行政院國家科學委員會專題研究計畫　成果報告

韌帶組織工程(二)：韌帶組織細胞外基質之強化研究

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Ⅰ、中文摘要：

韌帶或肌腱的斷裂缺損，在臨床上是一個令人非常困擾的問題，臨床醫師常面臨韌帶自體移植來源有限及韌帶修補處強度不夠的窘境。於是工程化培養再造韌帶組織之概念應運而升。目前組織工程韌帶之研究可以為人類帶來有下列好處：其一、取代韌帶自體移植：韌帶自體移植最大的缺點就是來源不足，人體可供給作韌帶移植的來源不多。其他的缺點尚包括：會有移植處併發症，規格無法訂製之缺點。二、改善斷裂韌帶在經過修補後，癒合處強度不足問題。

研究顯示，韌帶修補處之強度在完成修補一年後，只恢復 60-80％的拉力強度，而在一些斷端嚴重受創之韌帶，其強度甚至不到 40％，故韌帶接合處之癒合強度不足是造成其再斷裂或沾黏之最主要原因。故如何改善此強度，本研究能提供詳細答案。現階段的韌帶組織工程研究所遭遇的問題是細胞不易附著、細胞生長分裂能力有限及基質分泌不足等三項。

本實驗利用自原始培養韌帶細胞，純化出類胰島素基因片段，在將其殖入韌帶細胞中，以求能促進細胞生長、間質蛋白質分泌增加為目的。實驗結果發現經過強化後的基因轉殖韌帶細胞，其細胞分裂生長能力較沒有基因轉殖的韌帶細胞增加約 28%，而基質產生能力對 Collagen 而言約增加 58%，對 proteoglycan 而言，約增加 52%。如此可改善細胞生長分裂有限及基質分泌不足等問題，希望能對本領域的進步有所貢獻。

關鍵字: 組織工程，韌帶，肌腱，基因轉殖，insulin-like growth factor，gene activated matrix

Ⅱ、英文摘要：

According a study in San Diego Kaiser Health System, they estimated the incidence of knee ligament injuries in the general population was 178 injuries per 100,000 people per year. The insufficiency or rupture of tendon or ligament is a very challenging problem clinically. They included ACL rupture, PCL rupture, patella tendon rupture, and Achilles tendon rupture etc. If they are irreparable like ACL insufficiency or there is a gap between the two ends due to loss of tendon substance, the clinician must consider the autogenous tendon graft, allogenic tendon graft or artificial ligament for reconstruction. Artificial ligament and allogenic ligament is not widely used due to many complications happening. Autogenous tendon graft is the best and most frequent source for tendon reconstruction like the reconstruction of ACL insufficiency. But it has several potential problem unsolved. The problems included the limitation of source, donor site morbidity, and unable to be custom-made etc. The development of engineered ligament offers the alternative choice for antogenous tendon graft. We hope the engineered ligament can replace the traditional method and offer the knowledge to improve the strength of tendon or ligament healing.

Currently, the major problems of ligament tissue engineering include poor adhesion of tenocyte onto scaffold, limited ability of cell proliferation and insufficiency of cell-tissue matrix production. In my experiment, we try to clone the IGF gene from the primary cell cultures of tenocytes. Then we transfected this gene segment into tenocytes by the liposomal vector. We hope this procedure can promote the growth and proliferation of tenocytes and improve the production of matrix. According to our result, the proliferation rate of transfected tenocytes increases 28%. The ability of production of collagen increase 58%. The ability of production of proteoglycan increases 52%. Through this way, we can improve the both abilities of cell proliferation and matrix production.

Keyword: tissue engineering, ligament, tendon, gene transfer, insulin-like growth factor, gene-activated matrix

