筑　波　大　学

博士（医学）学位論文

Three Dimensional Alginate Culture Conditions Enhance the Chondrocytic Phenotype of Perichondrium – Derived Cells

（アルジネート三次元培養環境は、軟骨膜分離細胞
において軟骨特異的な表現型の発現を促進させる）

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三　島　　初

Contents

Chapter 1
INTRODUCTION

1-1. Background of this study

Lesions of the articular cartilage are a great challenge to the orthopedic surgeon because of the increasing number of articular traumas, longer life expectancy of the population with the concomitant aging of the locomotor system, and the development of degenerative osteoarthritis. Articular cartilage is frequently vulnerable to traumatic or degenerative conditions that may lead to osteoarthritis.

NORMAL ARTICULAR CARTILAGE

Articular cartilage contains chondrocytes embedded in an extracellular matrix composed primarily of type II collagen, proteoglycans, and other collagens and noncollagenous proteins [1]. The collagen architecture contributes to the tensile strength and stiffness of cartilage [2]; its compressibility is due to its proteoglycan component [3]. While type II collagen predominates (comprising about 90-95% of total collagen), smaller amounts of types V, VI, IX, X, and XI collagen are also present [2, 4]. Cartilage proteoglycans (PG) include hydrodynamically large, aggregating PG, with covalently linked sulfated glycosaminoglycans, as well as hydrodynamically smaller nonaggregating PG such as decorin, biglycan, and lumican. Among the noncollagenous proteins are thrombospondin and cartilage oligomeric matrix protein; their functions continue to be elucidated. The chondrocyte elaborates matrix components, degradative enzymes, and the activators and inactivators of these enzymes. This synthetic function

is influenced by a variety of cytokines and growth factors.

The repair response in vascularized tissues is triphasic, consisting of necrosis, inflammation, and repair [5]. The avascular nature of articular cartilage allows it to undergo necrosis in response to injury, but the inflammatory phase is largely absent. Therefore, if the damage is limited to the cartilage layer and does not involve the subchondral bone, there is no recruitment of undifferentiated cells to effect a repair. Chondrocytes in mature articular cartilage have little intrinsic potential for replication [5, 6] ; lesions may attempt repair by an "extrinsic" mechanism which depends on metaplasia of mesenchymal cells from para-articular connective tissues [7]. However, the population of mesenchymal stem cells declines with increasing age [1]. When the subchondral bone is penetrated, its vascular supply allows a triphasic repair to take place. The resulting tissue is usually mechanically sub-optimal fibrocartilage [5].

TREATMENT OPTIONS

Osteoarthritis is not simply a degenerative disorder. In the past, failure of symptomatic treatment, such as non-steroidal anti-inflammatory drug, and physical therapy, has left joint replacement as the principal option in the care of more sever affected patients. But loosening and wearing in prosthetic replacement are a major problem in total joint arthroplasty. And clinical success of articular prostheses is overshadowed by their poor long-term performance and there is a growing concern among surgeons regarding the economical and technical difficulties posed by their use.

And so, various kinds of operative intervention except of total joint replacement has

been tried, such as penetration of subchondral bone (microfracture, abrasion arthroplasty, subchondral drilling), osteochondral allografts, osteochondral autografts.

Surgical Options :

For many years, debridement of the joint was a widely used procedure for degenerative processes of the knee. Pridie and Insall described debridement along with drilling in the exposed subchondral bone to encourage fibrocartilagnous repair [8]. Ficat et al. introduced "spongialization" in which all diseased cartilage was resected enbloc to expose the underlying cancellous bony bed completely [9]. With the advent of arthroscopy, modifications in the procedures described above have been made, retaining the basic concepts of joint debridement and subchondral drilling to promote three phase tissue healing.

Chondral shaving of partial-thickness defects provides symptomatic relief for a brief period, but does not stimulate regeneration of cartilage [10]. At the surface, the cartilage instead had become fibrillated with occasional cracks and fissures evident in the surrounding cartilage [11]. This lack of regeneration has been attributed to the lack of induction of an inflammatory response since chondral shaving does not violate the tidemark or subchondral bone. Therefore, abrasion arthroplasty and subchondral drilling may be employed; this does result in fibrin clot formation and the ensuing inflammatory response [10, 12].

Abrasion arthroplasty yields variable results in the repair tissue produced, ranging from fibrous tissue with little type II collagen to hyaline-like cartilage with some type II collagen [10, 12]. When compared with subchondral drilling, abrasion arthroplasty appears to have worse long-term results with increasing breakdown of the

repair and subsequent degeneration [13]. Subchondral drilling seems to promote excellent early repair , with evidence of hyaline articular cartilage formation and increased chondrocyte mitotic and metabolic activity and increased proteoglycan staining [13, 14]. However, long term results in rabbits (8-12 months after treatment) showed degeneration and loss of cartilaginous structure with significant PG staining. The surface layer was more typical of fibrocartilage and the tangential orientation of collagen was lost [12, 15].

