行政院國家科學委員會專題研究計畫　期中進度報告

利用生物活性基質進行骨軟骨及　帶之組織工程(1/3)

計畫類別：整合型計畫
計畫編號：NSC91-2321-B-002-005-
執行期間：91 年 08 月 01 日至 92 年 12 月 31 日
執行單位：國立臺灣大學醫學院骨科

計畫主持人：劉華昌
共同主持人：楊台鴻，陳瑞明，侯勝茂，侯連團，林峰輝，黃義侑，洪士杰

報告類型：完整報告
報告附件：出席國際會議研究心得報告及發表論文
處理方式：本計畫可公開查詢

中　華　民　國 92 年 11 月 3 日

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摘要

膝關節的軟骨缺損或退化性關節炎常常會造成膝關節之疼痛。雖然在外科手術上有許多方法發展出來如鑽動術、骨膜皮瓣及軟骨細胞移植、馬賽克成形術等，試圖解決這方面的問題，但效果都不盡理想。關節置換手術、自體、異體或異種植體也用在臨床上，但關節置換手術所使用材料為非生物性，長期使用常有鬆脫的現象；自體移植須有額外的手術，供應位置的復原及受應力情形也會受到影響；異體或異種植體則有免疫及疾病傳染的危險。本研究擬利用組織工程技術培養出 autogenic osteochondral grafts 及骨-韌帶-骨的植體，研究中將骨髓幹細胞載入所發展的雙相支架中，上層為 gelatin/hyaluronan/chondroitin-6-sulfate 海綿共聚體，下層為多孔質氫氧基陶瓷體；生長激素將以表面接枝或共聚方式導入支架、微粒方式導入支架達到緩釋效果、或加入培養基中促進細胞的分化及增生。組織培養將在自行設計的生物反應器下進行。骨-韌帶-骨的植體也是利用相同觀念，將細胞值入載有生長激素的 PDLLA 纖維束中培養。

本計劃分三年進行。第一年為各種支架材料之製備與測試、幹細胞之分離培養及分化、微粒之開發、反應器之設計組裝及測試。

關鍵詞: 組織工程，骨軟骨植體

ABSTRACT

In case of extensive arthritis, avascular necrosis, or traumatic or tumor excision-caused loss of osteochondral joint tissues, joint replacement or osteochondral defect reconstruction is often require joint function. Although there are many techniques being developed to solve the problem such as drilling chondroplasty, mosaicplasty, or periosteal flap combined with autogenous chondrocyte transplantation, all of these treatments had their limitations. Prosthetic replacement, allogenic and autogenic osteochondral grafts have been performed for this purpose. However, prosthetic joint replacement may be compromised by the limited durability of non-biological materials used due to wearing and loosening of the implant. The availability of tissue and morbidity of the donor site have limited the use of autografts. The major problems for allograft or xenograft are the potential danger of infection with HIV or hepatitis, and the necessity for immunosuppression with possible serious complications. Therefore, suitable osteochondral grafts have long been one of the goals of orthopaedic research. Tissue engineering is a recently developed science with promising results reported for reconstruction of bone, cartilage, and small joints. The project try to use the tissue engineering technique to fabricate osteochondral grafts with bone marrow-derived mesenchymal stem cells seeded on the bi-phasic scaffold. The scaffold is a bi-layer structure with gelatin/Hyaluronan/chondroitin-6-sulfate copolymer sponge on the top and porous hydroxhapaptite beneath. The growth factors will be incorporated into the system by surface graft, microsphere delivery, and dripping into the cultural medium. All the cultured system will be performed in a new designed double-chamber bioreactor. The bone-ligament-bone graft will be prepared under the same philosophy as that of osteochondral graft.

The project is a three-year study. In the first year, all kinds of scaffold used into the study will be developed to mimic the extracellular matrix of real tissue as a viable matrix. The new designed bioreactor will be assembled and tested to fit the demands of future applications. Growth factors, TGF-β1, bFGF, and PDGF, will be encapsulated with biodegradable polylactide and liposome by lyophilization and intramolecular interaction. The MSCs will harvest from the bone marrow and then preliminarily seed on the viable matrix. A PLGA fiber seeded with ligament stem cell will prepare to cooperate into the system later. The tidemark at the junction of hyaline cartilage and subchondral bone will be developed by ameoblast-seeded bovine bone

Keywords: tissue engineering, osteochondral composite graft

個別分項計畫之報告分述如下：

雙腔室生物反應器之製造與雙相式支架之製作, 及軟骨於該支架上之生長

Progress Report: Development of Double-Chamber Bioreactor for Osteochondral Tissue Engineering and the Making of Biphasic Scaffold with Growing of the Cartilage on the Scaffold

Design of, and rationale for, the double chamber bioreactor

The double-chamber bioreactor consists of two glass chambers, each of which has three branch tubes for medium inflow, medium outflow, and oxygen ventilation. The bioreactor is made of glass, which is completely transparent. The outer diameter of the bioreactor is 48 mm, and the inner diameter is 45 mm. The chambers are separated by a silicon septum with multiple holes to hold the biphasic scaffold. The glass chamber can be sterilized by ethylene oxide and the silicon septum can be sterilized with 75% alcohol. Magnetic bar stirring provides the mechanical stimulation for the engineered cartilage. The whole setting can be put into a standard humidified incubator at 37°C and 5% CO_2. The bioreactor has two independent medium circulation systems (Fig. 1(a) and (b)) and thus can be used to supply different types of culture media to support different kinds of cells in co-cultures or different induction media to induce mesenchymal stem cells to differentiate into chondrocytes and osteoblasts in the same biphasic scaffold (e.g. medium containing beta-glycerophosphate and dexamethasone for induction of bone formation and medium containing TGF-beta 1 for induction of cartilage formation). We hypothesized that the different medium in the different chamber will not mixed up due

to the limited diffusibility in the biphasic scaffold. However, limited diffusion of the cytokines is supposed to occur inside the scaffold, which could facilitate micro-interactions between different cells at the interface, but little exchange will occur between the two chambers. In addition, as the cells secrete increasing amounts of matrix, diffusion would progressively decrease.

Fig 1 (a).
The design of Double-Chamber bioreactor: The double-chamber bioreactor consisted with two glass chambers. Each chamber contains three branch tubes for medium inflow, medium outflow and oxygen ventilation. The two chambers were separated with a silicon septum with multiple holes to hold the biphasic scaffold. Magnetic bar stirring provided the mechanical stimulation for the scaffold. The bioreactor contains two independent medium circulation systems.

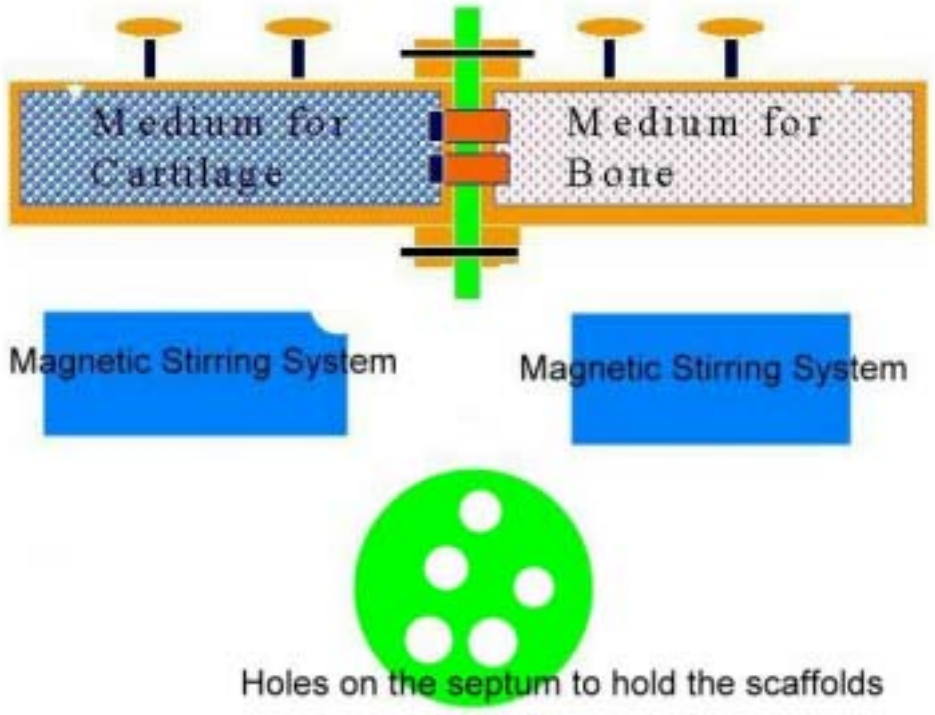


Fig 1 (b). The Double-chamber bioreactor.

Fabrication of biphasic scaffolds and SEM and confocal microscope examination:

Cancellous bone, obtained from calf femoral condyles, was cut into small cubes of approximately 1 cm^3. To avoid cracking and soot formation in the material during heat-treatment, the cubes was boiled in distilled water for 12 h. After boiling, the bone blocks were dehydrated in an alcohol series and dried at 70°C for 3 days. To remove organic matrix, the dried blocks were calcined in a conventional Ni-Cr coiled furnace by increasing the temperature to 800°C at a rate of 10°C/min and maintaining it at this temperature for 6 hours. The calcined blocks were placed on a platinum sheet and heated in a SiC heated furnace to 1350°C at a rate of 2.5°C/min and maintained at this temperature for 1 h to sinter the bone. The material, the so-called “sinbone”, was then cooled to room temperature by slow furnace cooling, then cut into a cylindrical shape with 20 mm in length and 10 mm in diameter.

Gelatin powder, extracted and purified from porcine skin, with an average molecular weight of about 60,000-100,000 daltons on SDS-PAGE (Sigma Chemical Co., USA) was dissolved as a 1% solution in deionized water in a 65°C water bath

with continuous stirring. A final concentration of 0.05 % (w/w) hyaluronan (HA) (Seikagaku Co., Tokyo, Japan) solution was then added, giving a gelatin/HA molar ratio of 9:1.

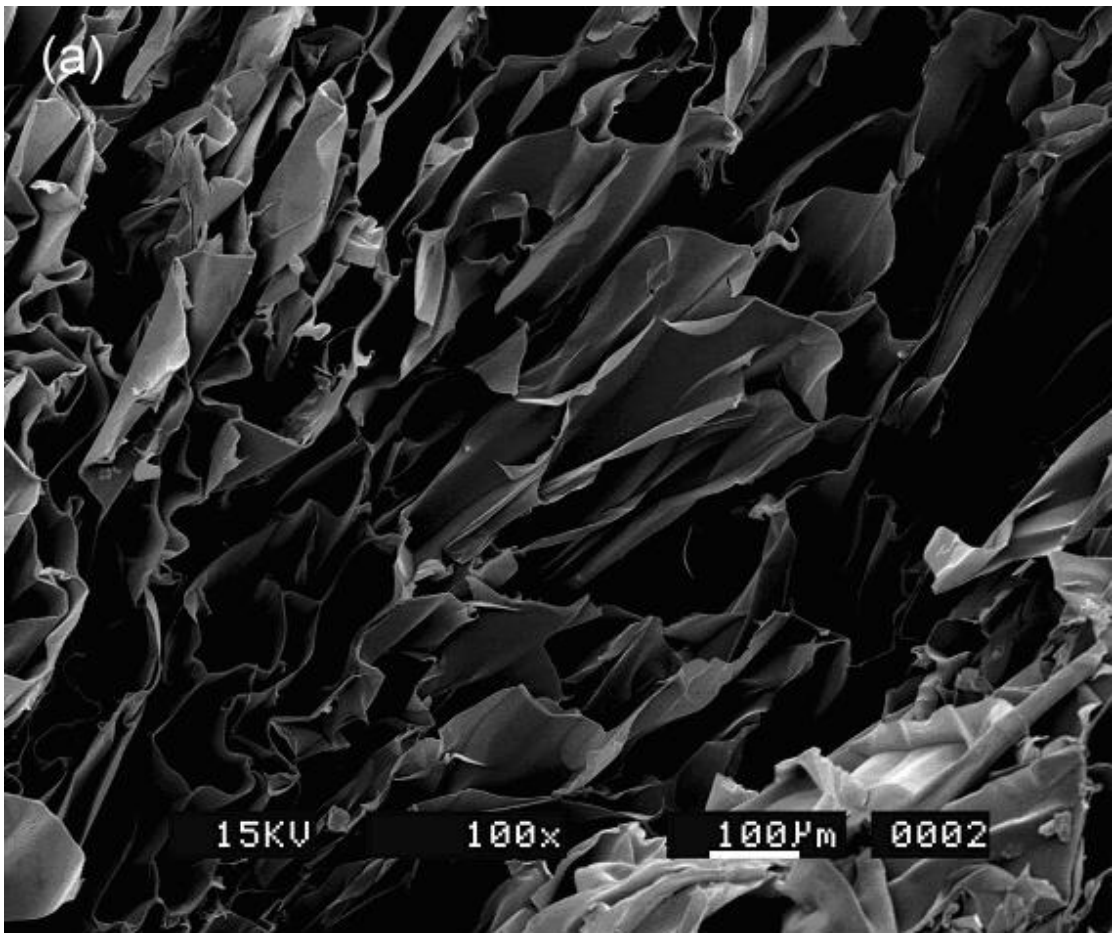
The sinbone block with 20 mm length and 10 mm diameter was soaked for 30 minutes in 5 ml of the gelatin/HA solution at a negative pressure of 10^{-1} torr, then the sinbone/gelatin was cross-linked overnight at room temperature with 0.01% glutaraldehyde and lyophilized for three days. The cross-linking and lyophilization process was repeated another two times. The sinbone/gelatin was then used as a bilayer scaffold for osteochondral graft tissue engineering. The thickness of the gelatin layer is about 2 mm (Fig. 3).

