretically offers a rich space for deposition of newly produced matrix.

- Fluid Water absorbing capacity of the gelatin scaffold

Average dry weights of the disc-shaped gelatin scaffolds was 28.6 ± 1.5 mg (range 26.6-30.8 mg). Average wet weight of the full-hydrated gelatin scaffolds was 177.9 ± 22.0 mg (range 136.4-203.8 mg). Through our processing method, the gelatin scaffolds were able to absorb 149.3 ± 21.8 mg (109.1-174.6 mg) culture medium. It was 5.2 ± 0.8 times (range 4.0-6.2) of their initial dry weight. Hopefully this will be able to offer enough nutrient supply in the in-vivo avascular situation.

- Number of cultured disc cells attached to the scaffold

By culturing $1x10^6$ human disc cells with the gelatin scaffolds for 2 hours, 4.4 ±0.7 x 10^5 cells (range 3.5-5.5 x 10^5) could attach onto the scaffold.

- Results of pilot studies

After 2-week and 6-week culture, we could find strong absorbance in the MTT test. It indicated the activities of cultured human disc cells were still very strong after the culture period. WE can say this gelatin scaffold provides a appropriate environment

for the survival and even proliferation of the cultured disc cells.
Histological examination also revealed that this newly designed scaffold was good for survival and even proliferation of cultured disc cells (figure 2, H-E stain). Blue stained substance in Alcian Blue staining indicated there was production of extracellular proteoglycans by the cultured disc cells (Figure 3).

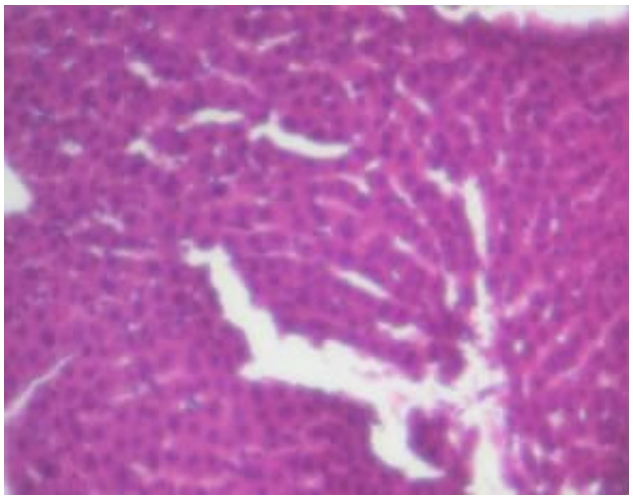

Figure 2. H-E stain of 3-dimensional culture of human nucleus pulposus cells in gelatin scaffold

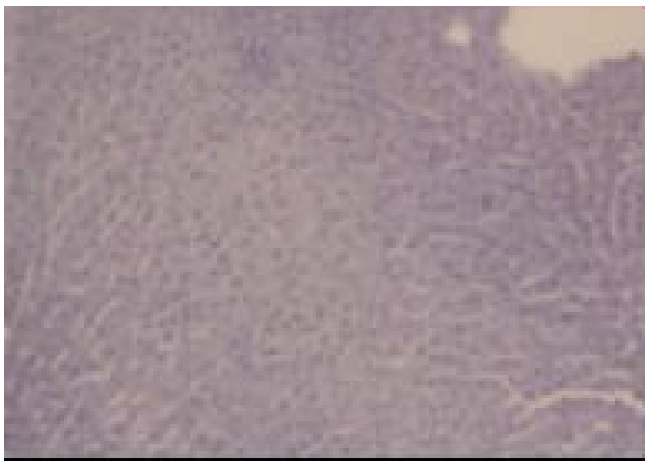

Figure 3. Alcian blue staining for proteoglycans

結論及計畫成果自評

From the results we obtained in the first year of this study, we prove this gelatin scaffold is very promising for regeneration of nucleus pulposus. We propose to conduct serial studies in the second year. Larger scale of 3-dimentional disc cell cultures will be performed in this gelatin scaffold. Nucleus pulposus cells obtained from the surgical specimens of ten to twenty patients will be used to establish the primary NP cell culture. MTT test will be used to demonstrate the activities of the cultured disc cells in the gelatin scaffolds. The contents of collagens and

glycoaminoglycans will be measured to demonstrate the ability of producing extracellular matrix. Histological and immunohistochemical staining will be performed to show the microscopic picture of this 3-dimentional culture system for disc cells.