III、報告內容

前言：

韌帶或肌腱的斷裂或缺損是臨床上常見的問題。在前十字韌帶斷裂、髕骨韌帶斷裂、手部肌腱斷裂或是阿基里斯腱斷裂時，如果面臨韌帶無法修補或是斷裂端粉碎狀況嚴重而導致有空窗的情形，便會考慮自體韌帶移植手術。韌帶自體移植最大的缺點就是來源不足，人體可供給作韌帶移植的來源不多。其他的缺點尚包括：會有移植處併發症，規格無法訂製之缺點。另一項嚴重的缺陷是韌帶斷裂後，即使韌帶斷裂處已癒合，但其強度往往無法回復到原本的生物力學特性。研究顯示，韌帶修補處之強度在完成修補一年後，只恢復60-80%的拉力強度，而在一些斷端嚴重受創之韌帶，其強度甚至不到40%，故韌帶接合處之癒合強度不足是造成其再斷裂或粘黏之最主要原因[10,11,13,14]。組織工程韌帶之研究不僅可以促進斷裂之肌腱或韌帶癒合速度，更可以使組織癒合後之機械性質表現，更貼近人體肌腱或韌帶的標準，如此便可以減少再斷裂之可能性，藉此使復建工作提早，以減少發生粘黏的併發症[6,18,19]。

研究目的：

本研究之主要是利用 Insulin-like Growth Factor (IGF)能促進細胞分裂生長的特性，首先嘗試找出屬於韌帶細胞獨特之 IGF Subgroup，並且將其基因轉殖入韌帶細胞中，進而再分析對照這些含有 IGF gene 之韌帶細胞的生物特性，以達到促進斷裂肌腱之癒合速度，改善斷裂肌腱癒合後的機械強度，最後再利用此一轉殖基因細胞去製造組織工程化韌帶，以取代及改善現有韌帶移植手術之困難與窘境。

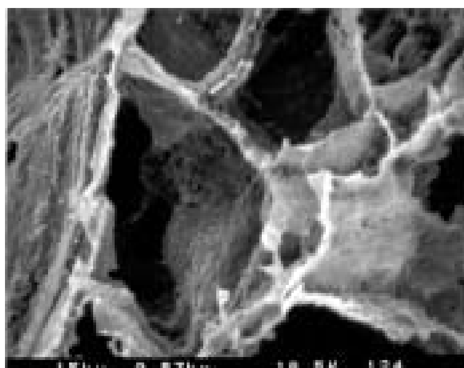
文獻探討：

組織工程科學不斷的進步，已使它成為近十年來發展最迅速的科學之一。近年來，真正符合人體各部分的組織已陸續問世，但組織工程韌帶技術尚屬於初步啟動的階段。其問題的癥結有二。其一為原始韌帶細胞約在分裂 12-16 代之後便失去分裂及分泌基質的能力，這樣的特性對培養組織韌帶細胞是關鍵性的瓶頸，故如何保持原始韌帶細胞之分裂及分泌基質的能力便成為極重要的課題。其二為分泌細胞外基質彼此之間鍵結連合之能力不夠，目前我們所培養出來的組織韌帶之強度不夠，不足以承擔韌帶所要負荷的機械強度。

Cao et. al.在 1995 年曾利用 PLGA 為支架[4,5]，把牛之韌帶細胞植於其上，並將其埋入老鼠之皮下組織，結果長成組織性質相當類似自然韌帶之結構，但是其對機械強度並沒有說明。Goulet et. al.在 1997 年曾嘗試以 collagen gel 來做支架，並在體外做培養，結果也長出了類似韌帶之組織，但其強度僅達到人體的 1%。故在 Scaffold 上培養 tenocyte 已不是問題，再來各位學者就嘗試改善細胞外基質強度的問題[12]。細胞外基質強度與細胞彼此的黏附性[1]及基質內容有關，就基質內容而言，主要分為兩個主軸，其一就是改善 scaffold 本身的結構與組成物質[15,17]，如各種 scaffold 的材質，例如 collagen、collagen-GAG、PLGA、carbon fiber 等等皆被研究過，如 Torres 在 2000 年[17]指出 Cross-linking 於

Collagen-GAG matrices 中對於收縮強度是有正相關的。其二就是使用 local plasmid-based gene transfer technology[7,8,9,16]，這類技術現已被普遍稱為 GAM (gene activated matrix)[2,3]。此類的學問已被發展成一個技術平台，專門用來研究甚至已達治療的目的。GAM 簡單而言，就是一種 DNA-based technology。藉由 gene transfer 之技術，使得細胞能夠持續的分裂生長及大量的製造我們想要的蛋白質。延長細胞的生命週期，大量分泌間質以便細胞與間質合組成組織，以達到我們製造韌帶的目的。IGF 為一種生長激素，普遍存在於各種細胞中。其本身兼具有 Autocrine 及 Paracrine 的作用方式。IGF 又有各種不同的 Subtype，如 IGF-1a、IGF-1b、IGF-1c、IGF-2 等等。但沒有文獻提到韌帶細胞的 IGF 是屬於哪一類的次族群。故於本實驗中，我們經由所培養的韌帶細胞(primary cells)去抓 IGF 的次族群，並將其轉殖入韌帶細胞中，觀察其生長及基質分泌的情形。