Treatment Modalities Utilizing Transplantation

Soft Tissue Grafts:

Fascia, tendon, muscle, periosteum, and perichondrium have all been used as graft material in soft-tissue athroplasties [16]. Perichondrial and periosteal grafts have had the best results. In fracture healing and embryonic limb development, mesenchymal progenitor cells present in the cambium layer of periosteum are capable of differentiation and proliferation [17]. Local low oxygen tension and nutrient accessibility encourage mesenchymal differentiation into chondrocytes [17]. Rib perichondrial grafts for full-thickness defects in rabbits have been reported to result in neo-cartilage that is morphologically and biochemically similar to hyaline cartilage [18, 19]. However, this tissue begins to degenerate after eight to twelve months of normal joint function [20]. Clinical trials in humans utilizing perichondrial grafts have also had mixed results. Engkvist and Johannson reported some cases with slightly improved motion and decreased pain, but an equal number with worsened complaints [21] Seradge has used perichondrial grafts in the metacarpophalangeal and interphalangeal joints in humans and found that the age of the patient seemed to influence the results, in

that no patient over the age of 40 had good results [22].

Some of the failures of this type of graft (and others) were deemed secondary to inadequate fixation and graft instability that led to loss of chondrocytes and subsequent graft degeneration [23, 24]. A variety of techniques and "adhesives" for stable fixation have been tested. Adhesives have a long history of use in orthopedic surgery, as they have been utilized in the past for fracture fixation. A good adhesive and delivery substance must be biocompatible, support the transplanted chondrocytes, and provide growth-promoting conditions while protecting the graft from the host immune response. Ossocol, a combination of collagen and fibrous tissue proteins, was one of the earliest adhesives employed. It, however, caused allergic reactions thereby negating its clinical use. Other substances utilized include mussel adhesive protein, collagen, chondronectin, and fibronectin. Other methods of graft fixation included use of a sutured periosteal flap, which is technically demanding and may cause micro-trauama to the adjacent tissue [23].

Osteochondral Transplantation:

Osteochondral transplants have been extensively studied in both animal and human models. Seligman et al. evaluated whole knee fresh autogenous grafts and frozen allografts in dogs [25]. The frozen allografts showed subchondral collapse (by radiography and histology) at one year. In contrast, the autogenous graft group maintained normal architecture for two years, although histologic evaluation showed survival of cells only in the superficial layer of the articular surface. No evidence of antibody response was found; the investigators thus suggested that the subchondral collapse altered the joint mechanics leading to cartilage destruction. This hypothesis

was supported by other studies, which reported on fresh osteochondral grafts that resulted in subchondral bone collapse and creeping substitution of grafted bone in the failed grafts with subsequent altered joint mechanics and deterioration of transplanted cartilage [24]. Rodrigo et al. found that 60% of dogs receiving allografts had cytotoxic antibodies present in their serum, although there was no evidence of graft rejection [26].

Due to long-term problems associated with prosthetic replacement in young patients, osteochondral allografts continue to be used for treatment of various osteochondral defects (especially when associated with tumors) despite the results of animal studies. Clinical studies have shown mixed results. McDermott et al reported on 100 cases of fresh small-fragment osteochondral allografts in femoral osteochondral defects [27]. At five-year follow-up, the cartilage showed fissuring, loss of matrix staining with safranin-O, and chondrocyte clumping and degeneration. Some specimens showed no evidence of hyaline cartilage; many had fibrocartilage, while others had denuded bone. There was no evidence of immune reaction; they hypothesized that weaking of the osseous portion of the graft during the revascularization phase, which takes from one to four years to complete, resulted in mechanical alterations leading to degeneration (mcdermott/langer). Kandel et al. reported necrosis of the subchondral bone and marrow with creeping substitution of host bone [28]. The response seen at the articular surface varied; some specimens showed viable chondrocytes even after 7 years, while others showed degenerative changes ranging from fibrillation to erosion.

Garrett performed a series of fresh osteochondral allografts in 24 patients with defects in the femoral condyles who had failed treatment with abrasion arthroplasty [29, 30]. At two-year follow-up, all patients had improved clinical symptoms with decreased pain,

buckling, and swelling. Eleven patients had subsequent arthroscopies after grafting and only 2 patients exhibited significant deterioration of the graft. No problems with graft extrusion or rejection were encountered. Others have also reported good clinical results with osteochondral allografts. Gross et al. reviewed ninety-two of cases using fresh osteochondral allografts to treat post-traumatic osteochondral defects in the knee [27, 28, 31]. They found good results at five (75%) and ten (64%) year follow-up.

A recently introduced related technique is mosaicplasty, which uses multiple small osteochondral autograft cylinders obtained from non-weight-bearing areas (i.e., the femoral trochlear groove) to resurface defects. The surface of the transplanted plugs maintain their hyaline nature in 60-80% of cases, 73 with fibrocartilaginous tissue acting as filler between the plugs, integrating the transplants with the host tissue. Small grafts are necessary to minimize donor site morbidity; these sites refill with cancellous bone and fibrocartilage [32]. Three-year follow-up data of 57 patients was reported by Hangody et al ., who found that patients who underwent condylar mosaicplasties had a more frequent repair result than those who had patellar mosaicplasties [33]. Biopsies showed that the tissue remained predominantly hyaline in nature.