The scaffolds were dehydrated by treatment with a series of graded ethanol solutions (50% for 12 h, 75% for 2 h, 85% for 2 h, and 95% for 2 h), then left overnight in a vacuum oven at 50℃ before coating with gold for scanning electron microscope (SEM) examination. One scaffold was examined using a Leica TCS-SP2 confocal microscope with an Ar/Kr laser with a wavelength of 568 nm.

Under the SEM, the dehydrated gelatin scaffold had a uniform pore size of 180 μm and an adequate porosity of 75 % (Fig. 4(a)). This highly porous structure of the scaffold would be expected to allow cell growth and proliferation, and the size of the pore should increase even further on soaking in culture medium, allowing cell penetration and adhesion. In the calcined bone part of the scaffold, the natural trabecular pattern was preserved (Fig. 4(b)). On confocal microscope examination (Fig. 5), the porous gelatin structure could be seen, and had infiltrated into the pores of the calcium phosphate. The pores in the gelatin and calcined bone are interconnected, which would facilitate micro-diffusion in the interface.

Fig 3.
The gross appearance of the biphasic scaffold.

Fig. 4 (a) Scanning electron microscopic examination of the gelatin scaffold showing a porous structure with a uniform pore size of about 180 μ m and porosity of 75 %. (b) Scanning electron microscopic axamination of the calcined bone showing the preservation of the trabecular pattern.



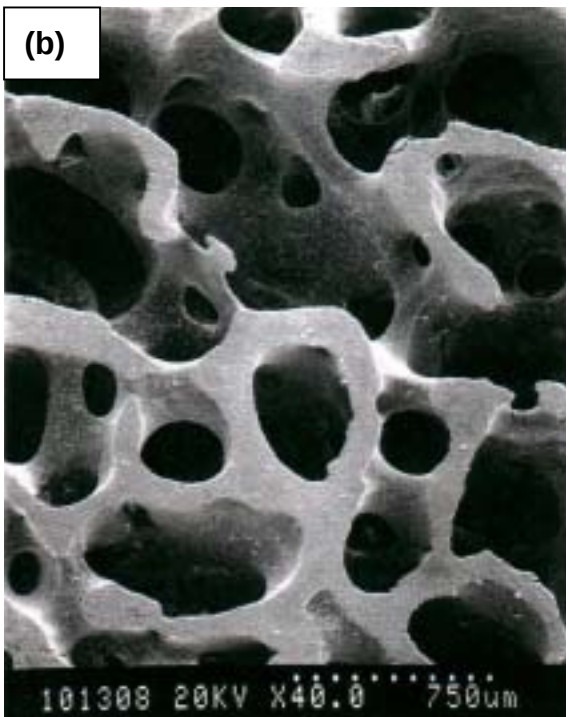
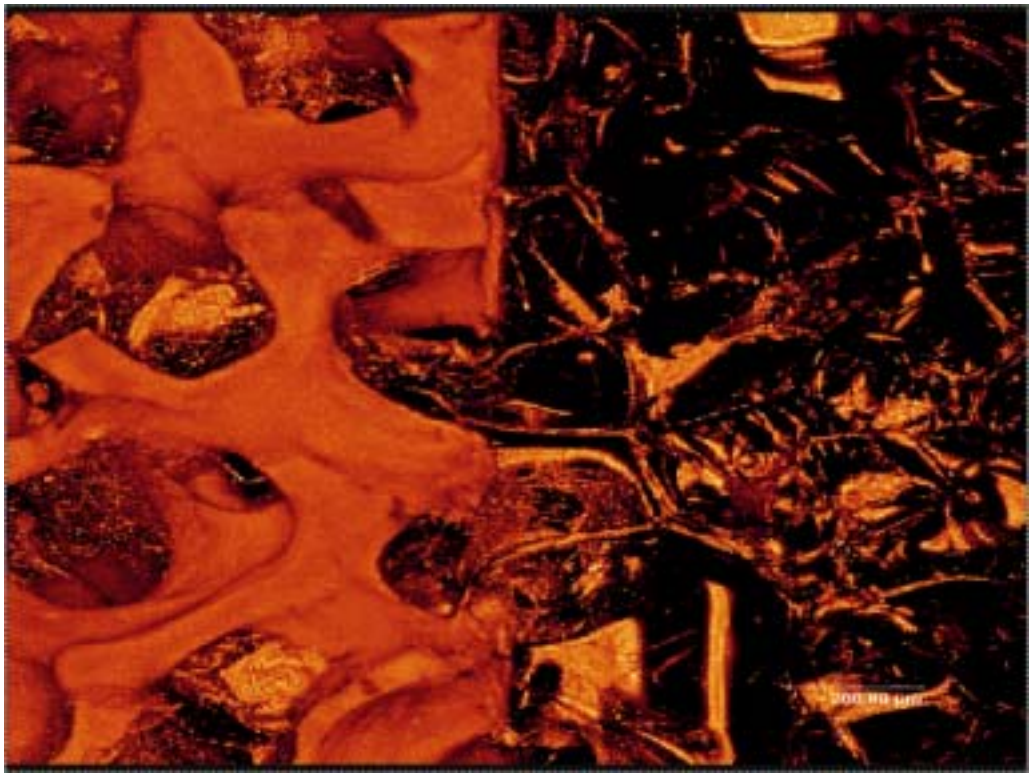


Fig. 5 Under confocal microscope examination, porous gelatin structure can be seen, and the gelatin structure had infiltrated into the pores of calcium phosphate. The integration of cartilage part and bony part of scaffold depends on this penetration.



Isolation of Condrocytes:

Full thickness articular cartilage was harvested aseptically from adult porcine knee joints within 12 h after slaughter and chondrocytes isolated by incubating the cartilage specimens for 12 – 16 h at 37°C in DMEM medium (Hyclone Co., Logan, Utah, USA) containing 0.2 % collagenase (Sigma Co., St. Louis, USA). The isolated chondrocytes were resuspended in phosphate-buffered saline (pH 7.4), washed, and counted using a hemocytometer. Chondrocyte viability was determined using Trypan blue dye exclusion. The monolayer culture is subcultured after a week. The chondrocytes were culture expanded for 2 weeks before seeding to the biphasic scaffold.

Seeding of Chondrocytes and Cultured in Double-Chamber Bioreactor

Biphasic scaffold cylinders (20 mm long, 10 mm diameter, 3 mm thick in cartilage

part) were sterilized with 75% ethanol. Chondrocytes were expanded in DMEM containing 10% fetal bovine serum (Biological Industries Ltd, Kibbutz Beit Haemek, Israel), 1 % penicillin/gentamicin (Sigma Co., St. Louis, USA), and 50 μg/ml of L-ascorbic acid (Sigma Co., St. Louis, USA). At confluence, the chondrocytes were trypsinized and resuspended at a concentration of 5.3 x 10^7 cells/ml DMEM, and then about 2 ml of cell suspension was injected into each scaffold (10^8 cells per scaffold). The cell-containing scaffolds were then cultured in double-chamber bioreactor for 2 and 4 weeks. The medium in the spinner flask was changed every week. The magnetic bars were stirred at 70 rpm for 10 hours to distribute the cells more evenly, then at 50 rpm during the rest of the culture period. Quadruplicate samples were removed at each time-point and fixed with formaldehyde, decalcified with 5% formic acid, and dehydrated by exposure to an ethanol gradient. The specimens were embedded in paraffin and cut into 5 μm slices then stained with H & E stain for histological examination. Alcian blue was used to stain glycosaminoglycan and antibodies against human S-100 protein (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) or type II collagen (Santa Cruz Biotech. Inc., CA. USA) to check the chondrocyte phenotype.

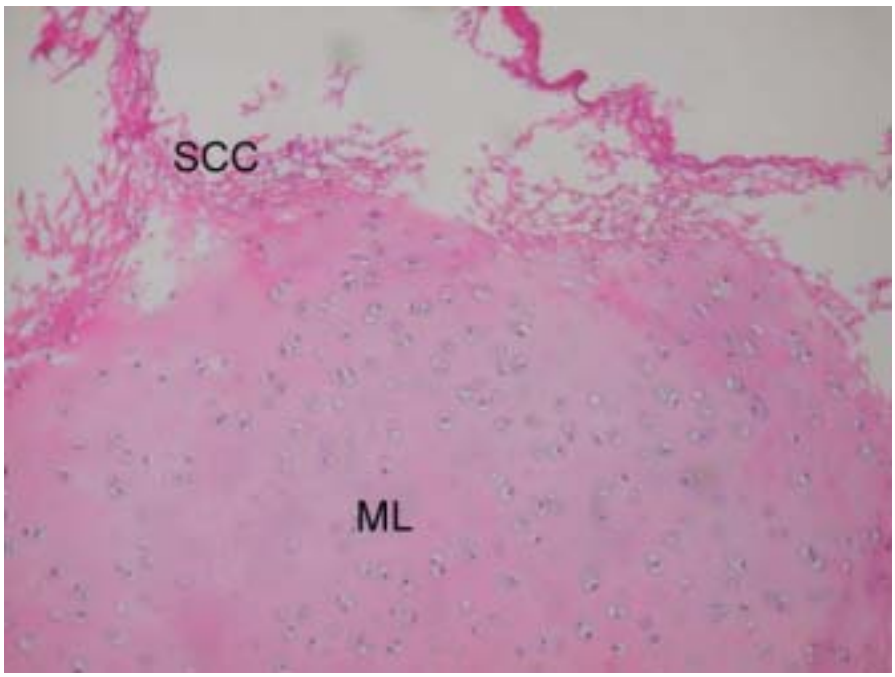
Culture results in Double-Chamber Bioreactor

After 2 weeks' cultivation of the chondrocytes loaded biphasic scaffolds in double-chamber bioreactor, hyaline like matrix production and lacuna formation around chondrocytes were observed. Some chondrocytes can be seen distributed in the scaffold pores without matrix production, indicating the early stage of engineered cartilage formation (Fig. 6(a)). Under Alcian Blue staining, the matrix in engineered cartilage showed different depth of blue colors, showing the different stages of sulfating of the matrix (Fig. 6(b)). After 4 weeks' cultivation in double-chamber bioreactor, the matrix stains bluer than the 2 weeks' matrix, indicating more glycosaminoglycan production by chondrocytes (Fig. 6(c)). On the section of the decalcified specimen, the interface between cartilage and calcium phosphate can be seen. Engineered cartilage with lacuna formation on the top of the calcium phosphate was observed (Fig. 6(d)). The cartilage is well integrated to the calcium phosphate scaffold through infiltration.

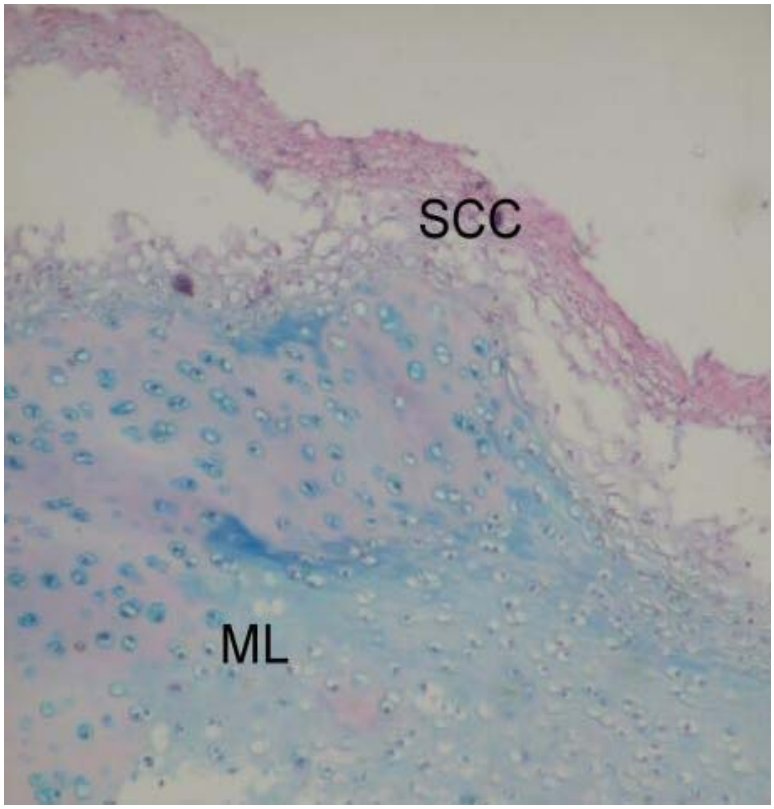
Immunohistochemical staining for S-100 protein and type II collagen showed that chondrocytes cultured in double-chamber bioreactor for 4 weeks retained their phenotype in the cartilage part of biphasic scaffold. As shown in Fig. 7(a), the cells retained their round shape and stained positive for S-100 protein. The round shape of chondrocytes is an indicator of phenotype retention and is essential for matrix

formation. S-100 protein is only found in cells of ectodermal origin and chondrocytes are the only cell of mesenchymal origin to express S-100 protein; S-100 expression is lost when chondrocytes lose their phenotype. As shown in Fig. 7(b), the chondrocytes were also stained with anti-type II collagen antibody. Type II collagen, which is essential for articular cartilage, is produced by chondrocytes; these results therefore show that the chondrocytes in the scaffold were functionally active.