Fig 1& 2: Primary tenocytes under microscope and H&E stain



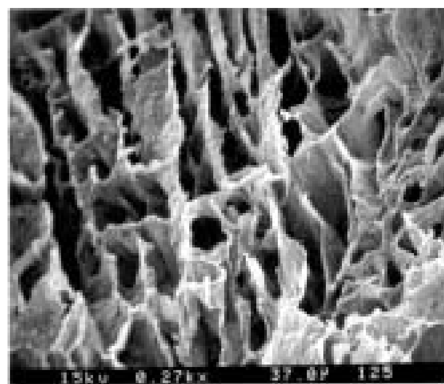


Fig 3 & 4: They show the scaffold pictures of PCL. The pore size is about 80-100 um.

結果與討論：

一、Insulin growth factor gene cloning：

IGF gene cloning procedure was followed the standard procedure of gene cloning. The tissue was taken from the live Wistar rats under anesthesia. We take the ligament portion of the patella tendon and Achilles tendon from hind legs. The rat is on average 6 weeks old and 250 gm weight. Then we rinsed and washed the tissue several times with PBS. We used the Polytron machine to homogenize the cells. We extract the total RNA using Trizol Reagent (Invitrogen) following the manufacture's protocol. Then one-step RT-PCR was performed using the primer we designed from NCBI website and some reference.

Electrophoresis was done repeatedly for confirmation of the segments we wanted. 2nd PCR was done for more accurate DNA product. Then gel elution was done for purification of the DNA. PCR product was ligated into the pGEM-Teasy vector (Promega). Sequencing was done for making sure of the collection of DNA. Plating and proliferation in the 3 cc LB solution for checking sense or antisense ligation. The sequence was checked using restriction enzyme correspondingly. Then we sent the sample for sequence confirmation again. After sequence confirmation, plasmid was prepared by using Concert High Purity Plasmid Maxiprep System (GIBCO BRL). Transfection was done using the Lipofectamine 2000 (Invitrogen). The comparative group was set-up using GFP marker. The 3-4 generation of ligament cells were used for transfection. Neomycin is used for screening the non-transfected cells. They are observed under the fluorescent microscope.

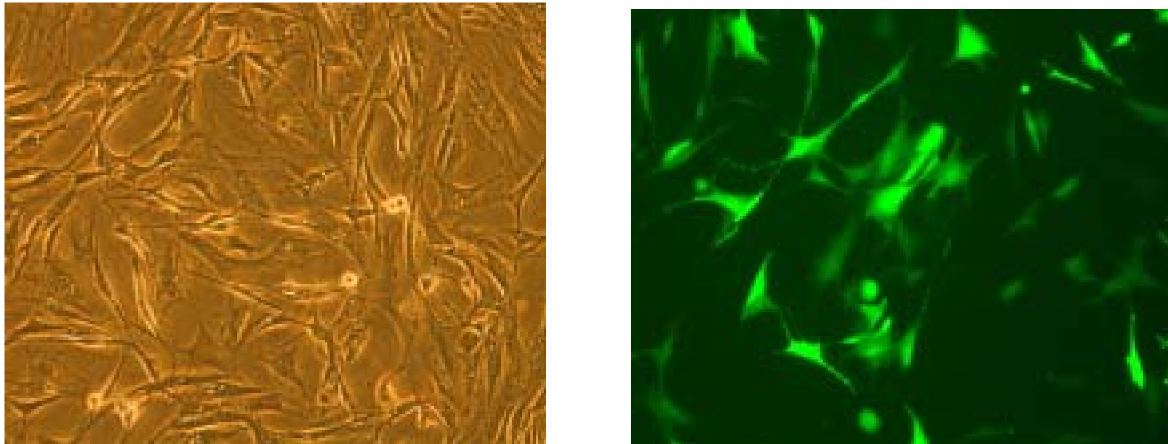


Fig 5 & 6:Under microscope with fluorescence, transfection rate is almost 60%.