The problem of surgical options up to now:

Various surgical technique have been tried, these methods have left some problem. [34-40]

1. Newly repair tissues are not "normal cartilage"; fibrocartilagenous and type I collagen rich matrix.
2. These fibrocartilages are not suitable for mechanical force in joint environment.

The problem lies in achieving spontaneous regeneration that enables the newly formed tissue to support the forces it has to bear. For this reason, numerous techniques for the repair of osteocartilaginous defects have been developed using both biological and synthetic materials. And so, to get normal characteristics on repair tissue, cell transplantation that include tissue engineering has been tried in recent years.

Chondrocyte Transplantation

For over thirty years, attempts have been made to stimulate repair by transplanting isolated chondrocytes. Several animal models have examined both allograft and and autogenous cells as transplant material.

Culture of isolated chondrocytes: Moskalewski noted that chondrocytes isolated from immature animals resulted in more regular arrangement of cells and matrix similar to that elicited by normal articular cartilage; cells isolated from mature animals produced a more irregular arrangement [41]. Other studies have also shown that isolated chondrocytes from immature animals had significantly better repair capacity than those isolated from mature donors [34].

Chondrocytes grown in collagen gels maintain normal morphology and evidence of GAG(glycosaminoglycans) and type II collagen synthesis in vitro for five weeks [42]. In monolayer cultures, isolated chondrocytes behave more like fibrous tissue and fibrocartilage, with production of a mixture of both type I and II collagen [43]. Approximately 50% of the newly synthesized glycosaminoglycans are lost into the medium; an increased proportion of hyaluronic acid relative to the total GAG content

reflected the dedifferentiation of the chondrocytes [42]. In clonal or re-aggregated cultures, however, chondrocytes produce a repair tissue which contains type II collagen and whose GAG profile resembles that of articular cartilage [43]. The chondrocytes remain differentiated and phenotypically stable and retain their ability to accumulate metachromatic matrix [42]. Thus, it would appear that cultured chondrocytes in a clonal or re-aggregated pattern are more appropriate for transplantation. Although type II collagen is the predominant type found in normal hyaline cartilage, type II collagen gels do not function as well as type I collagen as a delivery substance for cultured chondrocytes [44].

Allografted Isolated Chondrocytes

With the use of a carrier material, attempts at repair of articular defects with allografted chondrocytes appeared more successful. Itay et al. in 1987 used cultured embryonal chick chondrocytes embedded in a biological resorbable immobilization vehicle (BRIV) composed of fibrin to repair 1.5 mm full-thickness articular cartilage defects in the tibial condyles of roosters. Sixtyfour defects filled with a smooth, shiny surface which was maintained at 6 months. No sign of rejection was observed.

Wakitani et al. reported on a series of allografts which were used to repair defects slightly larger than in the Itay study [45]. Cryopreserved articular chondrocytes suspended in collagen gels were thawed and transplanted into 4mm diameter full-thickness articular cartilage defects in rabbit femurs. At one week, there was evidence of hyaline cartilage formation, which became more organized and was maintained up to twenty-four weeks. Chondrocytes in the repair site were determined to have been derived from the implanted chondrocytes, using autoradiographic analysis..

Immunologic response as determined by host direct and indirect blast formation reactions was insignificant [45].

Noguchi et al. compared the results of allografted and autografted chondrocytes in repair of osteochondral defects in the rat [46]. Cells were embedded in a collagen gel and implanted into 1.5 mm full-thickness defects in femurs. The animals were sacrificed at up to 52 weeks. There was slightly more lymphocytic infiltration noted in the allograft group, although there was no evidence of graft destruction. At twelve weeks, the articular surfaces of both groups were completely covered by hyaline cartilage, and subchondral ossification was noted in 100% of the isograft and 50% of the allograft groups respectively. At 52 weeks, healing was maintained in 100% of the autograft group and 75% of the allograft group.

Autogenous Chondrocyte Transplantation

Grande et al. (1987) reported the use of autologous cultured chondrocytes in articular defects in immature rabbits [7]. Cells were cultured and transplanted autogenously into 3mm diameter full-thickness defects in the patellae, using a sutured periosteal flap to hold the grafted cells in place. At six weeks the implanted area contained a hyaline-like regenerate [7, 23]. Brittberg et al. reported on the results of autogenous chondrocyte grafts in full-thickness defects in adult rabbit patellas [47]. Chondrocytes which had been expanded in vitro were injected under a sutured periosteal flap, with the patch sealed with fibrin. At 52 weeks, the repair tissue appeared to be fully mature with a high degree of chondrocyte columnarization and organized extracellular matrix formation. Control groups with only a periosteal flap sutured over the defect showed fragmented, dense, and disorganized fibrous repair tissue with mild

synovitis and osteophyte formation.