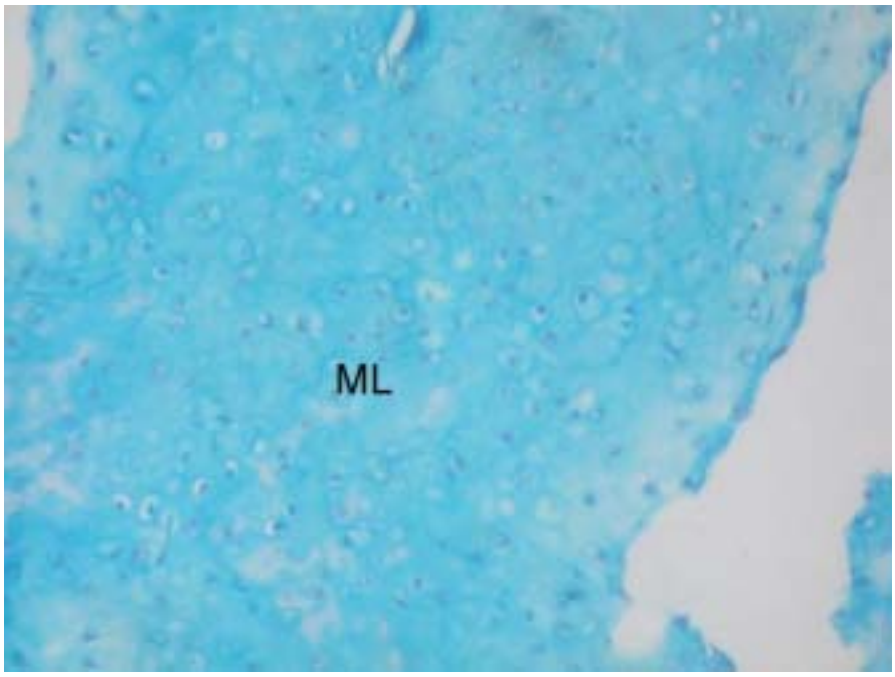
Fig. 6
(a)



(b)



(c)



(d)

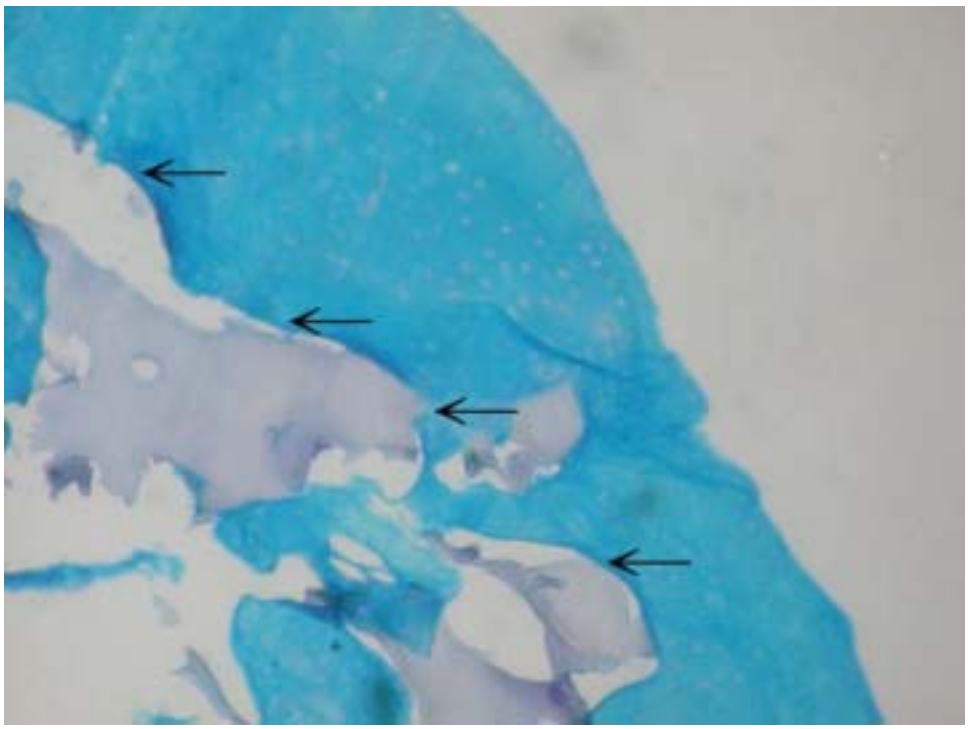
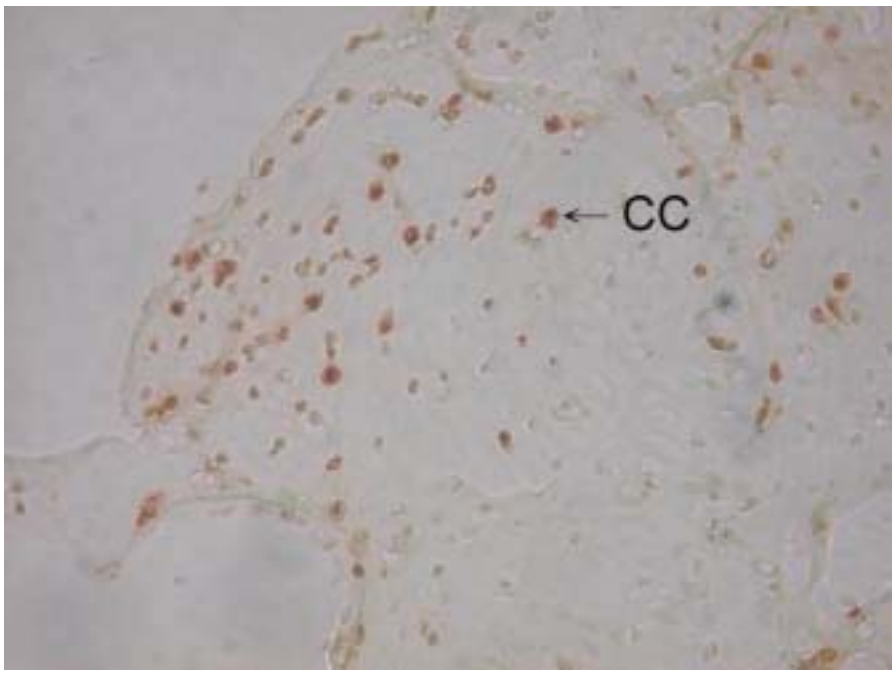
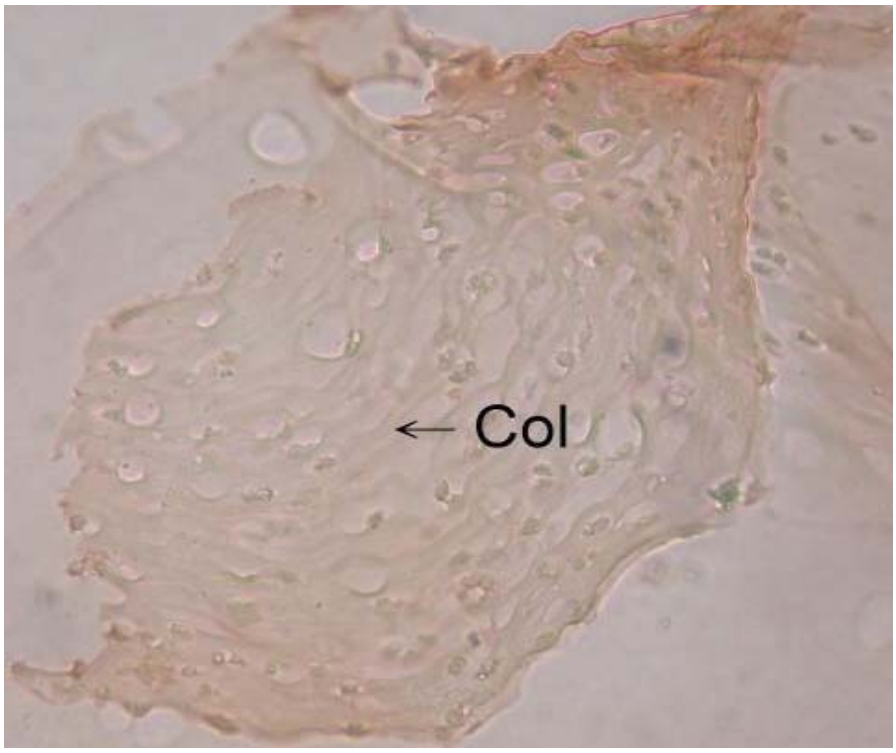


Fig. 7
(a)



(b)



BIPHASIC EFFECTS OF NITRIC OXIDE ON CHONDROCYTE ACTIVITIES

ABSTRACT

Chondrocytes play critical roles in cartilage formation. Promising new therapies based on tissue engineering have been recently developed for cartilage repair. The development of cartilaginous tissue is dependent on the environment that surrounds it. Nitric oxide contributes to the regulation of chondrocyte activities. This study is aimed to evaluate the roles of nitric oxide on human chondrocytes using sodium nitroprusside as a nitric oxide donor. Administration of human chondrocytes with low concentrations of sodium nitroprusside (<0.1 mM) significantly caused a 20-30 % increases in the levels of nitric oxide. In parallel to the increase of nitric oxide, the cell proliferation of chondrocytes was significantly enhanced. Immunoblotting analyses of collagen type I and II revealed that low concentrations of sodium nitroprusside increased the protein levels of these tow extracellular matrixes.

Exposure of chondrocytes to 1 mM sodium nitroprusside significantly augmented 2-fold nitric oxide and lead to cell injury and death. Apoptotic analysis showed that a high concentration of sodium nitroprusside significantly increased the percentage of chondrocytes undergoing apoptosis. This study has shown that the slight increases in nitric oxide (20-30 %) could promote chondrocyte proliferation and synthesis of extracellular matrix collagen type I and II. However, when the increases of nitric oxide are more than 2 folds, chondrocytes would be damaged and the death mechanism is via an apoptotic pathway.

INTRODUCTION

Chondrocytes play important roles in cartilage formation. The autologous in vitro expansion of chondrocytes is a new method for the treatment of localized cartilage defect zones in humans. The association of biomaterials with autologous chondrocytes expanded in vitro can represent a useful tool to regenerate this tissue. The development of cartilaginous tissue is dependent on the environment that surrounds it. Tissue engineers have taken a cue from normal cartilage physiology and incorporated the use of mechanical stimulation into their attempts to engineer functional cartilage. Promising new therapies based on tissue engineering have been

recently developed for cartilage repair (Shieh and Athanasiou, 2003). Recent studies demonstrate that chondrocytes in native articular cartilage and in tissue-engineered constructs respond to mechanical stimuli through multiple regulatory pathways. Responses of the cells are manifested by intra- and intercellular signalling, alterations in transcription level, protein translation, post-translational modifications, and synthesis of intracellular and extracellular macromolecules (Darling and Athanasiou, 2003).

Nitric oxide (NO), produced from L-arginine by nitric oxide synthase (NOS), is involved in various physiological and toxicological processes in many tissues (Moncada et al., 1991). Our previous study had shown that NO, released from sodium nitroprusside, caused osteoblast insults through the apoptotic mechanism according to several lines of evidence including morphological shrinkage, the increase of apoptotic cells and DNA fragmentation (nuclei TUNEL and DNA ladder assays) (Chen et al., 2002). However, low levels of NO could protect osteoblast from oxidative stress-induced cell damage (Chen et al., 2001). Chondrocytes can produce NO and this oxidant contributes to the cell activity (Terauchi et al., 2003; Yoon et al., 2003). This study is aimed to sutdy the roles of NO in chondrocyte activities, including cell proliferation, production of collagen types I and II, cell injuries.

MATERIALS AND METHODS

Cell culture and drug prepatration

Human chondrocytes were purchased from Cell Applications, Inc. (San Diego, CA, USA). These cells were seeded in chondrocyte growth medium (San Diego, CA, USA). All cultures were maintained at 37 ^{o}C in a humidified atmosphere of 5 % CO_2-95 % air. When chondrocytes grew to a confluent monolayer, they were sub-cultured for various experiments.

Sodium nitroprusside (SNP; Sigma, St. Louis, MO, USA) was dissolved in a PBS buffer (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) to 200 mM as a stock solution, protected from light, and stored at –20 ^{o}C for use in related experiments. Chondrocytes were treated with various concentrations of SNP for different time intervals. Cell morphologies were observed and photographed by a reverse-phase microscope.

Assay of nitrite in the culture medium

The amounts of NO in cultured medium were evaluated using enzymatic reduction of nitrate by nitrate reeducates and following the spectrophotometric

analysis of total nitrite with Greiss reagent according to the standard protocol of Bioxytech NO assay kit (OXIS International, Inc, Portland, OR, USA). In the kit, nitrate reductase was used to reduce nitrate to nitrite, and the amounts of total nitrite were determined to represent the amount of NO in the culture medium.