參考文獻

1. Anderson JAD. Back pain and occupation. In: Jayson MIV, ed. The lumbar spine and back pain. 3rd edition. London: Churchill Livingstone, 1987; 2-36.
2. Borenstein D. Epidemiology, etiology, diagnostic evaluation, and treatment of low back pain. Curr Opin Rheumatol 1992; 4: 226-32.
3. Greenberg SN, Bello RP. The work hardening program and subsequent return to work of a client with low back pain. J Orthop Sports Phys Ther 1996; 24: 37-45.
4. MacDonald MJ, Sorock GS, Volinn E, Hashemi L, Clancy EA, Webster B. A descriptive study of recurrenct low back pain claims. J Occup Environ Med 1997; 39: 35-43.
5. Waddell G. Low back apin: a twentieth century health care enigma. Spine 1996; 21: 2820-5.
6. Webster BS, Snoock SH. The cost of 1989 workers' compensation low back pain claims. Spine 1994; 19: 1111-5.
7. Adler JH, Schoenbaum M, Silberberg R. Early oonset of disk degeneration and spondylosis in sand rats (Psammomys obesus) Vet Pathol 1983; 20: 13-22.
8. Hirsch C, Schajowicz F. Studies on structural changes in the lumbar annulus fibrosus. Acta Orthop Scand 1953; 22: 184-231.
9. Silberberg R, Aufdermaur M, Adler JH. Degeneration of the intervertebral disks and spondylosis in aging sand rats. Arch Pathol Lab Med 1979; 103: 231-5.
10. Buckwalter JA. Aging and degeneration of the human intervertebral disc. Spine 1995; 20: 1307-14.
11. Pearce RH, Grimmer BJ, Adams ME. Degeneration and the chemical composition of the human intervertebral disc. J Orthop Res 1987; 5: 198-205.
12. Frymoyer JW, Selby DK. Segmental instability: rationale for treatment. Spine 1985; 10: 280-6.
13. Goel VK, Nishiyama K, Weinstein JN, Liu YK. Mechanical properties of lumbar spinal motion segments as affected by partial disc removal. Spine 1986; 11: 1008-12.
14. Lee CK, Langrana NA. Lumbosacral spinal fusion: a biomechanical study. Spine 1984; 9: 574-81.
15. Hirabayashi K, Maruyama T, Wakano K, Ikeda K, Ishii Y. Postoperative lumbar canal stenosis due to anterior spinal fusion. Keio J Med 1981; 30: 133-9.

16. Lipson SJ. Degenerative spinal stenosis following old lumbosacral fusion. Orthop Trans 1983; 7: 143.
17. Bao QB, McCullen GM, Higham PA, Dumbleton JH, Yuan HA. The artificial disc: theory, design and materials. [Review] Biomaterials 1996; 17: 1157-67.
18. Bao QB, Yuan HA. Prosthetic disc replacement: the future? Clin Orthp 2001; 394: 139-45
19. Garcia A, Lavignolle B, Morlier P, Ducassou D, Baquey C. Intradiscal polymerization: preliminary results of chemical and biomechanical studies. In: Brock M, Mayer HM, Weigel K ed. The Artificial Disc. Berlin: Springer, 1991: 39-43.
20. Kostuik JP. Intervertebral disc replacement. Experimental study. Clin Orthop 1997; 337: 27-41.
21. Kostuik JP. Alternatives to spinal fusion. Orthop Clin N Am 1998; 29: 701-15.
22. Gruber HE, Johnson TL, Leslie K, Ingram JA, Martin D, Hoelscher G, Banks D, Phieffer L, Coldham G, Hanley EN Jr. Autologous intervertebral disc cell implantation: a model using Psammomys obesus, the sand rat. Spine. 2002; 27: 1626-33.
23. Ganey TM, Meisel HJ. A potential role for cell-based therapeutics in the treatment of intervertebral disc herniation. European Spine Journal. 2002; 11: S206-14.
24. Nomura T, Mochida J, Okuma M, Nishimura K, Sakabe K. Nucleus pulposus allograft retards intervertebral disc degeneration. Clin Orthop 2001; 389: 94-101.
25. Nishimura K, Mochida J. Percutaneous reinsertion of the nucleus pulposus: an experimental study. Spine 1998; 23: 1531-8.
26. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 1983; 65: 55-63.
27. Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Research 1988; 48: 589-601.