行政院國家科學委員會專題研究計畫　成果報告

探討蛋白多醣對誘導小鼠胚胎幹細胞分化為軟骨細胞之影響及作用機轉

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附件一 **國家科學委員會補助專題研究計畫** ■ 成 果 報 告 □期中進度報告

探討蛋白多醣對誘導小鼠胚胎幹細胞分化為軟骨細胞之影響及作用機轉

Studies of the Effect of Proteoglycans on the Chondrogenic Differentiation of Clonal Mouse Embryonic Cell

計畫類別：■ 個別型計畫　□ 整合型計畫
計畫編號：NSC　－　93　－　2320 －　038 －　035　－
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計畫主持人：楊維中
共同主持人：黃德揚
計畫參與人員：劉為麟

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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□國際合作研究計畫國外研究報告書一份

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執行單位：台北醫學大學生物醫學材料研究所

中　華　民　國　94　年　10　月　27　日

現代生物醫學材料及組織工程領域發展的重點，包括皮膚，骨組織，及神經組織的再生與移植，及修復癌症或糖尿病患者身上難以癒合的傷口等。細胞外間質(extracellularmatrix, ECM)，誘導因子，及未分化幹細胞，被認為是研究組織工程技術探討組織修補及移植所必須考慮之三要素。**蛋白多醣(proteoglycan)**由一條或多條直鏈粘多糖(glycosaminoglycan, GAG)，如硫化軟骨膠(chondritin sulfate)，硫化肝素(heparin sulfate)等及一核心蛋白(core protein)，共同組合而成，存在於細胞外間質中與膠原蛋白(collagen)，纖維連接蛋白素(fibronectin)，纖維蛋白原(fibrinogen)，並列為結締組織中之主要成分。細胞外間質的組成，往往影響細胞的生長、形態、及分化。近年來膠原蛋白已應用為促進骨組織再生之生物材料，被認為可促進細胞附著與增生。如以蛋白多醣中之粘多糖成分如硫化軟骨膠與膠原蛋白並用，比單獨使用膠原蛋白能更加促進細胞的活化及再生。而一些研究亦證實蛋白多醣又比其組成分之一粘多糖，更能符合生物環境促進組織再生。因其不但能與其他細胞間質分子行交互作用，提供細胞附著及生長所需的生物物理特性，且可與周邊細胞組織分泌出的生長因子及細胞膜蛋白行交互作用，調節細胞內外之訊息傳遞，提供組織再生及修復工程中所需的化學性質。但是，蛋白多醣之萃取分析及製備技術，需要專業及有經驗之研究人員及設備方可施行。本計劃主持人在美具備多年研究蛋白多醣之經驗並於回國後積極建立製備蛋白多醣及醣質分析實驗室及設備並與國內外實驗室合作，於先期研究已發現蛋白多醣在誘導幹細胞分化確有比傳統使用其成分之一,粘多糖更具生物活性也更能製造接近體內細胞外間質之環境。此初步研究成果論文已被第七屆世界生物材料大會接受,將於 2004 年於澳洲雪梨大會中發表。

本計劃將以完整的蛋白多醣取代單純的粘多糖為研究對象，分析及研究其誘發未分化幹細胞株成軟骨細胞的效能，並探討蛋白多醣誘發軟骨細胞分化之機轉。此計畫將有助於研發促進關節軟骨組織再生及修補之生物醫學材料，並對於以幹細胞治療退化性關節炎之理論基礎作更深入之建立與探討。

A perfect work for tissue engineering is determined by three key factors, extracellular matrix (ECM), growth factors, and the stem cells. Modern biomaterials for tissue repair must consider the biocompatibility, bioresorbability/biodegradability, and bioactivity. Proteoglycan consisting of a core protein to which one or more linear polysaccharide, glycosaminoglycan (GAG) is covalently attached. Proteoglycans play a central role in ECM remodeling and tissue repair through their bindings of either core protein or GAG with the other matrix molecules, growth factors, cytokines, adhesion receptors, enzymes, and enzyme inhibitors in the extracellular matrix. The designed material mimic the ECM will provide physical and chemical stimuli for tissue regeneration. Previous reports have shown that the GAG, by itself, can be used as a carrier material for the transplant engineering of cartilage-like tissue. However, the GAG alone may not fully replace the activity of the intact proteoglycan including the core protein and the GAG provides both the structural and biochemical effect on reconstitution of the complicate matrix assembly for tissue repair and remodeling. Preliminary study from our laboratory has extracted and purified various proteoglycans and exaimed their inductive activity on the chondrogenic cells differentiation to the chondrocytes, suggesting that the intact proteoglycan may cooperate with the chondrogenic growth factors and regulate the chondrogenesis. Part of the data will be present in the 7th World Biomaterials Congress, Sydney, Australia in 2004.