Brittberg et al. (1994) utilized autogenous chondrocyte transplantation in human subjects [47]. Twenty-three patients ranging from 14 to 48 years of age who had previously diagnosed full-thickness defects of the articular surface in either the femur or patella secondary to trauma or osteochondritis dissecans and who had failed initial treatment such as arthroscopic shaving and debridement were included in this study. Previously isolated and cultured chondrocytes were transplanted into defects ranging from 1.6 to 6.5 cm in diameter with sutured periosteal flaps. Initially all patients improved. Arthroscopic evaluation at 3 months showed repair tissue in the defects although the borders of the repair had not become fully incorporated. At 12 months grafts were firmer and more closely resembled the normal surrounding articular cartilage. Histologic evaluation of samples obtained at 12 months showed that 11 of 15 femoral grafts had a hyaline-like appearance, with positive immunostaining for type II collagen. The results in patellar transplants were poorer; only 1 of 7 grafts exhibited hyaline-like regeneration. Biopsies of the remaining femoral and patellar specimens revealed central areas of fibrous tissue surrounded by occassional areas of hyaline-like tissue. Percentages of chondrocyte viability and type II collagen composition were not provided, and more detailed examination of the underlying repair tissue was not performed.

Tissue Engineering (Illustration A.)

Tissue engineering concepts have been applied to a variety of bio-materials to design chondrocyte-seeded or cell-free implants for articular cartilage repair. Among the materials in these engineered devices are demineralized or enzymatically treated

bone [48, 49], polylactic acid [50], polyglycolic acid [51, 52], hydroxyapatite/ Dacron composites [53], fibrin [54], collagen gels [45, 55], and collagen fibers [49, 56]. Some of these materials allow formation of a repair tissue that resembles normal cartilage; however, the repair is often accompanied by substantial fibrocartilage formation. Limited availability of donor chondrocytes has led to the use of alternative cell sources and techniques. Synovial tissue has been used with limited success [57]. Tissues rich in mesenchymal stem cells have also been tested as grafts. Wakitani et al. (1994) used osteochondral progenitor cells from either bone marrow or periosteal tissue, which they have termed "mesenchymal stem cells (MSCs)", to repair articular cartilage defects [55]. Adherent cells from bone marrow and periosteum were isolated , cultured in a type I collagen gel, and transplanted into full-thickness defects in rabbit femoral condyles. Similar results were seen with both types of progenitor cells. By 2 weeks, the autologous MSCs had differentiated into chondrocytes. At 12 weeks, the subchondral bone was completely restored and the defects were filled with hyaline-like cartilage, although in some cases there was a gap between the repair tissue edges and the surrounding normal cartilage. By 24 weeks, however, the repair tissue showed thinning. The repaired surface in the periosteal group appeared split and fibrillated. The bone marrow group had a smoother surface, although the cartilage had thinned. Both groups had areas of incomplete integration of the repair and host cartilage. Mechanically, the repair tissue was more compliant than normal cartilage [55].

Peptide growth factors are important regulators of cartilage growth and cell behavior (i.e., differentiation, migration, division, or matrix synthesis or breakdown) [58]. These factors are being investigated for their potential to induce host cartilage

repair without transplantation of cells, and are being incorporated into engineered devices for implantation.

Because growth factors are soluble proteins of relatively small molecular mass that are rapidly absorbed and/or degraded, making them available to cells in sufficient quantity and for a sufficient duration is a challenge. It may be desirable to have different factors present at the repair site during different parts of the developmental cycle, and for varying lengths of time. Delivery vehicles should ideally be biocompatible, resorbable, have appropriate mechanical properties, and release no harmful degradation products; the same criteria can be applied to cell-carrier or tissue scaffold devices. Several natural and synthetic materials (including those used as cell carriers) have been tested as growth factor carriers. While each has several positive features, the optimal material has not been identified. To date, these methods have been hindered by the lack of knowledge of the appropriate dosage, duration of treatment, and joint clearance of growth factors that are administered by these routes.

The problem of Tissue Engineering

The application of tissue engineering to the repair of articular surface defects in OA or after injury will require that viable cells be transplanted to the injured tissue site. Experimental and preliminary clinical studies have shown that both committed differentiated chondrocytes and undifferentiated chondroprogenitor cells placed in a supportive carrier when used to repair a defect, survive and produce an appropriate extracellular matrix(ECM). Bittberg et al. [47] reported on the use of autologous chondrocyte transplantation under a sutured periosteal flap to repair deep cartilage defects. The early outcome of these studies suggested that approximately 80% of the

patients were clinically improved and the biopsies of the repaired tissue have the appearance of viable hyaline cartilage. However, other experimental studies suggest that the long-term fate of these repaired articular surfaces is characterized by early degeneration and loss of structural integrity [15, 40, 59, 60]. Clearly the long-term durability of this treatment approach remains in doubt and additional studies will be required to define whether strategies such as these will be useful in the restoration of degenerated articular surfaces.

Isolated chondrocytes might be used to generate neo-cartilage for the repair of articular surfaces in the future. For clinical applications, the use of autologous cells is preferred. Because the amount of cells that can be harvested from a patient is limited, in-vitro multiplication of cells is required. During this multiplication process the cells loose their cartilage specific phenotype (dedifferentiation). These dedifferentiative cells are not useful for cell transplantation.

1-2. Purpose of this study

Because there are these above problems in operative options and in cell transplantation techniques, the repair of articular cartilage defects remains a challenge with several current therapeutic modalities based on the grafting of chondrogenic tissues [61, 62] or the implantation of cells capable of generating a neocartilage matrix[47, 55].