Determination of cell viability

Mitochondrial function of chondrocytes was assayed by the ability of viable cells to convert soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into an insoluble dark blue formazan according to the method of Carmichael et al. (1987). In the bulk cell photometric MTT assay, the bulk conversion of MTT in the well of a 96-well plate was measured photometrically. MTT was dissolved in PBS at a concentration of 5 mg/ml and sterilized by passage through a 0.22-μm filter. This stock solution was added (one part to 10 parts medium) to each well of a 96-well tissue culture plate, and the plate was incubated at 37 ^{o}C for 4 hr. DMSO is added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature, to ensure that all the crystals were dissolved, the plates were read on a microplate reader at a wavelength of 570 nm. A standard curve was set up using 200-50,000 cells/well, and the absorbency was directly proportional to the number of cells over this range.

Analysis of apoptotic cells

Using a flow cytometer, apoptotic chondrocytes were determined to detect DNA fragments in nuclei stained by propidium iodide according to the method of Nicoletti et al. (1991). After treatment, chondrocytes were harvested and fixed in cold 80% ethanol. Following a process of centrifugation and washing, the fixed cells were stained with propidium iodide and analyzed using a FACScan flow-cytometer (FACS Calibur, Becton Dickinson, San Joes, CA, USA) on the basis of a 560 nm dichroic mirror and a 600 nm band pass filter.

Immunoblotting analyses of Collagen type I and II proteins

Cells were washed with PBS and lysed in ice cold RIPA buffer (Tris-HCl pH 7.2, 25 mM; SDS 0.1%; Triton X-100 1%; sodium deoxycholate 1%; NaCl 0.15 M; EDTA 1 mM) containing 1 mM of phenyl methyl sulfonyl fluoride (PMSF), 10 μg/ml of aprotinin, 1 mM of sodium orthovanadate and 5 μg/ml of leupeptin. Protein concentrations were determined with the BCA method (pierce, Rockford, U.S.A.). Protein (50 μg) was resolved on 12.5% polyacrylamide gels and blotted onto nitrocellulose sheets using the semidry blot system (TE 70; Hoefer Scientific Instruments, San Francisco, CA) at 2 mA/cm2 for 60 min in 25 mM Tris-HCl, pH 8.3;

192 mM glycine; and 20% methanol. The membrane was blocked overnight at room temperature with a blocking reagent (20 mM Tris, pH 7.4; 125 mM NaCl; 0.2% Tween 20; 4% nonfat dry milk; and 0.1% sodium azide). Then it was incubated for 1 h with the mouse anti-human collagen type I and II antibodies, washed three times, and then incubated with alkaline phosphatase-conjugated rabbit anti-mouse in PBS and 0.5% Tween 20 for another 45 min with gentle shaking. After three final washes, the proteins were made visible by the Bio-Rad NBT-BCIP color development system.

Statistical Analysis

The statistical significance of the difference between control and treated groups was evaluated by Student's *t*-test. A P value < 0.05 was considered as statistically significant.

RESULTS

NO was detectable in untreated chondrocytes (Table 1). Exposure of chondrocytes to 0.1, 0.5 and 1 mM SNP significantly increased 30%, 3- and 5-fold nitrite. Administration of chondrocytes with 1 mM SNP for 6, 12 and 24 hours significantly augmented 2-, 5- and 7-fold nitrite, respectively.

Exposure of human chondrocytes to 0.1 mM SNP for 24 hours increased 40% viability of human chondrocytes (Table 2). SNP at 0.5 and 1.0 mM significantly decreased 30 and 50% cell viability, respectively. When human chondrocytes were exposed to 1 mM SNP for 6, 12 and 24 hours, the cell viability was decreased by 20, 50 and 60%, respectively.

Cell proliferation was assayed using the trypan blue exclusion method to analyse the permeality of the cell membrane (Table 3). Administration of human chondrocytes with 0.1 mM SNP for 24 hours significantly increase 34% cell proliferation. However, SNP at 0.5 and 1 mM decreased 29 and 52% cell proliferation. After exposing to 1 mM SNP for 6, 12 and 24 hours, the cell proliferation of human chondrocytes was suppressed by 27, 42 and 58%, respectively.

SNP at 0.1 mM did not cause chondrocyte apoptosis (Table 4). Exposure of human chondrocyte to 0.5 and 1.5 mM SNP for 24 hours significantly increased 31 and 57% chondrocytes undergoing apoptosis. The percentages of chondrocytes undergoing apoptosis reached to 12, 49 and 61% after exposing to 1 mM SNP for 6, 12 and 24 hours.

Immunoblotting analysis revealed that collagen type I and II are detectable in human chondrocytes (Fig. 1, lane 1). Administration of human chondrocytes with 0.1 mM SNP significantly increased the levels of collagen type I and II proteins (Fig. 1,

lane 2). Quantitative analyses using β-actin as the internal standard showed that SNP at 0.1 mM significantly increase 5- and 3-fold collagen type I and II protein levels, respectively.

CONCLUSSION

This study has shown that nitric oxide released from sodium nitroprusside has biphasic effects on human chondrocyte activities: low concentrations of nitric oxide production could promote chondrocyte proliferation and synthesis of collagen type I and II, however, high concentrations of nitric oxide would lead to cell insults and death through an apoptotic pathway.

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TABLES AND FIGURES

Table 1 Concentration- and time-dependent effects of SNP on nitrite production in human chondrocytes

SNP, mM	Nitrite, μM	Time, hr	Nitrite, μM
0	2.9 ± 0.2	0	2.9 ± 0.3
0.1	4.0 ± 0.3	6	7.9 ± 0.9*
0.5	8.1 ± 0.5*	12	16.5 ± 2.1*
1.0	16.2 ± 0.7*	24	19.4 ± 3.5*

Human chondrocytes were treated with 0, 0.1, 0.5 and 1.0 mM sodium nitroprusside (SNP) for 16 hours or with 1 mM SNP for 6, 12 and 24 hours. The levels of nitrite in culture medium were assayed by the Griess reaction. Each value was represented Mean ± SE for n=6. *Value significantly different from the respective control, $P < 0.05$.

Table 2 Concentration- and time-dependent

effects of SNP on viability of human chondrocytes analyzed by MTT assay

SNP, mM	Cell viability (OD value at 570nm)	Time, hr	Cell viability (OD value at 570nm)
0	0.86 ± 0.08	0	1.03 ± 0.09
0.1	1.20 ± 0.09*	6	0.84 ± 0.03*
0.5	0.61 ± 0.07*	12	0.52 ± 0.03*
1.0	0.46 ± 0.03*	24	0.42 ± 0.05*

Human chondrocytes were treated with 0, 0.1, 0.5 and 1.0 mM sodium nitroprusside (SNP) for 24 hours or with 1 mM SNP for 6, 12 and 24 hours. Cell viability was assayed by the colorimetric MTT test as described in Materials and methods. Each value was represented Mean ± SE for n=12. *Value significantly different from the respective control, $P < 0.05$.

Table 3 Concentration- and time-dependent effects of SNP on viability of human chondrocytes analyzed by trypan blue exclusion assay

SNP, mM	Cell viability (OD value at 570nm)	Time, hr	Cell viability (OD value at 570nm)
0	100	0	100
0.1	134 ± 16*	6	73 ± 17*
0.5	71 ± 20*	12	56 ± 17*
1.0	48 ± 12*	24	42 ± 14*

Human chondrocytes were treated with 0, 0.1, 0.5 and 1.0 mM sodium nitroprusside (SNP) for 24 hours or with 1 mM SNP for 6, 12 and 24 hours. Cell viability was assayed by the trypan blue exclusion method as described in Materials and methods. Each value was represented Mean ± SE for n=5. *Value significantly different from the respective control, $P < 0.05$.

Table 4 Concentration- and time-dependent effects of SNP on human chondrocyte apoptosis

Apoptotic cells, %			
mM		hour	
0	2.1 ± 0.4	0	1.3 ± 0.4
0.1	1.9 ± 0.3	8	12.1 ± 2.7*
0.5	31.9 ± 12.1*	12	48.7 ± 10.3*
1.0	57.1 ± 13.4*	24	60.6 ± 12.5*

Human chondrocytes were treated with 0, 0.1, 0.5 and 1.0 mM sodium nitroprusside (SNP) for 24 hours or with 1 mM SNP for 6, 12 and 24 hours. The percentages of apoptotic cells in human chondrocytes were determined by a flow cytometric method as described in Materials and Methods. Each value was represented Mean ± SE for n=6. *Value significantly different from the respective control, $P < 0.05$.

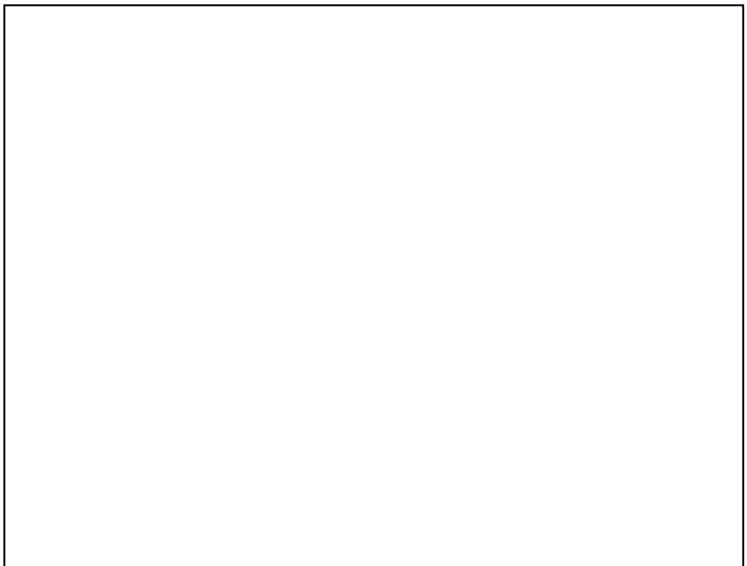

Fig. 1 Effects of SNP on collagen type I and II protein. Human chondrocytes were treated with 0.1 mM SNP for 24 hours and cytosolic proteins were isolated. Immunoblotting analyses of collagen type I and II were carried out using monoclonal antibodies against human collagen type I and II protein.

利用生物活性基質進行軟骨級韌帶之組織工程

Using hydrogel as drug carrier and scaffold for cartilage regeneration

一、中文摘要（關鍵詞：聚乳酸、微小球、幾丁聚醣、藥物釋放、軟骨再生）

組織工程中生長因子的傳遞與制放在組織再生及組織培養中扮演著相當重要的地位。近年使用的基材偏向使用方便的水膠，因此本實驗模擬組織在體內培養的狀態，利用聚乳酸微小球包覆藥物樣本 lidocaine，並將之分散於去乙醯幾丁聚醣凝膠中以研究藥物釋放的情形及其機制，並探討幾丁聚醣凝膠作為細胞培養基材的潛力。實驗結果顯示經由幾丁聚醣基材之藥物樣本在凝膠中可減緩其釋放速率，達到長效性藥物釋放之目的，而細胞在幾丁聚醣凝膠中的生長情形良好並可建構為立體的結構。因此幾丁聚醣凝膠是一個良好的控制釋藥與組織重建的材料。

Growth factor delivery in tissue engineering affects the success of tissue repair. Recent studies turned to choose hydrogel as matrix for its convenience in use. Our experiments mimic the conditions of tissue growing in human body; therefore, the mechanisms of drug delivery system by using PLLA microspheres encapsulating lidocaine were studied; each of which was mixed in chitosan gel, and the potential of chitosan as tissue matrix was also investigated. The results demonstrate that sample drugs in chitosan gel make the release rate diminish and achieve long-term release. In addition, chondrocytes performed well in chitosan gel and constructed 3D structure with matrix. In conclusion, chitosan gel is an appropriate material for both controlled release and tissue regeneration.