This research aims to characterize the effect of proteoglycans on induction of chondrogenesis using an embryonic stem cell derived ATDC5 cells as a research model. The induction mechanism of the proteoglycan in chondrogenesis will be also studied. The obtained information will be helpful on developing a biomimic material for cartilage repair and for better understanding of the stem cell therapy in cartilage repair. The specific aims are listed as follows,

I. Extraction, purification, and characterization of various proteoglycans from tissues.

II. Study the induction of proteoglycans on chondrogenesis of ATDC5 cells.

III. Identifications of the pathway of typical proteoglycan induced chondrogenesis.

Introduction

The ability of cells to adhere to the extracellular matrix (ECM) is an essential effect of tissue regeneration. Previously reported biomaterials including hydroxapatite, polyphosphoesters, collagens, fibrin clot, derivatives of hyaleuonan, polylactic acid, and polyglycolic acid, etc. for dental/orthopedic applications are in an attempt to provide physically mimic ECM environment for tissue regeneration.

Extracellular matrix (ECM) molecules, including collagens, structural glycoproteins such as fibronectin, fibrinogen, and laminin, and proteoglycans associate to each other and form a well-organized network surrounding the cells. These tightly associated ECM molecules build the connective tissue that protects organs and maintains the body shape. In addition, the ECM plays as a reservoir preserve the soluble molecules such as cytokines, growth factors, and ions and regulate the release. Proteoglycan, a class of glycoconjugates, is composed of a core protein and at least one glycosaminoglycan (GAG) chain that is covalently attached to the core protein. The GAG chain is a linear unbranched polymer consisting of repeating disaccharide units. The components of the disaccharide unit in GAG chain are: the iduronic acid (IdUA)-N-acetylgalactosamine (GalNAc) unit in dermatan sulfate (DS); the glucuronic acid (GlcUA)-GalNAc unit in chondrotin sulfate (CS); the GlcUA-N-acetylglucosamine (GlcNAc) unit in heparan sulfate (HS); or the galactose (Gal)-GlcNAc unit in keratan sulfate (KS), in addition, hyaluronic acid (HA), consisting of alternating glucuronic acid and N-acetylglucosamine, is grouped as a type of GAG chain, it is not covalently attached to a core protein and has no sulfate substituents. Proteoglycans, extensively distributed in connective tissue, are not only secreted in ECM but also associated with the cell membrane or stay inside the cells. The extracellular proteoglycans were found to maintain a hydrated environment in tissues and provide resistance to compressive, tensile, and shear force, additionally, it plays an important role to regulating the functions of the other matrix proteins such as soluble growth factors for cell adhesion, differentiation, and proliferation [1-3].

Tissues like articular cartilage and neurons that show little or no capability to be repaired or regenerated in response to injury or disease caused lesions. The development of tissue engineering has brought up a bright hope to combine implanted **cells**, **extracellular matrix**, and **biologically active molecules** to repair and regenerate the injured tissues. The predominant proteoglycan present in cartilage is the large chondroitin sulfate proteoglycan 'aggrecan'. Other proteoglycans expressed during chondrogenesis and in cartilage include the cell surface syndecans and glypican, the small leucine-rich proteoglycans decorin, biglycan, fibromodulin, lumican and epiphycan and the basement membrane proteoglycan, perlecan. [4]. PG composition and structure has been reported to alter with age, therefore, a proper matrix stoichiometry is critical for metabolism of PGs [5]. The defect of proteoglycan composition may associate with the severity of bone and cartilage degeneration. Joint pain due to cartilage degeneration such as osteoarthritis (OA) is a serious problem, affecting people of all ages. There are differences between the matrix components and water content during the progression of

cartilage degeneration; these properties correspond to a higher equilibrium modulus and dynamic stiffness but lower hydraulic permeability and serve to make the ankle cartilage stiffer, slowing movement of molecules through the cartilage [6]. Several reports have shown that the GAGs can be used as an effective component in a biocompatible scaffold for supporting the chondrocytes or nerve cells to grow *in vitro*. [7-12]. In the studies of bone formation, several reports have shown that proteoglycans are the key components in bone matrix and secreted by osteoblastic cells, suggesting that proteoglycans regulate the bone formation [13-22]. It has been implied that growth factor induced expression of intact proteoglycan, including both corer protein and GAGs, accelerate the cartilage tissue repair and regeneration [23]. Additionally, the intact proteoglycan showed greater binding to hydroxyapatite with higher adsorption maxima than the constituent core protein or GAG alone [24, 25]. Yet, it has not been reported that the intact proteoglycan containing scaffold was used for cartilage or bone regeneration system.

At present, there is no well-established procedure for the repair of cartilage defect with articular cartilage, which has the same biochemical and biomechanical properties as the surrounding normal intact cartilage. Transplantation of human autologous chondrocytes in suspension, as reported by Brittberg et al. in 1994, provided a potential procedure for articular cartilage repair. Many studies focused on developing strategies for cultivating autologous chondrocytes a period in vitro for transplantation (Solchaga, Yoo et al. 2000; Suh and Matthew 2000; Uchio, Ochi et al. 2000; Wyre and Downes 2000; Grigolo, Roseti et al. 2001). The stimuli of growth factors have been considered to enhance the cartilage tissue repair [26-28]. The induction of growth factors and cytokines on the chondrogenesis of mesenchymal stem cells have also been reported [29-31]. Growing of stem cell on desired scaffold in vitro as well as for implantation in cartilage repair is an ideal approach and has been examined [32-39], yet, the genes associated with chondrogenesis of stem cells remain unclear. Some studies have reported that addition of the matrix molecules may induce the chondrogenesis of stem cells for the cartilage repair [40, 41].