二、Transfected Tenocyte 定性及定量

The proliferation of tenocytes was assayed by Bride assay. The 35S praline assay was used for proteoglycan assay. We compared two groups of cell cultures. The first group is the transfected tenocyte. The seaward group is the control group, the primacy cell culture. We culture the cells with the cell density of $1x10^5$ cells/cm^2. The cells were cultured with adding the C^{14}-glycine in 10 uCi/ml concentration and S^{35}-proline in 10 uCi/ml concentration in the culture mediun. After adding the radioactive regents, the cells were cultured for 3 days. Then we collected the condition mediun and cells for collagen and proteoglycan analysis. The results as in Table.1.

	Proliferation	Collagen	Proteoglycan
Transfected Tenocyte	2.5 ± 0.4 $x10^5$	198 ± 40 $x10^{-4}$ dpm/cell	35 ± 8 $x10^{-4}$ dpm/cell
Normal Tenocyte	1.8 ± 0.3 $x10^5$	125 ± 32 $x10^{-4}$ dpm/cell	23 ± 6 $x10^{-4}$ dpm/cell

Table 1: The data shows the different rate of proliferation of cells, secretion of collagen and proteoglycan between transfected tenocytes and regular tenocytes.

三、討論：

1.在我們 clone IGF gene 的過程中，我們分別以三種 IGF-1 之 Subtype: IGF-1a、IGF-1b、IGF-1c 三種 primer 來夾，我們發現這三種 Subtype 中，其中以 IGF-1a 在 Tenocyte 中最顯著表現。IGF-1b 其次，但量不多。而 IGF-1c 幾乎沒有辦法夾到這一段基因(根據 IGF-1c 所設計的 primer)。故實驗數據顯示，在 tenocyte 中以 IGF-1a 之表現為最顯著。

2.關於 Transfection rate 的問題，用 primary cells 來做 Transfection，本身的效率就會高低起伏不定。我們固定用 6 週大的老鼠取下 tendon 組織後以 Enzyme Digestion，之後固定用第一代的 primary cell 來做 transfection。幸運的是我們的 transfection rate 約可達 60-70%，這與國外一流研究單位的效能差不多。於是我們直接用這些 transfected tenocytes 去做接下來的定性及定量工作。

3.Tenocytes 在經過轉殖入一段 IGF-1a 基因後，其生長能力分泌基質能力均有明顯顯著的增加。在生長方面，我們之前的研究顯示，原始 Culture 的 Tenocyte 在經過 13 代左右的分裂生長後，就會停止分裂生長，甚至死亡。但經過強化 Transfection 後之 Tenocyte 之生長分裂能力大為加強，約增加 28%左右，故大可增加 Tenocyte 在支架上的增值能力。在分泌 Collagen 及 Proteoglycan 方面，經過強化 Transfection 後之 Tenocyte 之基質分泌能力對 collagen 而言約增加 58%，對 proteoglycan 而言約增加 52%。有了此一特性，故其一旦黏附上支架後，便可立即大量分泌 Collagen 及 proteoglycan 等基質，以便更快速形成組織。有了上述二種優點，本實驗證明了 IGF 在韌帶組織工程之可用性及實用性，下一步的話就是將之用於實際上的操作，由 in vitro 進入 in vivo 的階段。

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V、計畫成果自評：

1. 研究內容與原計畫相符程度：
   本計畫是嘗試以基本轉殖技術改良現有韌帶組織工程之細胞生長與基質合成。本人在這一年中，成功的 clone 出韌帶細胞之類胰島素生長因子之基因片段，確定其 subtype 存在之類型，並加以轉殖入韌帶細胞中，然後對於這些經過基因強化之細胞加以定性及定量，測定其生長分裂速率及 Collagen、proteoglycan 這兩個基質主要成份之定量測試。故研究內容與原計畫是完全相符的。
2. 達成預期目標：
   已達預期目標 80%以上，未完成部分是以此細胞殖入各式不同的支架上，看細胞在支架上附著與再生的情形。
3. 已投稿於雜誌審稿中。本成果將實際應用於人體之治療，短程目標為應用於韌帶扭傷及韌帶斷裂癒合後之機械強度，中程目標為申請人體試驗，以達到實際應用的目的。