Keywords: PLLA, microsphere, chitosan, drug delivery, cartilage regeneration

二、計畫的緣由與目的

利用組織工程的技術，重建修補身體的組織缺陷是目前研究發展的目標。組織重建所需要的元素包括細胞、胞外間質及生長因子[1]。生物可分解性高分子可以在生物體內自行分解並代謝，一些特定的材料如聚乳酸[1]亦有良好的生物適應性。

近年的組織工程研究趨向於使用在低溫為液狀、升溫可成膠狀的材料，優點是可填覆各種形狀的傷口、避免複雜手術的操作，亦可輕易結合藥物治療。本計畫是以水膠 PluronicF-127 及幾丁聚醣凝膠作為熱可逆性支架探討其熱成膠現象，並加入聚乳酸微粒當作藥物載體，包覆水溶性與油溶性作為控制釋藥，此外，將軟骨細胞培養於凝膠，以評估水膠作為細胞培養及控制釋藥基材的能力。

三、研究方法

實驗所選用的水膠是一種熱成膠性凝膠，因此須先決定材料最適當的濃度及酸鹼值。首先我們測定 Pluronic F-127 以及幾丁聚醣成膠溫度。幾丁聚醣凝膠是將 36%的

Glycerophosphate 溶液滴入 2%的 chitosan/HCl 溶液而形成 pH=7.2 的熱敏感性凝膠。另外配置不同濃度的 Pluronic F-127 水溶液，利用黏度計測定不同溫度下兩種溶液黏度的變化值。

在藥物釋放實驗方面，以 PLLA 包覆油溶性藥物 lidocaine，混合在凝膠中進行釋放，另外則以 free drug 從基材釋放、PLLA 微小球包覆藥物從透析膜釋放作為對照，每種條件皆加入固定量 PBS 溶液。在固定時間測定藥物(lidocaine at UV 215nm)的濃度。

我們使用豬軟骨細胞在較低溫下與幾丁聚醣凝膠混合均勻，置入細胞培養箱中使之形成失去流動性的人工組織，以評估水膠作為組織工程支架的可能性。以 DMEM+10%FBS 為培養液，培養 3 週後觀察細胞的型態。

四、結果與討論

(一)熱成膠現象探討

Pluronic-F127 是經 FDA 核准且在藥劑中常被用到的一種高分子材料，具有 sol-gel 的特性，在低溫時溶液呈液狀；而在接近體溫時會變成水膠狀，不同濃度 Pluronic-F127 溫度對黏度的關係如圖一，顯示濃度越高，成膠溫度越低，如表一所示。形成凝膠的過程一般認為是親水性的 PEO 鏈在外部，疏水性的 PPO 鏈在核心形成微小球。當微小球不斷累積糾結即形成大塊的膠狀物。在體溫 37^{o}C 下成膠最適當濃度為 15%至 20%，因為過高濃度造成操作不易，在液態下與細胞混和時會因控制的溫度過低而造成細胞的死亡。然而，因為 Pluronic F-127 優良的親水性，使其於水溶液中極易溶解，無法維持固定的形狀。因此根據評估的結果，Pluronic F-127 水膠不適宜作為體外藥物釋放或體內組織重建的基材。

幾丁聚醣是一種半結晶狀的高分子，在 pH＞6.2 時會和陰離子交聯而沉澱，因而限制了其用途。低 pH 值的幾丁聚醣溶液培養細胞或直接注入體內皆因酸度過高會造成細胞的死亡。Chenite [2]製作出在 pH=7 時仍為液體，且當溫度升高時，溶膠轉變為凝膠的幾丁聚醣溶液，僅須將調整 chitosan 溶液酸鹼度的溶液改為弱鹼性的 Glycerophosphate 即可。造成幾丁聚醣凝膠成膠的原因推測為隨著溫度的上昇，幾丁聚醣分子疏水性的作用力大於分子內氫鍵力，使幾丁聚醣分子彼此及與 Glycerophosphate 分子之間聚集形成凝塊[2]。實驗所選用的去乙醯化程度為 85%，以黏度計測量所得的成膠溫度為 26.8℃，如圖二，且在 PBS 溶液及培養液中均能維持其形狀，因此可以應用在人體進行藥物釋放或組織工程基材。

(二)體外藥物釋放實驗

實驗中選用的代表藥物為油溶性的 lidocaine。Lidocaine 是一種具有抗發炎效果的油溶液藥物，可利用 lidocaine 在 UV 215nm 處的吸光值測量固定時間下藥物的釋放情形。首先利用 PLLA 包覆 lidocaine 後，於幾丁聚醣凝膠中進行藥物釋放實驗。控制釋藥的能力是 drug in PLLA from matrix ＞free drug from matrix＞drug in PLLA from dialysis membrane，如圖三。Burst release 的現象在 48 小時內發生，但從基材釋放出來的藥較少，因凝膠本身就可當成控制釋藥的基材。可減緩其釋放速率、增加藥物滯留時間。

藥物初始釋放量皆很明顯上升，主要是因為幾丁聚醣在成膠過程中，發生體積驟縮現象，使得原本懸浮在 PLLA 微小球內的藥物或懸浮藥物因體積的變化而游離出來，因此造成最初的 24 小時內就達到高的釋放量。

(三)幾丁聚醣凝膠的細胞培養評估

軟骨的組織中最主要胞外間質為 GAGs，而幾丁聚醣的和 GAGs 的分子結構相當類似，因此將此材料用於模擬體內軟骨細胞的生長環境是一個合理的方法。

在培養 2、7、14 天時測試細胞增殖 MTS test 以評估材料的生物適應性，初始豬軟骨細胞數為 1.2 ×10^6 cells/ml。在 2 天內，培養於細胞培養盤的細胞較多，但之後因細胞凋亡使數量劇減，而培養在凝膠內的細胞在過了適應期後，細胞數明顯增加，顯示幾丁聚醣凝膠對於軟骨細胞培養有正面的影響，如圖四。

此外，如圖五所示，培養於凝膠中的軟骨細胞保持圓形，因此仍具有軟骨細胞的表型與功能，但在培養盤上的細胞則呈細長紡錘型而可能喪失了軟骨細胞的特性。而細胞在凝膠中形成了立體的結構，圖六即為同一個視野不同的焦距的連續圖，在不同位置的焦距可觀察到不同的細胞，顯示這樣的組織是一個良好的 3D 結構，可應用在真實的軟骨傷害中。

五、結論

綜合上述，幾丁聚醣凝膠是一極優的長效性藥物釋放系統，而細胞在幾丁聚醣凝膠中的生長情形良好並建構為立體的結構。因此幾丁聚醣凝膠是一個良好的控制釋藥與組織重建的材料。

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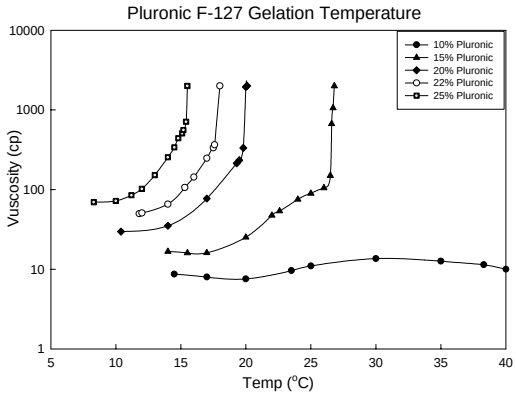
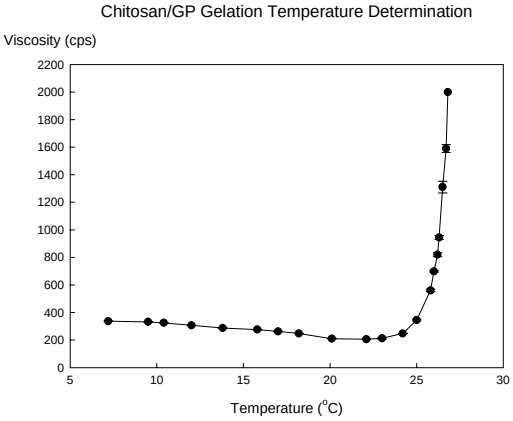
七、圖表



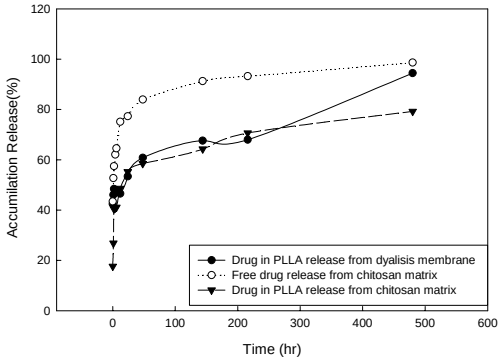
圖一、不同濃度 Pluronic-F127 溫度對黏度的關係

表一、Pluronic-F127 之成膠溫度

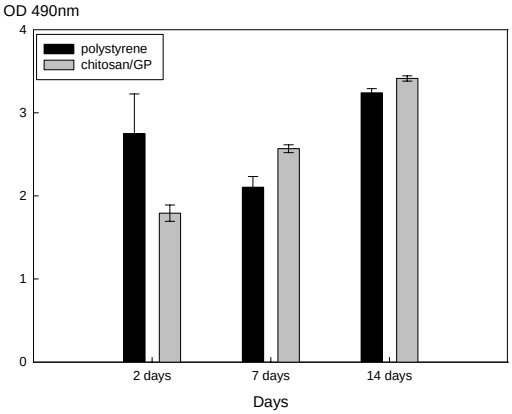
Pluronic-F127 溶液濃度	10%	15%	20%	22%	25%
成膠溫度(°C)	no	26.7	20.1	18.0	15.5



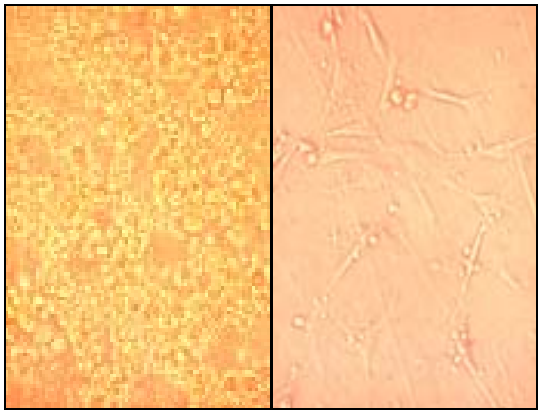
圖二、pH 7.2 幾丁聚醣凝膠黏度與溫度變化圖



圖三、Lidocaine in PLLA 藥物釋放圖

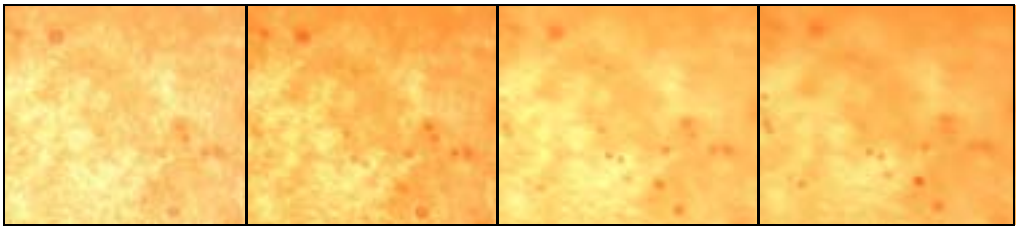


圖四、細胞增殖 MTS Test



(a) (b)

圖五、細胞型態培養於 (a)幾丁聚醣凝膠 (b)細胞培養盤



圖六、不同焦距相同視野的軟骨細胞，顯示整體構成了立體的結構

韌帶組織工程之研究

I.簡介（Introduction）與背景說明

根據 Langer 的統計，美國每年至少有十二萬的病人需要進行肌腱或韌帶置換的手術，在台灣也因為機車為普遍之交通工具，導致車禍前十字韌帶斷裂之情形較國外更為常見。目前臨床上處理韌帶或肌腱的斷裂大多是採用自體或異體移植的方式，或是以人工合成之材料作為取代物。然而目前為止，以合成材料作為取代物之手術雖然在臨床上相當普及，但材料經過長時間之後仍會有老化等問題發生，而異體移植目前仍處於臨床試驗之階段，主要的問題為仍無法有效抑制免疫及發炎反應的發生。因此，找尋合適的方法幫助撕裂肌腱及韌帶之修復仍是臨床上亟欲解決之課題。

一般生醫材料多為身體組織損壞時之替代品，因此需具有良好之穩定與持久性、適當的物理化學性質，及良好的生物相容性。並於生醫材料植入人體後能維持其生理機能。近幾年來由於生醫材料大部分需植入人體，因此多往生物裂解方面研究，許多脂肪族聚酯類(aliphatic polyesters)聚合物諸如聚乳酸(polylatic acid，PLA)、聚甘醇酸(polyglycolic acid，PGA)、聚己內酯等均具有生物可降解性及生物可吸收性，而此類聚合物已發展在止血材料、可降解之手術縫線、固定用之骨釘、骨板等牙科及骨科材料上，可作為修補及促進軟骨再生材料，待組織修復後材料易被分解吸收。

近年來國外專家學者對細胞培養的研究結果顯示，生醫材料的應用中，生物體往往只接觸到生醫材料的表面，故材料表面構造的重要性遠大於整體（bulk）構造，（整體構造與機械性質或其他性質較有關聯），而根據過去本實驗室的研究也發現，材料表面結構的改變確實會對於細胞的貼附及生長有很大的影響，因此實驗的初步將先探討不同表面結構之聚乳酸薄膜對於韌帶及肌腱細胞貼附及生長的影響。

組織中的活細胞是藉由一層細胞外基質(extracellular matrices, ECMs)進行貼附和生長行為，ECM 大部分是由蛋白質(protein) 和聚葡萄胺(glycosaminoglycans)所構成。而 ECM 已經被確認能調節細胞的多種行為，例如細胞的貼附、生長、移動和分化等等。而構成這些 ECM 的生物分子成份包含有如 fibronectin, collagen, vitronectin, polylysine 和 laminine 諸如此類具有黏著性的蛋白質。而有鑑於過去學者認為聚乳酸為疏水性之生醫材料，其生物適合性並不佳，因此本計畫在聚乳酸的表面作改質，塗佈較適合韌帶及肌腱細胞生長之細胞外間質(Extracellular matrix 簡稱 ECM)。根據文獻，肌腱為連接骨骼與肌肉的組織，而韌帶則為連接骨骼與骨骼之間的組織，在肌腱及韌帶的周圍都被大量的第一型膠原蛋白(type-I collagen)及少量的聚葡萄胺(glycosaminoglycans)所環繞。因此，本研究探討在聚乳酸薄膜表面塗佈不同的 ECM 如 type I collagen、fibronectin 等，找尋最適於內側副韌帶細胞及前十字韌帶細胞之 ECM。

II 關鍵材料及方法(Subject and Methods)