Materials and Methods

Isolation of proteoglycans

Sliced tissue was washed with 0.15M NaCl, sodium phosphate buffer, pH7.4 (1:20, w/v). The minced tissue was then re-suspended in 4M guanidine-HCl (15:20, w/v) containing 0.5% CHAPS and protease inhibitors (1mM PMSF, 5mM diisopropyl fluorophosphates in propanol) with 2% β-mercaptol ethanol or 10 mM DTT. Proteoglycans were extracted at 0-4 °C and incubated end-over-end for 12-24 hr. Followed by centrifugation at 16 000 x g for 60 min at 4 °C. The suspension was dialyzed to 7M Urea in 10 mM Tris-HCl, pH 8.3 at 4 °C with buffer change every 4-6 hrs for three times. The extract was stored at -20°C for further analyses.

Purification of the proteoglycan extract

The proteoglycan extract in 7M Urea in 10 mM Tris-HCl, pH 8.0, was diluted with buffer A

(10 mM Tris-HCl, pH 8.3)(1:1, v/v) and subjected to a DEAE Sephrose Fast Flow column (HR 5/10, Amersham Biosciences), which has been pre-equilibrated with buffer A in a liquid chromatography system (AKTA Basic 10, Amersham Biosciences). After the extensively wash with buffer A, the bound material was eluted with a salt gradient from 0 to 100% buffer B (1M NaCl in 10 mM Tris-HCl, pH 8.0). According to the corresponding UV absorbance and the elution profiles, the fractions corresponding to the individual peak were collected and poured together for further analyses. The protein concentration of each peak was determined based on the Lawry method.

Cell culture

The ATDC5 cells were kindly provided by Dr. Tamayuki Shinomura. The culture condition was followed as described previously[42, 43]. Briefly, the cells were maintained in DMEM/Ham's F12 hybrid medium (GIBCO BRL, Gaithersburg, MD, U.S.A) containing 5% (V/V) FBS (Hyclone, U.S.A), and 3 X 10^{-8} M sodium selenite (Sigma, St. Louis, MO, U.S.A) at 37°C in a humidified 5% CO2/95% air atmosphere. Chondrogenesis and cartilage nodule formation was induced when cells were cultured in the medium supplemented with 10 mg/ml bovine insulin (Sigma, St. Louis, MO, U.S.A). The cells were treated for the indicated various doses of the test substances in the presence and absence of insulin.

MTT assay: The long-term viability of photoencapsulated osteoblasts, as measured by mitochondrial activity, was analyzed with an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay after 1 day, 1 week, and 2 weeks of culture. A 1% MTT in serum-free media solution was added to each well containing the cell–hydrogel construct. Active mitochondria metabolize the tetrazolium salt to form an insoluble formazin dye. After 4 h of incubation, the constructs were placed in 1.5 ml polypropylene tubes, and 0.04 n HCl in spectrograde isopropanol was added. The constructs were broken apart using a tissue homogenizer and placed on an orbital shaker for 30 min for dissolution of the formazin. The absorbance was read at 560 nm.

Characterization of chondrogenic phenotypes

Morpholoy of the cultured ATDC5 cells. Based on the previous description, the undifferentiated ATDC5 cells are a fibroblast like cell line. While the cells get confluent and cultured in the medium contained insulin, the cells start differentiate and grow as rounded shape and form the cartilage nodule, which can be stained by alcian blue.

Expression of cartilage markers. The cells cultured in various substances for different time period will be collected for chondrogenesis marker charaterization. Total RNA will be extracted and reverse transcribed with desired primers to investigate the mRNA expression of collagen I, collagen II, collagen X, and aggrecan by RT-PCR.

Characterization of osteoblast

Alkaline phosphatase activity: The method of Lowry [45] was used to assay alkaline

phosphatase activity in the cell lysates.

Ouantification of extracellular calcium: The extracellular matrix of each preparation will be treated with 0.6N HCl at 37°C overnight. The amount f calcium present in the acidic supernatant will be spectrophotometrically quantified using a commercially avalible kit (Sigma). The measurement protocol provided by the manufacture is followed.