Chondrocyte is usually used to cell transplantation; it has been needed to isolate chondrocyte from normal lesion. And also, the amount of cells that can be harvested from a patient is limited. And chondrocyte has limitation of proliferation and lose it's

phenotype in the condition of several passage cells.

Another approach to cell-based treatment strategies is to experimentally employ chondroprogenitor cells isolated from periosteum and bone marrow to repair large full and partial thickness defects of articular cartilage [17, 20, 61, 63-66]. Perichondrium is also recognized as a tissue containing cells with chondrogenic potential [21, 67-69] **(Figure 1)**, and has been used as a tissue source for isolation and monolayer culture of cells for subsequent implantation into an osteochondral defect as a bioengineered method of repair [50, 70, 71].

With the goal of effecting functional repair of an osteochondral defect, the chondrogenicity of implanted cells is an important parameter. Various studies have made it evident that specific conditions of culturing can be critical to the phenotypic expression of cells with chondrogenic potential since dedifferentiation of chondrocytic cells frequently occurs with monolayer cultures [72-75]. Thus, it is important to optimize the culture conditions in order to maintain the chondrocytic phenotype.

It has been found that certain compounds can modulate the chondrogenicity of cells *in vitro*. For instance, in a recent study Goomer et al demonstrated that the chondrocytic phenotype in explant cultures of mature perichondrium–derived cells was enhanced by the addition of TGF–β1[76] and Klein et al demonstrated that differentiation of cartilage cells was stimulated by the osteogenic cytokine, OP-1/ BMP-7, *in vitro*[77]. However, the usefulness of such compounds in repair of osteochondral defects can be compromized by such problems as immunogenic response and difficulty in controlling growth factor concentration and activity.

In monolayer culture, chondrocytes are grown with the addition of serum.

After a short culture period they dedifferentiate to fibroblast-like cells, form collagen type I, change the pattern of their proteoglycan synthesis and they are overgrown by fibroblast-like cells [75]. Recent studies have suggested that three–dimensional culture of cells with chondrogenic potential can be beneficial in terms of promoting and maintaining a phenotype characteristic of chondrocytic cells, i.e. type II collagen gene expression that is a specific marker of hyaline cartilage, and high proteoglycan synthesis rates [78-82]. In the current study we have chosen to examine one such system employing alginate as the method for generating a three dimensional mileau **(Figure 2)**. In such an application alginate offers an important advantage over other potential compounds: an alginate gel can be readily dissolved by chelating substances so that cells may be easily isolated for analysis or subsequent implantation [83, 84]. Although the effects of three-dimensional culture of chondrocytes on maintenance of their condrocytic phenotype have been investigated [80, 82], no studies have been carried out on pluropotential cells such as those derived from perichondrium.

The present study, therefore, examines and compares the culturing of mature perichondrium–derived cells under two-dimensional (monolayer) conditions and under three-dimensional conditions employing alginate as gel compound. Assessments include cell morphology, cell proliferation, proteoglycan synthesis and collagen gene expression.

Chapter 2

Study

2-1. MATERIALS AND METHODS

2-1-1. Preparation and culture of perichondrium-derived cells

Primary culture: Perichondrium was obtained from the costal cartilage of New Zealand White rabbits (mature: 8-10 mos old). All procedures conformed with the guidelines of the University Animal Subjects Committee and the American Association for Accreditation of Laboratory Animal Care. Cells were isolated from tissue with enzymatic digestion [50]. Using sterile procedures the costal ribs were dissected, adhering tissue was removed and the perichondrium layer was isolated. The perichondrium was then washed three times in sterile buffered salt solution containing antibiotics and incubated overnight in 0.1% collagenase (CLS-2, Worthington Biochemical, Freehold, NJ) solution at 37°C under 5% CO_2. The collagenase digest was passed through a sterile 0.45μm filter and the remaining cells and tissue debris were further digested with 0.1μg/ml hyaluronidase (Sigma) and trypsin (Irvine Scientific) for 2 hours. This digest was then passed through an 80μm filter to isolate the cells. Primary cell cultures were established on 10 cm tissue culture plates (Fisher Scientific Inc.) by incubation in Ham's F12/DMEM: 50/50 containing 10% fetal bovine serum (FBS) and antibiotics at 37°C under 5% CO_2. Culture media were changed every 2-3 days and the cultures were maintained until 80-90 % confluency was achieved (approximately for 1 week).

3-D alginate culture: (Figure 3.)

After 7 days in primary monolayer culture, the cells were trypsinized and suspended in 1.2% alginate, 0.15M NaCl (2×10^6/ml of cell suspension) with stirring for 1-2 hours. After passing through a 0.22μm filter, the cell suspension was added dropwise into a solution containing 0.102M $CaCl_2$ [78, 79]. After polymerization (about for 10 min), the $CaCl_2$ solution was removed by washing the bead-cell composites three times with sterile 0.15M NaCl and twice more with culture medium (Ham's F-12/DMEM: 50:50, 10% FBS, 25 μg/ml ascorbate, 50 μg/ml gentamicin). The alginate bead-perichondrial cell composites were then seeded onto 24-well plates (9 beads/well, 6 wells/each time point) and cultured at 37°C under 5% CO_2. Media was changed every three days. The 3-D alginate cultures were maintained for up to 60 days.