※薄膜製備

本研究使用相轉變法（phase inversion）來製備薄膜，聚乳酸高分子於二氯甲烷，於室溫下充分攪拌約一天，使其充分溶解均勻。

將混合均勻的高分子溶液，取一玻璃，將此溶液倒至玻璃板，以刮刀將溶液均勻塗在玻璃板上，再將此玻璃板置於bath槽中或室溫下一天後脫膜。

※膜性質測試

- 掃描式電子顯微鏡（SEM）觀察

將薄膜表面以雙面膠或銀膠黏於載台上，將薄膜浸泡在液氮中淬斷，也以雙面膠黏於載台上，以掃描式電子顯微鏡觀察試片表面和斷切面的形態。

※ 生物適應性

- 細胞培養

細胞培養主要分為細胞貼附（cell adhesion）和細胞成長（cell growth）兩種。而細胞培養乃是評估材料生物適合性主要的方法之一。

若材料表面的生物適合性不佳，則細胞不易與材料的表面產生交互作用，也就是細胞不易貼附及成長。反之，若材料表面的生物適合性較佳，則材料表面會貼附細胞並進而增生。

實驗使用的細胞為取自純種紐西蘭白兔的前十字韌帶細胞及內側副韌帶細胞。細胞培養基乃為市售的 MEM 培養液，混合 10％胎牛血清 FBS（Fetal Bovine Serum）並加入 1%的 sodium pyruvate 以及 1％的三合一抗生素（antibiotics）所調配而成。

初代培養步驟：此初代培養步驟之主要目的是取得前十字韌帶(Anterior Cruciate Ligament)ACL 之韌帶細胞，並將其種在不同表面結構之薄膜上。此韌帶細胞是取自膝關節處，其過程如下：

- 韌帶及肌腱細胞之取得：

韌帶或肌腱的纖維細胞培養，採用 Nagineni CN etal 的方法加以改進而來。膝前十字韌帶組織取自人工膝關節置換的病人所切除的前十字韌帶或前十字韌帶斷裂病人，肌腱組織取自接受前十字韌帶重建的病人的髖股韌帶或大腿後方內側的半腱狀肌，係手術中修剪剩下的肌腱、清除相連的肌肉和結締組織，在培養盤上切成小塊的 explant cultures。

- 純化細胞：

將取得之組織以 PBS 潤洗 2 次，切碎至 1mm^2，置放於培養盤上。置放於 37℃，5%CO_2 之培養箱中。待細胞長出後，移除小塊的組織，讓細胞繼續增生，直到滿盤。每三天更換新鮮之培養基，直到有足夠之韌帶細胞，再種至準備好之材料上。

將細胞"撒"入有試片的細胞培養皿上，細胞數量控制為 8 ㄨ10^4/ml，置於細胞培養箱中培養。其每個 well 培養基為 1 ml/well。每組薄膜試片均重覆 6 次實驗。

- 評估肌腱及韌帶細胞生長情形：
- 光學顯微鏡觀察

因為光學顯微鏡只能觀察光線可通過的樣本，所以只能用光學顯微鏡觀察培養在對照組和乾式膜上神經細胞生長的情形。

- MTT 試驗

MTT test 是利用活細胞中的粒線體可以將黃色的 MTT 代謝為藍色的 formazan 晶體，可以用來測量細胞的存活率，利用 Mosmann et al 所使用之方法，經改善後如下：

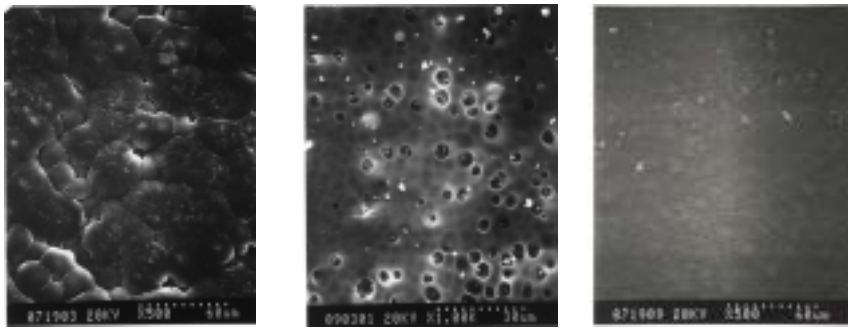
1. 將培養盤中的 medium 抽出並以 PBS 溶液沖洗兩次，將 0.1g 的 MTT 粉末溶於 50ml 的 PBS 溶液中，過濾滅菌，然後在每一個 well 中加入 0.1ml 的 MTT 溶液，置於 incubator 中作用 3 小時，整個過程需避光。
2. 在每個 well 中加入 0.2ml DMSO 溶液，在搖盪器(shaker)上搖 15 分鐘，使其均勻。將細胞內的 MTT 產物 formazan 溶出。
3. 取 0.1ml 溶液置於 96well 的培養盤中，以 ELISA Reader 讀取波長 570 nm 的吸光值。

- 電子顯微鏡觀察：

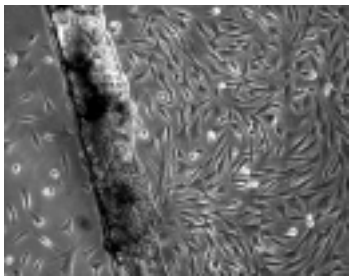
掃瞄式電子顯微鏡觀察前(SEM)必須先作細胞固定。培養箱裡，培養皿中薄膜試片上的細胞，從細胞培養箱中拿出，去除培養基，先用 PBS 清洗約兩次，以戊二醛(Glutaraldehyde)固定，然後，進行臨界點乾燥（Critical Point Dryer，CPD）處理，

以防細胞變形。將其表面用鍍金處理，鍍金後以掃描式電子顯微鏡觀察細胞形態。

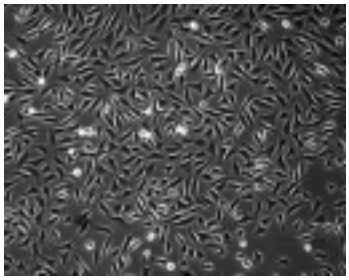
III.重要之結果 (Results)



圖一三種不同結構之PLLA薄膜之上表面SEM圖(a)顆粒膜(b)多孔膜(c)緻密膜



內側副韌帶細胞(100X)



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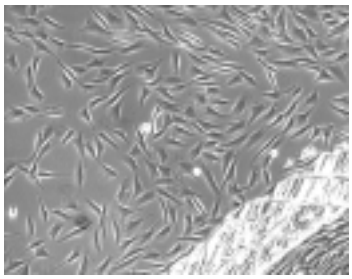
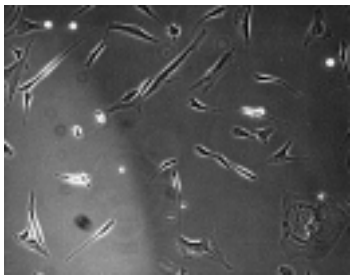


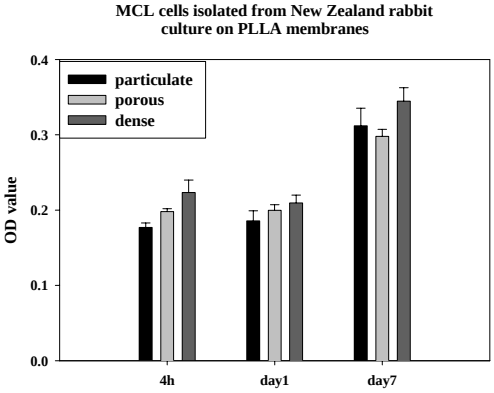
前十字韌帶細胞(100X)

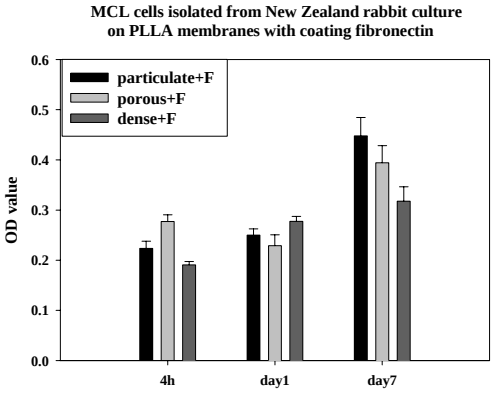


前十字韌帶細胞(100X)

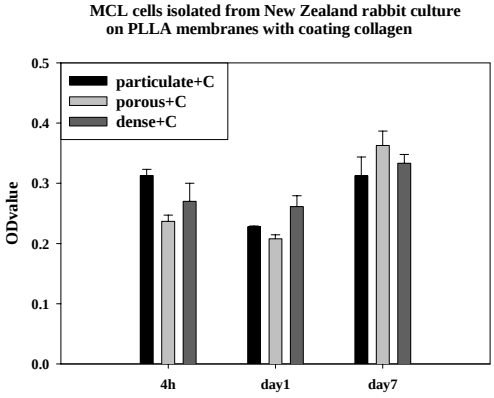
圖二、內側副韌帶細胞(MCL cell)與前十字韌帶細胞(ACL cell)之光學顯微鏡照片

由光學顯微鏡的照片可看出細胞會自組織的周圍遷徙出來，組織的周圍有較多的細胞，距離組織愈遠則細胞呈現愈稀疏的狀態。照片顯示內側副韌帶的細胞較為細而小，而前十字韌帶的細胞則是較為細而長，若觀察其自組織遷徙出來的速度是MCL較ACL快速，此情形均與paper所描述雷同(9)，因此可以確定我們所取得之組織確實是內側副韌帶及前十字韌帶 。



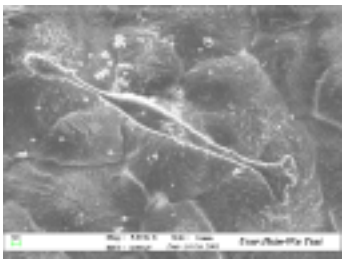


(a)未塗佈ECM　　(b)塗佈fibronectin

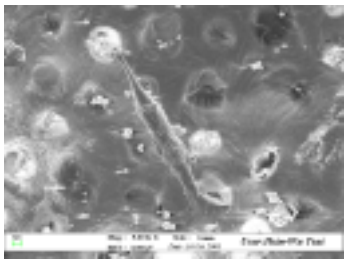


(c)塗佈collagen

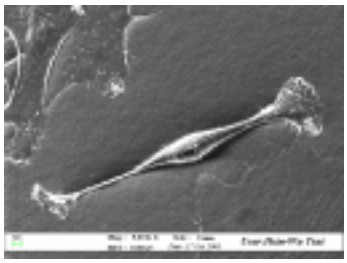
圖三、內側副韌帶細胞(MCL)於不同表面結構之聚乳酸薄膜上培養4小時、一天、及七天的MTT結果（n=4）



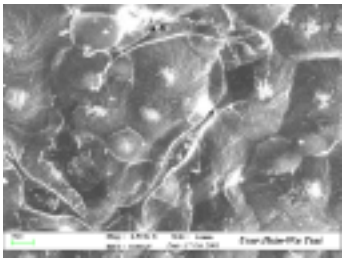
(a)未塗佈之顆粒膜

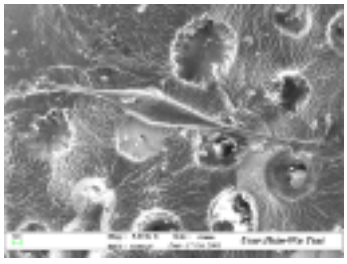


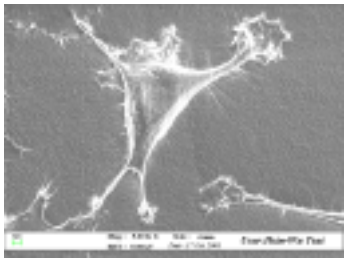
(b)未塗佈之多孔膜



(c)未塗佈之緻密膜







(d)塗佈collagen之顆粒膜　(e)塗佈collagen之多孔膜　(f)塗佈collagen之緻密膜

圖四、內側副韌帶細胞(MCL)於不同表面結構之聚乳酸薄膜上培養4小時之SEM

由圖三之(a)可以看出在MCL細胞的貼附及生長上三種不同結構的薄膜並沒有明顯

之差異，甚至有多孔膜較差的趨勢。由(b)圖可以看出添加fibronectin(纖維網蛋白)對於初期的貼附並沒有明顯的幫助，但由第七天的結果可以看出其MTT讀值都超過0.4，而(a)圖的第七天讀值均不到0.4，因此可以推測添加纖維網蛋白有助於內側副韌帶細胞後期之生長。若比較三種薄膜在二(b)圖的差異，可以看出第七天的顆粒型薄膜讀值最高，推測可能是因為顆粒型薄膜的表面積最高，因此可塗佈之面積最大的關係。由圖二(c)可以看出塗佈collagen(膠原蛋白)對於內側副韌帶細胞的四小時貼附有較為明顯的幫助，但對於後期的生長似乎並無助益。而顆粒型薄膜之所以有較高的讀值，我們推測其原因也是因為表面積較大的關係。

由圖四的SEM可以看出內側副韌帶細胞在未塗佈任何ECM的三種薄膜上，其貼附的型態並無明顯的差異，都是呈細長型的型態，但在塗佈collagen之後，可以看出細胞呈現更為攤平的貼附型態，與MTT結果相吻合。由此可以再次證明在四小時的時間點上塗佈collagen是有幫助的。