Results

We firstly studied the effect of various matrix molecules on the proliferation of ATDC5 cells. The results indicated that the coated ECM molecules in absence of insulin did not significantly affect the proliferation of ATDC5 cells compared to the control (Figre 1). Ten to 14 days after induction with insulin, the proliferation rate of ATDC5 cells on the coated wells with collagen I and decorin were greatly decreased compared to the cells grow on the HSPG and control (Figure 1). Morphology of a cartilage nodule-like aggregate of ATDC5 cells was observed after insulin induction (Figure 2). However, the nodule-like aggregation of ATDC5 cells is not essential phenotype of chondrogenesis. Hence, we measured the amount of GAG expressed in the cells by following the stained intensity of alcian blue (Figure 3). The results showed that in the 14 day culture, decorin and collagen I significantly enhanced the accumulation of alcian blue positive matrix in the cultured cells at insulin-dependent manner , whereas the cells grow in the presence of insulin in the HSPG coated wells were stained with alcian blue more intensively compared to the control (Figure 4). We also examined whether the ATDC5 cells with chondrogenesis potential expressed the moleculr markers of chondrocyte. RT-PCR analyses indicated that higher mRNA levels of collagen type II and aggrecan were induced while the cells grow in the wells coated with decorin in the absence or presence of insulin, suggesting that decorin may induce chondrogenesis potential of ATDC5 cells without insulin induction (Figure 5).

Discussion

The factor of stiffness should be taken into account. Additionally, it has been reported that through its core protein or glycosaminoglycans, proteoglycan can either directly or indirectly interact with growth factors to regulate cell growth [1, 46-49]. The intact proteoglycan, which we used in this study replace previous reported GAG only, was more effective on maintaining the cell growth and prolonging their growth in the *in vitro* culture system.

Previous studies have claimed that the clonal cell line ATDC5 enables the monitoring of the early- and late-phase chondrogenic differentiation in a single culture. Undifferentiated ATDC5 cells differentiate into type II collagen expressing chondrocytes through a cellular condensation stage (early-phase differentiation) and then to type X collagen-expressing hypertrophic chondrocytes (late-phase differentiation). ATDC5 cells expressed transcripts for at least four members of the BMP family. The BMP-4 transcripts were expressed in all stages of differentiation. In contrast, transcripts for BMP-6 were induced during the formation of cartilage nodules, and declined as the differentiated ATDC5 cells became hypertrophic, and BMP-7 transcripts were only detected after cells became calcified; Exogenous addition of BMP-4 indeed promoted the early-phase differentiation. Late-phase differentiation of cells was also stimulated by BMP-4 and

BMP-6 [50]. The condroitin sulfate proteoglycan has been suggested to involving in the BMP2 and BMP4 signaling during chondrogenesis of mesenchymal progenitor cells [51]. Various small leucine rich proteoglycan deficient mice and double knockout mice resulted musculoskeletal disorders such as biglycan deficient osteoporosis with age, severe bone mass loss in biglycan/decorin double knockout, and biglycan/fibromodulin double knockout resulting in osteoarthritis [52]. The results indicated that the rescue by introducing the functional chondrotin sulfate proteoglycan may attenuate the progression of the bone and cartilage defect. Additionally, heparin sulfate proteoglycan has been indicated in the induction of derived mesenchymal C3H10T1/2 cells to enter a chondrogenic differentiation pathway [53]. We in this study used the brain extract, which predominantly contains heparan sulfate proteoglycan, has also moderate activity to induce the differentiation of ATDC5 cells to perform chondrogenic phenotypes. Both HSPG and CSPG could enhance the inductive activity of insulin on the chondrogenic differentiation of ATDC5 cells, whereas CSPG decorin enabled the differentiation of ATDC5 cells in an insulin-independent manner, suggesting that GAG only may be insufficient to replace the intact proteoglycan play not only structural but regulatory roles in a scaffold to support the cell growth. In addition, the effect of induced chondrogenesis of ATDC5 cells by CSPG and HSPG in collaborating with insulin may be different. It suggested that the inductive pathway may be different. It required further studies to elucidate.

References

1. Kresse, H. and E. Schonherr, *Proteoglycans of the extracellular matrix and growth control*, in *J Cell Physiol*. 2001. p. 266-74.
2. Hocking, A.M., T. Shinomura, and D.J. McQuillan, *Leucine-rich repeat glycoproteins of the extracellular matrix*, in *Matrix Biol*. 1998. p. 1-19.
3. Iozzo, R.V., *Matrix proteoglycans: from molecular design to cellular function*, in *Annu Rev Biochem*. 1998. p. 609-52.
4. Knudson, C.B. and W. Knudson, *Cartilage proteoglycans*, in *Semin Cell Dev Biol*. 2001. p. 69-78.
5. Grzesik, W.J., et al., *Age-related changes in human bone proteoglycan structure: impact of osteogenesis imperfecta*, in *J. Biol. Chem.* 2002. p. M202124200.
6. Cole, A.A. and K.E. Kuettner, *Molecular basis for differences between human joints*, in *Cell Mol Life Sci*. 2002. p. 19-26.
7. Butler, C.E., F.A. Navarro, and D.P. Orgill, *Reduction of abdominal adhesions using composite collagen-GAG implants for ventral hernia repair*, in *J Biomed Mater Res*. 2001. p. 75-80.
8. Denuziere, A., et al., *Chitosan-chondroitin sulfate and chitosan-hyaluronate polyelectrolyte complexes: biological properties*, in *Biomaterials*. 1998. p. 1275-85.
9. Denuziere, A., D. Ferrier, and A. Domard, *Interactions between chitosan and glycosaminoglycans (chondroitin sulfate and hyaluronic acid): physicochemical and biological studies*, in *Ann Pharm Fr*. 2000. p. 47-53.
10. Ellis, D.L. and I.V. Yannas, *Recent advances in tissue synthesis in vivo by use of*