Monolayer culture: Passaged monolayer cultures were established from primary cultures in 6-well plates (0.5×10^5 cells/well) for assessments of proliferation and proteoglycan synthesis and in 10cm culture plates (2×10^5 cells/plate) for gene expression studies, i.e. RT-PCR. After cultures were grown to confluence, the cells were trypsinized and passaged for periods up to 60 days (8 passages). All cells were kept in DMEM/F12 with 10% fetal bovine serum (FBS) and 25μg/ml ascorbate.

2-1-2. Histology and Immunohistochemistry

At days 0(primary), 7, 14, 30, 60 alginate beads were collected (9 beads/each time point) and fixed in 4% paraformaldehyde, 0.1M cacodylate buffer containing 10mM $CaCl_2$, pH=7.4, for 4 hours at 20°C. They were washed overnight at 4°C in 0.1M

cacodylate buffer containing $BaCl_2$, pH=7.4[85]. The fixed beads were then embedded in paraffin, sectioned to 5μm thickness and mounted on poly-L-lysine coated glass slides. After deparaffinization in xylene, serial sections from each time point were stained with alcian blue to detect proteoglycan molecules and submitted to immunohistochemistry for antibody detection of chondroitin-6 sulfate or types I and II collagens. Monolayer cultures were also examined at days 0, 7, 14, 30 and 60. Analyses were performed in triplicate for each time point.

Alcian blue staining

Alcian blue staining was performed with 0.05% Alcian blue in 3% acetic acid, pH=2.5, plus 0.3M $MgCl_2$ for 24 hours. After through rinsing in 5% acetic acid, the sections were dehydrated in ethanol and xylene and coverslipped with Permount for examination by light microscopy.

Immunohistochemistry of chondroitin-6 sulfate and types I and II collagens

Monoclonal antibodies to chondroitin-6-sulfate (Seikagaku Kogyo, Tokyo, Japan) and type I collagen (Sigma Chemical, St Louis, MO) and type II collagen (Oncogen, Inc, Cambridge, MA) were used to assess the expression of these molecules. The sections were pretreated with 0.3% H_2O_2 in methanol at room temperature for 20 min and incubated in 5% normal goat serum at room temperature for 20 min. Primary antibodies were applied and allowed to incubate overnight at 4°C. The following primary antibody dilutions were employed; type I collagen 1:100, type II collagen 1:100, chondroitin-6-sulfate 1:50. The slides were then washed with tris-buffer three times for 15 min each and incubated for 30 min at room temperature with biotinylated goat anti-mouse IgG (Vector Lab, Burlingame, CA). Visualization of the antibody-antigen complex was

achieved using the avidin/biotin peroxidase (ABC) method (ABC Kit, Vector Lab, Burlingame, CA). The negative control consisted of nonimmune goat serum as a substitute for the primary antibody.

2-1-3. ^{3}H-Thymidine Incorporation Assay for Cell Proliferation

At 0,1,3,5,7,14 and 23 days (n=6 for each time point) cell proliferation rate were determined by measuring thymidine incorporation. Cells were pulsed with ^{3}H-thymidine (2 μCi/ml solution) in medium (Ham's F-12/DMEM: 50:50, 10% FBS, 25 μg/ml ascorbate, 50 μg/ml gentamicin) and incorporation was allowed to proceed for 12 hours. At the end of this period media was discarded and the cells were washed twice with cold(4°C) phosphate buffered saline (PBS) to remove unincorporated radioactive thymidine. Cells cultured in monolayer were then harvested with trypsin-EDTA. The cells embedded in alginate were dissolved with 55mM Na citrate, 0.15 M NaCl, 30mM disodium-EDTA, pH=6.8[84]. After centrifugation, cell pellets from the monolayer and alginate cultures were washed twice with cold PBS to remove any remaining unincorporated tritium. The cell pellets were then digested with papain (20μg/ml in 0.1 M Na acetate, 50mM EDTA, 5mM cycteine-HCl, pH=5.53) overnight at 60°C. One hundred μl of cell lysates was added to 10 ml of scintillation cocktail (Fisher Scientific) and radioactivity was counted in a liquid scintillation spectrometer. One hundred μl of cell lysates was also taken for determination of total DNA[86]. After trypsinization the number of viable and dead cells was counted manually with a hemocytometer using trypan blue staining. There were no differences in cell viability among the different wells at each time point; therefore, thymidine incorporation was

normalized for the corresponding DNA amount in each well. Relative proliferation rates were expressed as cpm/μg DNA.

2-1-4. ^{35}S-Sulfate Incorporation Assay for Proteoglycan Synthesis

At 0,1,3,5,7,14 and 23 days (n=6 for each time point) relative rates of proteoglycan synthesis were determined by measuring the rates of sulfate incorporation into the cells. Cells were pulsed with ^{35}S-sulfate (10 μCi/ml solution) in medium (Ham's F-12/DMEM: 50:50, 10% FBS, 25 μg/ml ascorbate, 50 μg/ml gentamicin) and incorporation was allowed to proceed for 4 hours. The cells were collected, digested, washed and counted as described above for thymidine incorporation. Results were expressed as cpm/μg DNA.