VI.討論 (Discussion)與成果之貢獻

由以上的實驗結果可以發現將內側副韌帶細胞培養在三種不同型態的聚乳酸薄膜上並無明顯的差異，推測多孔膜略差的原因可能是因為此細胞一顆約25μm，但孔洞卻只有8μm，因此細胞在多孔膜上的貼附情形反而不如緻密膜。而較少被人討論到的顆粒型薄膜雖然在未塗佈ECM時並沒有較佳的貼附及生長效果，但因具有較大表面積的優勢，因此對於塗佈collagen時有助於細胞的初期貼附及塗佈fibronectin時有助於細胞的後期生長都有優於其他兩種薄膜的結果。至於為何collagen有助於此細胞之貼附，而fibronectin有助於後期的生長呢?我們推測原因是type I collagen本來就是內側副韌帶細胞周遭最重要的一種細胞外間質，因此初期細胞尚未自我分泌collagen時，塗佈collagen有模擬體內韌帶環境的效果。而後期由於韌帶細胞本身即具有分泌collagen的功能，因此塗佈collagen並沒有太大的幫助。而韌帶細胞有可以和纖維網蛋白結合的domain，因此塗佈纖維網蛋白有幫助韌帶細胞後期生長之效果。

此研究發展出一種較為新式的顆粒型薄膜，可以提供更大的表面積以吸附更多的細胞外間質，並發現collagen有助於細胞之貼附，fibronectin而有助於細胞之後期生長，此機制可以進一步應用在斷裂韌帶之輔助修復上。

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Summary

Previous reports debated on the effects of differentiation on adenoviral vectors (AdV) transduction efficiency and Coxsackie-adenovirus receptor (CAR) expression. This prompted us to investigate the efficiency of AdV transduction and CAR expression in human mesenchymal stem cells (hMSC) and their differentiated progeny. Current results revealed high efficiency (> 90%) of AdV transduction and a consistent level of CAR expression in hMSC by the use of AdV carrying the green fluorescent protein (GFP) reporter gene. Competition of CAR with blocking monoclonal antibody RmcB resulted in a reduction in transduction efficiency; indicating the CAR involvement in transduction of hMSC. The cells were then induced to differentiate into bone, fat or neural cells, and results demonstrated that the differentiation was accompanied with a consistent decline in AdV transduction and a decrement in CAR expression. According to the present investigation, undifferentiated hMSC can serve as a gene-delivering system and gene transfer into hMSC before differentiation can resolve the difficulties in transduction of their differentiated progeny.

Key Words

Human mesenchymal stem cells (hMSC), adenoviral vectors (AdV), Coxsackie-adenovirus

receptor (CAR), Green fluorescent protein (GFP), gene transfer

Introduction

Adenoviral vectors (AdV) are well known in its stability, ease handling and highly uptake by many cell types including non-cycling cells. In addition, AdV can be concentrated to extremely high titers as well.[1-3] Recent advances in AdV modifications offer a less immunogenic strain (defective recombinants) and an increase in the recombinant genome insert size.[4,5] Therefore, the application of AdV is more relevant for human gene therapy than other means.

Since the efficacy of virus entry is restricted by interaction of viral fibers and cell surface receptors, the abundance and the variety of viral associated surface molecules expressed in cells are particularly important. The entry pathway for AdV consists of initial binding to the cells, which is mediated by the association of the adenoviral fiber protein and a 46 kDa membrane protein known as Coxsackie-adenovirus receptor (CAR),[6] followed by internalization, which is through an interaction of viral penton arginine-glycine-aspartate (RGD) sequence to the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins.[7]

The CAR is a primary passage mediated the uptake of adenovirus in 293 cell line, HeLa cell line and the cells derived from airways. However, cellular function of CAR is not clear and CAR is only expressed in some types of cells and tissues. It has been shown that CAR expression correlated not only with the undifferentiated state, but also with efficiency of AdV transduction in oropharyngeal epithelial cells that the superficial layer (more developed) had less CAR expression than basal layer (less developed).[8] In contrast, CAR expression was only

demonstrated in a small subset of $CD34^+$ bone marrow (BM) and mobilized blood cells,[9,10] while AdV transduction efficiency and CAR expression increase as the cells differentiate into erythroid and myeloid haematopoietic cells.[11] CAR is expressed in human mesenchymal stem cells (hMSC),[12] but not expressed in certain kind of mesenchymal cells, such as primary human fibroblast.[13]

Since hMSC can be induced to differentiate into certain kinds of mesenchymal and nonmesenchymal tissues, they provide a model to study the effects of differentiation on AdV transduction and CAR expression. Therefore, we investigated AdV tranduction efficiency and CAR expression in hMSC and their differentiated progeny. Efforts were also made to characterize the tranduced hMSC in the differentiation potential and to know the persistence of transgene expression into hMSC progeny.

Results

In vitro AdV-mediated gene transfer into hMSC

For these studies, gene transfer was demonstrated by AdV transduction with the enhanced green fluorescent protein (GFP) gene. The hMSC cultures were infected with Ad-PGK-GFP; and 2 days later, transduction was assessed by transgene expression. As seen in Figure 1, after exposure to Ad-PGK-GFP for 2 days, a population of transduced cells was detected under both fluorescent microscope (Figure 1a & b) and by flow cytometry analysis (Figure 1c), displaying high fluorescent signal for GFP. At this time, transduced cells encoding GFP fluorescence were found to have a good viability and no disturbance in adherence. Transgene was retained and expressed by a small fraction of hMSC progeny even up to 4 weeks after infection (data not shown). A strong green fluorescence in the infected hMSC indicated a high efficiency of virus particles entering in cells. The efficiency of AdV-mediated gene transfer into hMSC as analyzed by flow cytometry was 91.7 ± 6.6% (Table 1). High AdV transduction efficiency was also attained by the use of Ad-CMV-GFP, a vector directing the expression of GFP under the control of a cytomegalovirus (CMV) promoter (data not shown).

Expression and participation of CAR in AdV-mediated gene transfer into hMSC

Conget and Minguell[12] have reported that the attachment of CAR receptor but not integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ is required for AdV-mediated gene transfer into hMSC. Therefore,

we investigated by flow cytometry whether these cells express CAR. As shown in Figure 1d, CAR expression was demonstrated in hMSC by using CAR specific monoclonal antibody RmcB. The pattern of CAR expression was homogeneous, and near half of the cells had moderate expression of CAR receptor. To determine the participation of CAR in AdV infection, hMSC were infected with Ad-PGK-GFP in either presence or absence of monoclonal antibody RmcB for competing CAR receptor. As shown in Figure 2, incubating specific monoclonal antibody RmcB against CAR receptor immediately before AdV infection was able to inhibit AdV infection by nearly 40%. Moreover, the effect of CAR competition by specific monoclonal antibody to inhibit AdV infection in hMSC was a dose-dependent manner.

Reduced AdV transduction efficiency and CAR expression in osteogenic differentiated hMSC

The hMSC culture was induced to differentiate along the osteogenic, adipogenic or neurogenic lineage as previously described.[14,15] As shown in Figure 3 and Table 1, hMSC had a relatively low efficiency of AdV transduction with 31%, 14% and 59% at 10 days, 17 days and 23 days respectively after osteogenic induction. The low efficiency of AdV transduction in the differentiated cells was correlated with a decrease in CAR expression after 10 days of osteogenic induction. The level of CAR expression was undetectable at 17 days after

induction, but a regain of CAR was observed in 23-days induced cells.

Reduced AdV transduction efficiency and CAR expression in adipogenic differentiated hMSC

The AdV transduction efficiency was measured in hMSC on day 3, 7 and 10 after adipogenic induction. As shown in Figure 4, the adipogenic differentiation in hMSC was accompanied by a decrease in AdV transduction efficiency and a decrement in CAR expression. There was an inverse correlation between the maturation of adipogenic differentiation and the efficiency of adenoviral transduction. The observed efficiency was 44%, 30% and 19% at 3 days, 7 days and 10 days respectively after adipogenic induction (Table 1). CAR expression was only observed in a subpopulation of adipogenic differentiated hMSC, equal to 5% or less at 3 days after adipogenic induction, but CAR expression was undetectable if the induction was continued more than 7 days.

Reduced AdV transduction efficiency and CAR expression in neurogenic differentiated hMSC

Unlike osteogenic and adipogenic inductions, neurogenic induction of hMSC had only minor influence on AdV transduction efficiency. As shown in Figure 5 and Table 1, the efficiency corresponding to 5 hr and 5 days after neurogenic induction was 93% and 89%,

respectively. However, the prolonged neurogenic culture for 5 days had a lower fluorescent intensity compared to those had neurogenic induction for 5 hours. In addition, hMSC undergone neurogenic induction had less CAR expression in total population as compared to the undifferentiated status.

Discussion

We confirm in this report that AdV efficiently infect hMSC, and that transduced sequences are retained and expressed by a small fraction of hMSC progeny up to 4 weeks after infection. The 90% transduction rate we have obtained, assessed by transgene expression, is much higher than that observed by Conget et al in hMSC (19% transduction rate even at MOI 2000)[12] and also higher than bone marrow,[9] cord blood, and mobilized blood CD34+ cells.[10] Moreover, the fluorescence intensity of the transduced cell population is high, and maintained over several days in culture. This high expression rate associated with both Ad-PGK-GFP and Ad-CMV-GFP indicate that efficient key factor in AdV-mediated transduction is probably not on the vector or the promoter the vector used, but dependent on the content or purity of the cells. Bone marrow, a mixture of many cell populations, contained haematopoietic stem cells and nonhaematopoietic stem cells that include MSC. Previous reports debated on the homogeneity of hMSC, but the purity of hMSC depends on the method used to isolate and enrich them. The hMSC that we have isolated and characterized in previous report has attained 98% of homogeneity.[14] In contrast to undifferentiated hMSC, we did not achieve a high ratio of transgene expression in hMSC that have been treated by a different induction medium, where the cells underwent lineage-specific differentiation. Furthermore, several attempts to improve upon this infection efficiency by manipulating serum and divalention concentration achieved no increase in the transduction rate in induction medium-treated

hMSC.

Factors determining the susceptibility of the target undifferentiated and induced cells to the AdV infection are not entirely known. AdV utilizes fiber protein to attach to a recently identified cell surface CAR receptor,[6] and vitronectin-binding αvβ3 and αvβ5 integrins function as secondary receptors to mediate virus internalization.[7] The level of CAR expression but not integrins expression on the cell surface of hMSC determined the efficiency of AdV-mediated gene transfer[12] and the induction of CAR expression on fibroblast promoted the binding of penton base protein, and facilitated AdV-mediated gene delivery.[13] Based on these observations, we have evaluated CAR expression on hMSC surface before and after incubation with different induction mediums, and attempted to relate CAR expression with transgene expression. We reasoned that the initiation of differentiation in hMSC should affect the level of CAR expression and indirectly have effects on AdV-mediated gene transfer into the target cells. We observed that, before any induction, hMSC expressed a high level of CAR expression and more than 90% of hMSC were permissive for AdV infection; moreover, treatment with any induction medium effectively decreased surface expression of CAR receptor, and reduced expression of the transgene. Our results suggest that, as reported in other cell types,[6] CAR expression is necessarily predictive of the extent of transgene expression in hMSC. We also observed that incubation of hMSC with CAR specific monoclonal antibody RmcB significantly reduced about 40% of AdV-mediated gene transfer,

confirming the participation of CAR on AdV entry and transgene expression in hMSCs.[12] Similar with other reports, however, competition of CAR receptor with monoclonal antibody did not totally hinder the AdV-mediated gene transfer, suggesting that the necessity of CAR to infection may be relative rather than absolute, and possibly other, not yet defined, mechanisms have participated in AdV-mediated gene transfer into hMSC.

The degree of commitment to lineage-specific differentiation coincided with a reduction in CAR expression in hMSC, suggesting the role of CAR in differentiation-related pathway. CAR expression is also reciprocally correlated with the maturity of cellular differentiation in oropharyngeal cells that the superficial layer (more developed) had less CAR expression than the basal layer (less developed).[8] But CAR expression is upregulated during maturation and lineage-specific differentiation in haematopoietic cells. Cellular function of CAR is not investigated here and slight expression of CAR was observed again in the late osteogenic differentiation of hMSC. Therefore, efforts still should be made to know more about the involvement of CAR in cell proliferation and differentiation by the use of overexpression or deletion of CAR in hMSC.

According to our previous data, hMSC isolated from 5-10 ml of bone marrow, could be expanded to 10^{14} in an additional 15 weeks of culture, equal to the total cells of an individual human body.[14] The hMSC could be efficiently induced to undergo differentiation by different induction mediums into bone, fat, cartilage and electrically active neural cells, representing an

optimal source for cell therapy and tissue engineering.[14,15,16] From a clinical point of view, in contrast with retrovirus vectors that require extensive culture of target cells and long incubation times,[17] AdV can infect hMSC at extremely favorable conditions. The hMSC infected by AdV at a 1000:1 virus:target ratio with only 0.5 ml of supernatant, while 200 ml of a retrovirus supernatant with a 10^6 p.f.u./ml titer would be needed for the same purpose. This dramatic simplification of infection conditions supports the utilization of AdV for those clinical conditions in which permanent gene transfer into hMSC is not required. We can envisage two of these conditions, one being the autografting of hMSC genetically modified by transduction of a cancer therapy gene(s), in which hMSC should express high levels of the therapy gene just for the time period the cancer is evidenced in the body. Others have shown that the tumor microenvironment preferentially promotes the engraftment of hMSC as compared with other tissues.[18] The hMSC with forced expression of IFN-β inhibited the growth of malignant cells *in vivo*. Importantly, this effect required the integration of hMSC into the tumors and could not be achieved by systemically delivered IFN-β or by IFN-β produced by hMSC at a site distant from the tumors. These results indicated that hMSC might serve as a platform for delivery of biological agents in tumors. The second circumstance in which AdV-mediated *ex vivo* transduction of hMSC could be useful is the implementation of regeneration programs for tissue repair. Others have demonstrated that BMP2 expressed mesenchymal cells or osteoprogenitor cells can promote bone formation and help fracture healing.[19,20] AdV could

thus advantageously be utilized as a vehicle for delivering growth factor gene(s) into hMSC.