collagen-glycosaminoglycan copolymers, in *Biomaterials*. 1996. p. 291-9.
11. Spilker, M.H., et al., *Contraction of collagen-glycosaminoglycan matrices by peripheral nerve cells in vitro*, in *Biomaterials*. 2001. p. 1085-93.
12. Wojcik, S.M., D.S. Bundman, and D.R. Roop, *Delayed wound healing in keratin 6a knockout mice*, in *Mol Cell Biol*. 2000. p. 5248-55.
13. Raouf, A., et al., *Lumican is a major proteoglycan component of the bone matrix*, in *Matrix Biol*. 2002. p. 361-7.
14. Kamiya, N., K. Shigemasa, and M. Takagi, *Gene expression and immunohistochemical localization of decorin and biglycan in association with early bone formation in the developing mandible*, in *J Oral Sci*. 2001. p. 179-88.
15. Hoshi, K., S. Ejiri, and H. Ozawa, *Ultrastructural analysis of bone calcification by using energy-filtering transmission electron microscopy*, in *Ital J Anat Embryol*. 2001. p. 141-50.
16. Grave, B., *Localization of TGF-Bs and perlecan in mouse skull development*, in *Ann R Australas Coll Dent Surg*. 2000. p. 352-6.
17. Yamada, S., et al., *Expression profile of active genes in human periodontal ligament and isolation of PLAP-1, a novel SLRP family gene*, in *Gene*. 2001. p. 279-86.
18. Gori, F., E. Schipani, and M.B. Demay, *Fibromodulin is expressed by both chondrocytes and osteoblasts during fetal bone development*, in *J Cell Biochem*. 2001. p. 46-57.
19. Nakamura, H., et al., *Immunolocalization of keratan sulfate proteoglycan in rat calvaria*, in *Arch Histol Cytol*. 2001. p. 109-18.
20. Aigner, T., et al., *Cell differentiation and matrix gene expression in mesenchymal chondrosarcomas*, in *Am J Pathol*. 2000. p. 1327-35.
21. Lee, I., et al., *Immunocytochemical localization and biochemical characterization of large proteoglycans in developing rat bone*, in *J Oral Sci*. 1998. p. 77-87.
22. Sommarin, Y., et al., *Osteoadherin, a cell-binding keratan sulfate proteoglycan in bone, belongs to the family of leucine-rich repeat proteins of the extracellular matrix*, in *J Biol Chem*. 1998. p. 16723-9.
23. Fujisato, T., et al., *Effect of basic fibroblast growth factor on cartilage regeneration in chondrocyte-seeded collagen sponge scaffold*, in *Biomaterials*. 1996. p. 155-62.
24. Rees, S.G., et al., *Interaction of bone proteoglycans and proteoglycan components with hydroxyapatite*, in *Biochim Biophys Acta*. 2001. p. 118-28.
25. Klinger, M.M., et al., *Proteoglycans at the bone-implant interface*, in *Crit Rev Oral Biol Med*. 1998. p. 449-63.
26. Martin, I., et al., *Enhanced cartilage tissue engineering by sequential exposure of chondrocytes to FGF-2 during 2D expansion and BMP-2 during 3D cultivation*, in *J Cell Biochem*. 2001. p. 121-8.
27. Mason, J.M., et al., *Cartilage and bone regeneration using gene-enhanced tissue engineering*, in *Clin Orthop*. 2000. p. S171-8.
28. Bradham, D.M. and W.E. Horton, Jr., *In vivo cartilage formation from growth factor modulated articular chondrocytes*, in *Clin Orthop*. 1998. p. 239-49.