2-1-5. Collagen Gene Expression

Relative levels of types I and II collagen mRNA expressed in perichondrial cells grown in monolayer and 3D-alginate were determined by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). RNA was xtracted and analyzed at 0, 14, 30 and 60 days (n=3 for each time point). Cells were released from alginate beads using 55mM Na citrate, 0.15M NaCl, 30mM disodium-EDTA, pH=6.8. After centrifugation, the cell pellets were washed in sterile PBS as were cells cultured in monolayer. Total RNA was then extracted from the cells using the Tryzol RNA extraction regent (Gibco, Life-Technology Inc.) according to the manufacture's protocol.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To ensure that PCR amplification of contaminating genomic DNA would not affect the data, total extracted RNA was treated with 10 units/ml of RNase free DNase (RQ 1,

Promega, Madison WI). One μg of total RNA was then reverse-transcribed to cDNA in a final volume of 25μl containing 5μl of 5×Mg free PCR buffer, 5mM $MgCl_2$, 1mM each dNTP, 2.5μg oligo$(dT)_{16}$ primer, 200 units ribonuclease inhibitor and 200 units M-MLV reverse transcriptase (Promega, Madison, WI). The mixture was incubated at 25°C for 10 min and at 42°C for 60 min, heated to 95°C for 5 min, and stored at -20°C. First strand cDNA was then amplified by polymerase chain reaction. In order to monitor the quality of the RNA preparation and to normalize type I and type II collagens data, the glyceraldehyde 6-phosphate dehydrogenase (GAPDH) house-keeping gene was also subjected to RT-PCR in all extracts. One μl of cDNA was added to a 50μl reaction mixture containing 5μl 10×Mg free PCR buffer, 2.5mM $MgCl_2$, 5 units taq DNA polymerase (Promega), and 20pM of each primer. PCR primers specific to selected coding regions of GAPDH (5'-TCCATGCCATCACTGCCA-3'& 5'-CATACCAGGAAATGAGCT-3') [76], type I collagen(5'-TTGCACCTTTGGACATCG-3'& 5`-GACATTTCCCACATTAGG-3`) and type II collagen (5'-GACCCCATGCAGTACATG-3'& 5`-GACGGTCTTGCCCCACTT-3`) [87] were constructed (Retrogen, San Diego CA) on the basis of gene sequences reported in the MCBI genebank. PCR was performed using a DNA thermal cycler (GeneAmp PCR System 2400, Perkin Elmer, Norwalk CT). A cycle profile consisted of 30sec at 94°C for denaturation, 30sec at 58°C for annealing, and 45sec at 72°C for extension. All specimens in a given group were subjected to reverse transcription and polymerase chain reaction at the same time to minimize variations in efficiency between experiments. Electrophoresis of 10μl of reaction mixture on a 2% agarose (Fisher Scientific) gel containing ethidium bromide was

performed to evaluate amplification and size of generated fragments. One hundred bp DNA ladder (GIBCO BRL) was used as a standard size marker. National Institute of Health analysis software (NIH version 1.62; National Institute of Health, Bethesda, MD) was used to scan the PCR agarose gels after photographic documentation. The NIH software measures relative mean density over a fixed gray scale range after correction for background. Regression analysis of the relative mean densities over 12-32 cycles was performed to determine the linear range of PCR amplification for each gene. On the basis of the regression analysis a cycle number of 30 was deemed appropriate for all three genes. Relative levels of expression for type I and type II collagens were normalized to GAPDH for quantitative comparisons.

2-1-6. Statistical Analysis:

All quantitative data were presented as mean ± standard deviation(SD) of the mean. Differences between monolayer and 3-D alginate conditions for each quantitative parameter were established by one-way analysis of variance (ANOVA). Statistical significance was set at $p<0.05$.

2-2. Results

2-2-1. Light microscope and Immunohistochemistry

Perichondrium-derived cells grew as rounded-polygonal shaped cells in monolayer culture and became elongated and fibroblastic in appearance during subcultivation. They exhibited a slightly elongated polygonal shape at low density and became polygonal and then spherical when confuent. When cells were encapsulated into alginate beads, cells became evenly dispersed within the semi-solid beads and rapidly exhibited the typical spherical morphology of chondrocytes. The beads were stable for up to 60 days and the cells formed clumps of various sizes, indicative of continued cell division.

By 7 days cells cultured in alginate formed cell-associated extracellular-matrix that stained positively with alcian blue and antibody to chondroitin-6-sulfate indicative of proteoglycan. This proteoglycan-rich pericellular matrix increased with time through the study period. By 30 days alcian blue staining increased around the cells and many cell clusters appeared. Directly around the cells in the clusters, the produced matrix showed a more irregular pattern, as if the produced components were aggregated.(**Figure 4-A**)

By contrast, perichondrial cells grown under monolayer culture condition exhibited little staining to alcian blue or chondroitin-6 sulfate through the study period up through 60 days.

Similar to what was observed with proteoglycan staining, the chondrocytic phenotype of perichondrial cells grown in alginate was exhibited by the relatively

strong immunohistochemical staining of type II collagen and weaker staining of type I collagen. (**Figure 4-B**) Conversely, perichondrial cells grown in monolayer culture exhibited a more fibroblastic phenotype as evidenced by the relatively stronger staining of type I collagen at 30 and 60 days.