The present study demonstrates that CAR is rate limiting for AdV infection of hMSC, and importantly, it demonstrates that CAR loss and reduced AdV-mediated gene transfer in induction medium-treated hMSC. In addition, we have proved in AdV-transduced hMSC the maintenance of differentiation potentials and the persistence of transgene expression into induction medium-treated hMSC progeny. Accordingly, a strategy is designed to use AdV for delivering therapy gene or growth factor gene to undifferentiated hMSC, and has the gene persistently expressed after cell differentiation. In conclusion, the hMSC is potentially utilizable in clinical protocols of cell therapy and tissue engineering, and AdV is a useful tool for gene transduction into hMSC, particularly for those applications in which high transgene expression for limited period of time is required.

Materials and methods

Cell Culture

The hMSC was isolated from human bone marrow as previously reported.[14] In brief, bone marrow aspirates, washed twice with phosphate-buffered saline (PBS), suspended in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 μg/ml amphotericin B, were plated on a 10-cm plastic culture dish comprising a plate with 3-μm pores at a density of 10^6 mononuclear cells/cm^2. Cells that adhered to the pore-containing plate were recovered at 7 days after initial plating, and the hMSC culture was developed by plating the cells at about 6,000 cells/cm^2, and subculturing at a ratio of 1:3 when cells reached more than 80% confluence.

Recombinant adenovirus and AdV infection

An EGFP cDNA from pEGFP-N1 (Clonetech) was subcloned into the adenovirus shuttle plasmid vector, pAd-PGK, which contains a promoter of the human phosphoglycerate kinase (PGK) and a polyadenylation signal of bovine growth hormone. The Ad-PGK-GFP, with E1 and E3 deletions, was constructed by homologous recombination and amplified in human embryonic kidney 293 cells as previously described.[21] Viruses were purified by CsCl density gradient centrifugation, and viral titers were determined by plaque-forming assay. Purified

virus was stored in 10 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, and 10% (vol/vol) glycerol at -80 °C until used for the experiments. For infection, ~2×10^5 cells were seeded on a 10-cm^2 petri-dish in 0.5 ml of serum-free DMEM-LG, and virus was added at a multiplicity of infection (MOI) of 1000. After two hours, 2ml of DMEM-LG supplemented with 10% FBS or corresponding induction medium was added. Cells were harvested for GFP detection at 48 hr post infection.

GFP transgene detection and CAR expression determination

Expression of GFP transgene in hMSC was detected by visualization of cells with a phase contrast microscope equipped with a fluorescence filter set. Alternately, GFP transgene expression was quantified by flow cytometry after exclusion of dead cells and debris by staining of propidium iodide (PI) and analyzed using a standard filter set up for FITC detection (525 nm bandpass filter; FACScan, Becton Dickinson).

For determination of CAR expression, cells were washed twice with PBS and stained with monoclonal antibody RmcB (anti-CAR, kindly provided by Dr. R. Finberg, Harvard Medical School, Boston, MA) as hybridoma supernatant or control IgG for 30 min at 4℃, in PBS supplemented with 0.1% bovine serum albumin (BSA). After incubation, the cells were washed twice, and then incubated for 30 min at 4℃ in the dark with a fluorescence-conjugated secondary antibody in PBS. Cells were then washed, suspended in

PBS, and finally measured for fluorescence by flow cytometry analysis.

Competition of CAR receptor by monoclonal antibody

For competition experiments, suspensions of hMSC, either in culture medium alone (control) or in medium containing control IgG or the indicated blocking monoclonal antibody RmcB at different concentrations were incubated for 1 hr at 4℃, and then seeded on dishes and used for AdV infection. Cells were furthered incubated for 48 hr and the percentage of transduced cells was scored by flow cytometry.

Induction of Multilineage Differentiation

The hMSC culture was maintained in DMEM-LG supplemented with 10% FBS (as undifferentiated hMSC) or treated in one of the following formulas: osteogenic differentiation medium:[14] DMEM-LG supplemented with 10% FBS, 50 μg/ml ascorbate-2 phosphate (Nacalai; Kyoto, Japan), 10^{-8} M dexamethasone (Sigma), and 10 mM ß-glycerophosphate (Sigma); adipogenic differentiation medium:[14] DMEM-LG supplemented with 10% FBS, 50 μg/ml ascorbate-2 phosphate, 10^{-7} M dexamethasone, and 50 μg/ml indomethacin (Sigma); or neuron differentiation medium:[15] cells pretreated with DMEM-LG supplemented with 10% FBS, 10^{-7} M Retinoic acid (Sigma) and 10 ng/ml b-FGF (GIBCO) for 24 hrs, then serum is removed and continue culture for 5 hrs to 5 days. The medium was changed every 3 days, the differentiation status of culture was identified by histochemical and immunofluorescence

study, and cells were used for AdV infection or for CAR expression determination.

Histochemical and Immunofluorescence Study

The medium was removed from the culture, and cells were washed twice with PBS. Cells were fixed in 3.7% paraformaldehyde for 10 minutes at room temperature and washed twice with PBS. The cells treated by osteogenic formula were stained with alkaline phosphatase staining to reveal osteogenic differentiation.[14] Those treated by adipogenic formula were stained with Oil red-O to show adipogenic differentiation.[14] Immunofluorescence study for neuron marker, with an anti-β-tubulin III polyclonal antibody (PRB-435p, COVANC, Princeton, NJ) was also done to demonstrate neural differentiation.[15]

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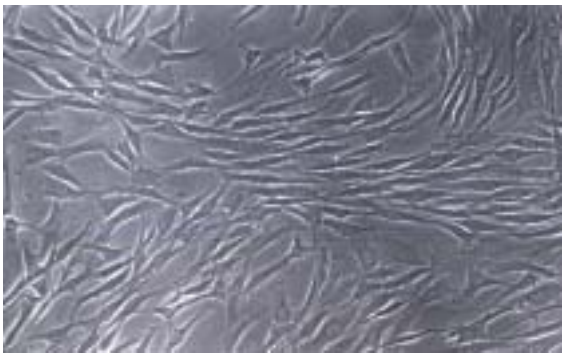
利用生物活性基質進行骨軟骨及韌帶之組織工程

Ⅰ：Ligament cells preparation

1. Ligament cells were obtained from anterior cruciate ligament and Achilles tendon of Wistar rats. The rats are average 6 weeks old and 250 gm. The ligament and tendon tissues were digested with collagenase 0.2%/DMEM for 1.5 hours. After washing and centrifugation 3 times, the solution was shifted to the culture flask. We culture the cells from the tendon substance and the epitenon tissue. The cells were grown in tissue culture flasks containing Dulbecco's modified Eagle mediun (DMEM) and 10% fetal calf serum, supplemented with nonessential amino acids (0.1mM), L-glutamine (4 mM), penicillin (100U/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml) (Amphotericin B; Gibco Grand Island, NY, USA). The cultures were maintained in an incubator at 37C with 5% CO2. Ligament cells from both tissues were trypsinized for 5 minutes with the use of a trypsin-Versene (Gibco) mixture (1:250 trypsin , 200 μg/ml Versene EDTA), washed two times with Dulbecco's modified Eagle medium, resuspended, and rotated in complete medium until ready for seeding. The medium was changed every 3 days.

2. Fig : They show the primary culture of ligament cells.



Ⅱ：Scaffold preparation and design:

1. The cylindrical polymer scaffolds, which were about 10cm long and 5mm diameter, were made from PCL and dioxane. The PCL/dioxane concentration is 5% ideally. We have ever tried the 3%, 5%, and 7% three different concentrations. The 5% of PCL/dioxane is most adequate for strength, resolvable time, and porocity size. It must be mixed thoroughly and blended overnight. Then put the solution into the cylinder shape of glass tube and put at the -20C refrigerator. It needs overnight as

the same. Then, take out of the polymer from the glass tube and put it into the -20C 50% alcohol solution for substitution. The substitution needs 1-2 days. Then the PCL polymer is ready to use. As you use it, you just need to take it out and wait for the alcohol evaporation. The pore size is about 100 nm diameter.

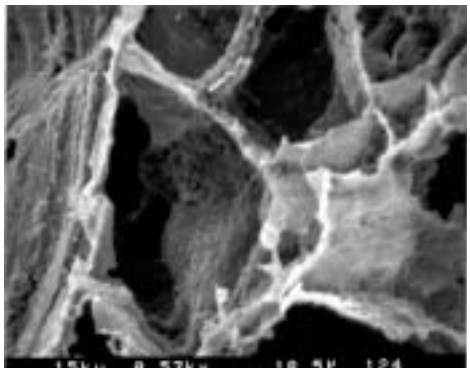
2. 製作過程圖片

5% PCL/dioxane mix thoroughly and blend overnitght.

Put into -20C refrigerator and wait for overnight.
50% alcohol solution was put into -20C refrigerator even the same.

Put the PCL/Dioxane polymer into 50% alcohol solution for substitution.

3. 結果



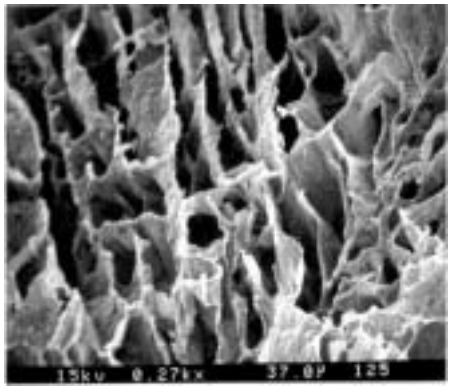


Fig 3 & 4: They show the scaffold pictures of PCL. The pore size is about 80-100 um.

III：Cultures in bioreactors supplying with cyclic mechanical force

1. The cells were seeded on the scaffold with the concentration of 1,000,000 cells/ml. The culture medium was monitored with glucose concentration and PH value per day. The medium was changed one half of volume every 3.5 days. There are two groups for study. The first group was simply cultivated without cyclic mechanical force adding. The second group was cultivated with cyclic mechanical force adding. The cycle period was 5 sec/cycle. The strain of the scaffold was 0.05. We take samples every week after cultivation for 3 weeks. Then we sent the engineered ligament for H&E staining and Alcian Blue staining. We also check the DNA content in the standard specimen.

2. Cultures in bioreactors supplying with cyclic mechanical force

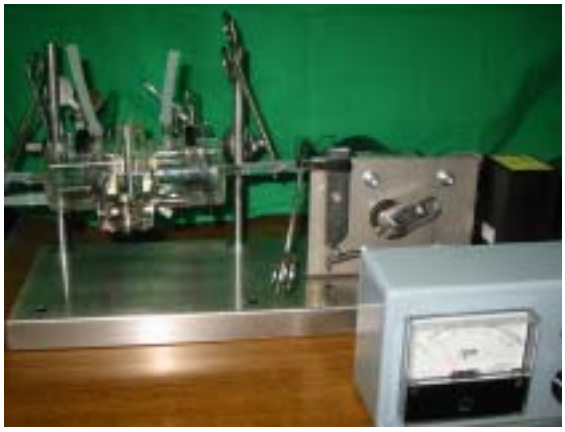
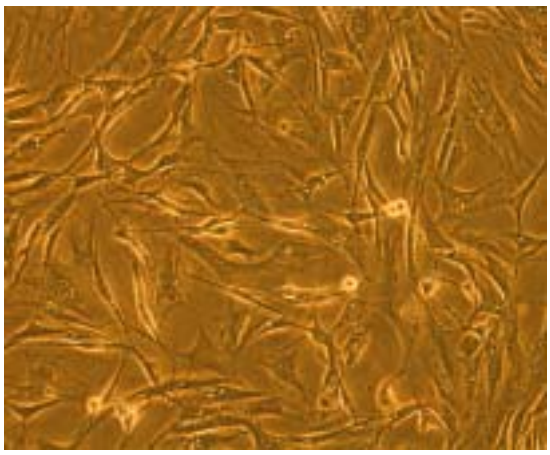


Fig showed the machine that affords stable, periodic, cyclic and longitudinal tension force. The direction of force is parallel to scaffold and longitudinal traction.

IV：IGF cloning and transfection

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.



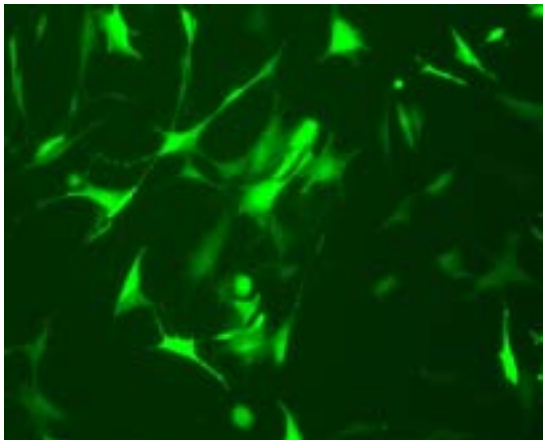


Fig 7 & 8：Under microscope with fluorescence, transfection rate is almost 60%.