29. Longobardi, L., et al., *A novel insulin-like growth factor (IGF)-independent role for IGF binding protein-3 in mesenchymal chondroprogenitor cell apoptosis*, in *Endocrinology*. 2003. p. 1695-702.
30. Sekiya, I., D.C. Colter, and D.J. Prockop, *BMP-6 enhances chondrogenesis in a subpopulation of human marrow stromal cells*, in *Biochem Biophys Res Commun*. 2001. p. 411-8.
31. Majumdar, M.K., E. Wang, and E.A. Morris, *BMP-2 and BMP-9 promotes chondrogenic differentiation of human multipotential mesenchymal cells and overcomes the inhibitory effect of IL-1*, in *J Cell Physiol*. 2001. p. 275-84.
32. Radice, M., et al., *Hyaluronan-based biopolymers as delivery vehicles for bone-marrow-derived mesenchymal progenitors*, in *J Biomed Mater Res*. 2000. p. 101-9.
33. Solchaga, L.A., et al., *Hyaluronan-based polymers in the treatment of osteochondral defects*, in *J Orthop Res*. 2000. p. 773-80.
34. Uchio, Y., et al., *Human chondrocyte proliferation and matrix synthesis cultured in Atelocollagen gel*, in *J Biomed Mater Res*. 2000. p. 138-43.
35. Suh, J.K. and H.W. Matthew, *Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review*, in *Biomaterials*. 2000. p. 2589-98.
36. Wyre, R.M. and S. Downes, *An in vitro investigation of the PEMA/THFMA polymer system as a biomaterial for cartilage repair*, in *Biomaterials*. 2000. p. 335-43.
37. Grigolo, B., et al., *Transplantation of chondrocytes seeded on a hyaluronan derivative (hyaff-11) into cartilage defects in rabbits*, in *Biomaterials*. 2001. p. 2417-24.
38. Kavalkovich, K.W., et al., *Chondrogenic differentiation of human mesenchymal stem cells within an alginate layer culture system*, in *In Vitro Cell Dev Biol Anim*. 2002. p. 457-66.
39. Aung, T., et al., *Chondroinduction of mouse mesenchymal stem cells in three-dimensional highly porous matrix scaffolds*, in *J Biomed Mater Res*. 2002. p. 75-82.
40. Louwerse, R.T., et al., *Use of recombinant human osteogenic protein-1 for the repair of subchondral defects in articular cartilage in goats*, in *J Biomed Mater Res*. 2000. p. 506-16.
41. Atkinson, B.L., et al., *Combination of osteoinductive bone proteins differentiates mesenchymal C3H/10T1/2 cells specifically to the cartilage lineage*, in *J Cell Biochem*. 1997. p. 325-39.
42. Atsumi, T., et al., *A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells.* Cell Differ Dev, 1990. **30**(2): p. 109-16.
43. Shukunami, C., et al., *Chondrogenic differentiation of clonal mouse embryonic cell line ATDC5 in vitro: differentiation-dependent gene expression of parathyroid hormone (PTH)/PTH-related peptide receptor.* J Cell Biol, 1996. **133**(2): p. 457-68.
44. Brown, A.N., et al., *Combining chondrocytes and smooth muscle cells to engineer hybrid soft tissue constructs*, in *Tissue Eng*. 2000. p. 297-305.
45. Lowry OH, R.N., Wu M, Hixon WS, Crawford EJ, *The quanitative histochemistry of the brain III. Enzyme measurements.*, in *J. Biol Chem*. 1954. p. 19-37.
46. Brucato, S., J. Bocquet, and C. Villers, *Cell surface heparan sulfate proteoglycans: target*

and partners of the basic fibroblast growth factor in rat Sertoli cells, in *Eur J Biochem*. 2002. p. 502-11.

47. Wang, L., et al., *Control of extracellular matrix homeostasis of normal cartilage by a TGFbeta autocrine pathway. Validation of flow cytometry as a tool to study chondrocyte metabolism in vitro,* in *Osteoarthritis Cartilage*. 2002. p. 188-98.
48. Noth, U., et al., *In vitro engineered cartilage constructs produced by press-coating biodegradable polymer with human mesenchymal stem cells*, in *Tissue Eng*. 2002. p. 131-44.
49. Xu, G., et al., *Control of proliferation, migration, and invasiveness of human extravillous trophoblast by decorin, a decidual product*, in *Biol Reprod*. 2002. p. 681-9.
50. Akiyama, H., et al., *Differential expressions of BMP family genes during chondrogenic differentiation of mouse ATDC5 cells.* Cell Struct Funct, 2000. **25**(3): p. 195-204.
51. Steinert, A., et al., *Chondrogenic differentiation of mesenchymal progenitor cells encapsulated in ultrahigh-viscosity alginate.* J Orthop Res, 2003. **21**(6): p. 1090-7.
52. Young, M.F., et al., *Biglycan knockout mice: new models for musculoskeletal diseases.* Glycoconj J, 2002. **19**(4-5): p. 257-62.
53. French, M.M., et al., *Chondrogenic activity of the heparan sulfate proteoglycan perlecan maps to the N-terminal domain I.* J Bone Miner Res, 2002. **17**(1): p. 48-55.

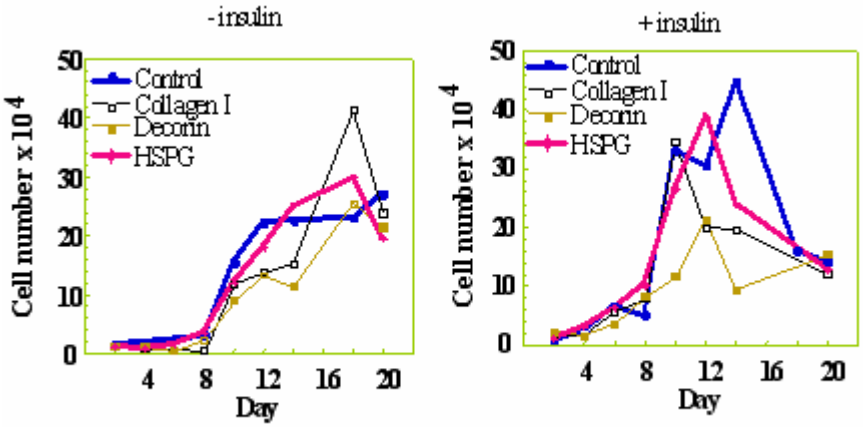

Figure 1. Effect of proteoglycan on the proliferation of ATDC5 cells. The ATDC5 cells were seeded on the wells precoated with collagen, decorin, heparan sulfate proteoglycan, respectively, and incubated in the absence and presence of insulin. The results indicated that the coated ECM molecules in the presence or absence of insulin did not significantly affect the proliferation of ATDC5 cell compared to the control.

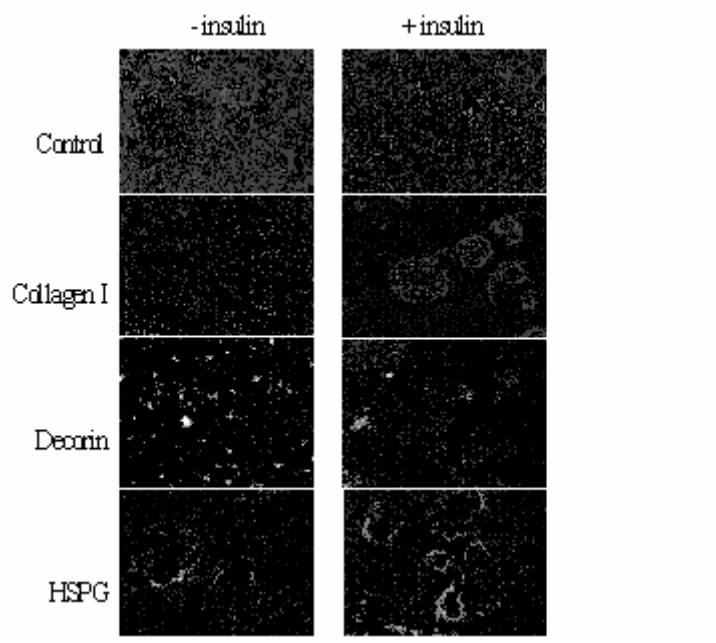

Figure 2. Effect of proteoglycan on the clustering of ATDC5 cells induced by insulin. ATDC5 cells were seeded on the wells precoated with the indicated proteoglycan and incubated with and without insulin for 12 days. The insulin induced chondrogenesis of ATDC5 cells resulted in the clustering phenotype. Heparan sulfate proteoglycan induced the clustering of ATDC5 cells even in the absence of insulin.

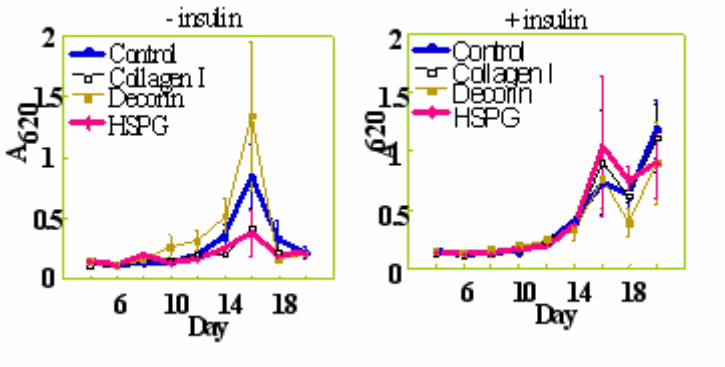

Figure 3. Effect of proteoglycan on the chondrogenesis of ATDC5 cells. The ATDC5 cells were seeded on the wells precoated with collagen, decorin, heparan sulfate proteoglycan, respectively, and incubated in the absence and presence of insulin. The results indicated that decorin induced proteoglycan expression as chondrogenic phenotype even in the absence of insulin.

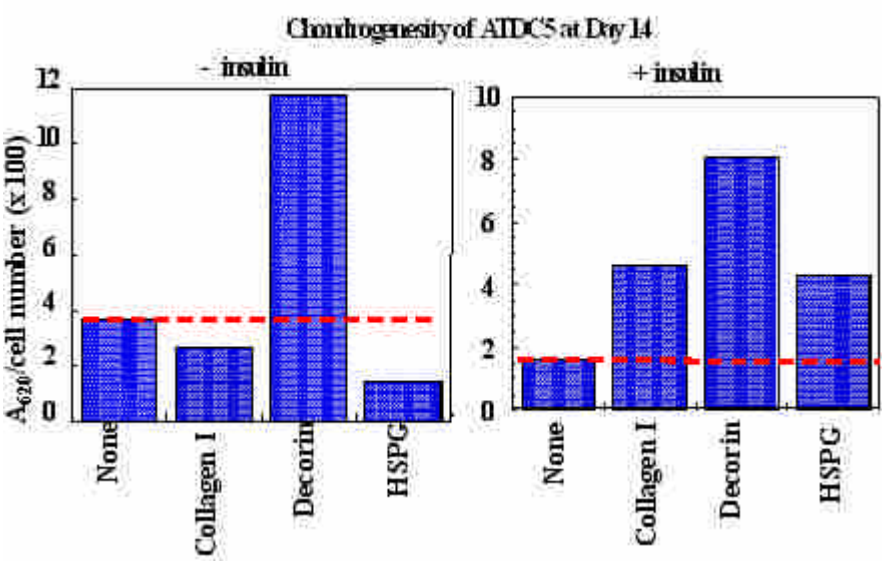

Figure 4. Alcian blue staining of ATDC5 cells cultured in proteoglycan coated wells with and without insulin induction. ATDC5 cells were seeded on the wells precoated with the indicated proteoglycan or collagen I and incubated with and without insulin for 14 days. Decorin significantly induced the extent of chondrogenic differentiation of ATDC5 cells in the absent of insulin. Both decorin and HSPG and collagen type I enhanced the insulin induction on chondrogenic differentiation of ATDC5 cells.

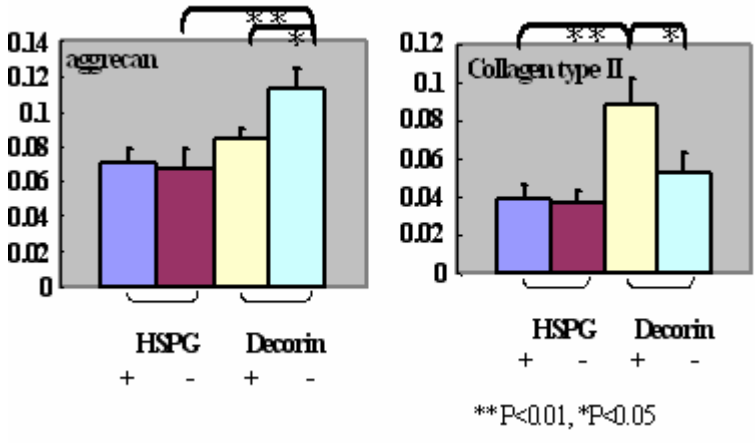

Figure 5. The mRNA expression of the chondrogenic markers in ATDC5 cells cultured in different proteoglycan coated wells with and without insulin induction. Quantitative data showed that aggrecan (AGN) and collagen I (COL I) were expressed in the cells both in the presence and absence of insulin. The level of collagen II (COL II) was higher in the cells cultured in decorin coated wells compared to cultured in the heparan siulfate proteoglycan (HSPG) wells.

附件二 此研究部份成果發表於 The 7th World Congress for Biomterials, May 17-21, 2004, Sydney, Australia.

Effect Of Proteoglycans On The Differentiation of ATDC5 Cells To Chondrocytes

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Abstract

Proteoglycan is composed of a core protein and various numbers of glycosaminoglycan (GAG). It is known that proteoglycans play a central role in ECM remodelling and tissue repair through their interaction by either core protein or GAG with the other extracellular matrix molecules, growth factors, cytokines, adhesion receptors, enzymes, and enzyme inhibitors. Literature reports have suggested that intact proteoglycan compared to GAG alone may be able to express the best effect in the interaction with other matrix molecules to create a complete matrix assembly environment for tissue repair and regeneration. However, the effect of intact proteoglycan as a material for supporting the cell growth and tissue regeneration has little been discussed. It may due to the limited availability of intact proteoglycans. This study aims to evaluate the efficacy of proteoglycan on the regulation of cell proliferation and differentiation by introducing the intact proteoglycan instead of using the GAG or core protein alone in an *in vitro* cultural system. Various proteoglycans from different sources were prepared and their effect on supporting the growth of ATDC5 cells in an *in vitro* cultural system was investigated. ATDC5 cell is an undifferentiated cell line derived from embryonic ectoderm cells, which can be induced by insulin to differentiate into chondrocytes. ATDC5 cells were cultured in the presence and absence of insulin on the wells that have been pre-coated with different proteoglycans. The cell morphology was observed and the molecular markers of chondrocyte were followed. The results indicated that proteoglycans could induce the differentiation of ATDC5 cells to chondrocytes. Both heparan sulfate proteoglycan (HSPG) and chondroitin/dermatan sulfate proteoglycan (CS/DSPG) could enhance the induced differentiation of ATDC5 cells to chondrocytes by insulin. Furthermore, decorin, a small chondroitin/dermatan sulfate proteoglycan can induce the differentiation of ATDC5 cells in the absence of insulin. It suggests that the regulatory mechanism of HSPG and CS/DSPG on the differentiation of ATDC5 cells may be different. The obtained information from this study implicates that proteoglycan may play both structural and regulatory roles as an effective component in a scaffold for supporting the cell growth. The dual effect of proteoglycan shows its potential to be used in a newly developed bio-mimic material for cartilage repair and regeneration in the future.