2-2-2. Proliferation:

These above morphological findings were in agreement with the cell proliferation data, which showed that perichondrial cells incorporated ^{3}H-thymidine throughout the experimental period.

^{3}H-thymidine incorporation was significantly higher in monolayer cultures as compared to alginate cultures at days 0, 1, 3, 5 and 7. At 14 days there was no significant difference between monolayer and alginate cultures. At 23 days thymidine incorporation by perichondrial cells cultured in alginate was significantly higher than for perichondrial cells grown in monolayer(**Figure 5**). At this time point, cells reached confluence in monolayer culture.

The incorporaion rate of cells in alginate culture increased with time gradually, reaching a level just above the initial rate by the end of culture period. This continued cell proliferation resulted in a slow, but measurable, increase in DNA content.

2-2-3. Proteoglycan synthesis :

Proteoglycan synthesis of perichondrium-derived cells was studied in order to understand the extracellular matrix production under 3-D culture condition that slowed proliferation and, thus, supported the expression of the differentiated chondrocyte

phenotype. ^{35}S-sulfate was significantly higher in alginate cultured perichondrial cells when compared to monolayer cultured cells at all time points beginning at 1 day and extending through 23 days of culture. (**Figure 6**). The newly synthesised proteoglycan continued to accumulate within the beads matrix, as evidenced by the progressive increase in ^{35}S-sulfate incorporation during 23days of culture. Remarkably, ^{35}S-Sulfate uptake increased immediately at day 1 in alginate culture and increased at 23 days. At the contrast, very lower uptake was observed at any time point in monolayer condition.

2-2-4. Analysis of collagen mRNAs in monolayer and alginate cultures

RT-PCR was used to analyze mRNAs for type I, II collagens in monolayer and alginate cultures. To rapidly screen for the expression of these matrix genes presentative of the chondrocytic phenotype, we used a semi-quantitative RT-PCR method. Relative to cells grown in monolayer, perichondrial cells grown in alginate displayed a higher level of expression of type II collagen mRNA. (**Figure 7-A, 7-B**). This figure shows that over the study period (60 days) the type II/type I mRNA ratio increased in alginate cultures while this ratio decreased in monolayer cultures. At 14, 30 and 60 days the type II/type I ratio was significantly higher in RNA extracted from alginate cultured perichondral cells compared to RNA from cells cultured in monolayer. There has been no studies about type II mRNA expression in perichondrial cells under 3-D culture condition, other recent findings supported this data that the reversal of gene expression of human chondrocytes after passage in monolayer culture and redifferentiation after transfer into alginate beads is time dependent [88].

2-3. Discussion

The present study has demonstrated that 3-D culture of perichondrial cells can modulate the phenotypic expression of those cells.

This study was initiated based on the hypothesis that perichondrial cells cultured in three-dimensional mileau can be stimulated to assume a chondrocytic phenotype characterized by upregulation of two key chondrocytic markers: type II collagen and proteoglycan synthesis. The results of this study have demonstrated both greater proteogrycan synthesis (^{35}S-sulfate incorporation and chondroitin-6-sulfate immunoreactivity) and increased type II collagen expression (type II collagen mRNA and type II immunoreactivity) in cultured cells in alginate compared with cells cultured in monolayer. A particularly interesting observation was that while the initial cell proliferation rates were low in alginate cultures relative to monolayer cultures, the sulfate incorporation rates (proteoglycan synthesis) were more than 5-fold greater in alginate cultured cells than in monolayer cultured cells as early as day 1 and those rates were maintained throughout the culturing period of 23 days. These results indicate that even though the alginate cultured cells were not multiplying rapidly, they were metabolically very active in terms of synthesizing proteoglycan-rich extracellular matrix.

Perichondrium has for some years been recognized as a tissue with chondrogenic potential yielding cells, which after culturing, can be used for implantation and repair of osteochondral defects [19, 21, 62, 89-93]. Last few years, some investigators have demonstrated advantages of perichondrial grafting in the treatment of osteochondral defects [50, 70, 71, 94]. With the goal of effecting functional repair of an osteochondral defect, the chondrogenicity of implanted cells is an important parameter. Preservation

of a chondrogenic phenotype (type II collagen) is particularly important to the ultimate success of repaired defect because tissue matrix characteristics, hyaline cartilage, are crucial to the proper functioning of the articulating surface. Thus it is important to optimize the culture condition in order to manintain the chondrocytic phenotype.

However, few *in vitro* studies using perichondrial cells have been performed. Upton et al. [92] cultured isolated cells derived from perichondium of the rabbit ear after collagenase digestion and found that even isolated perichondrocytes were able to produce hyaline-like cartilage. Bulstra et al. [90] corroborated the *in vitro* capacity for human rib perichondrium to form hyaline-like cartilage. In a recent study Goomer et al. demonstrated that the chondrocytic phenotype in explant cultures of mature perichondrium–derived cells was enhanced by the exogenous addition of TGF–β1 at specific concentrations [76] and Klein et al. [77] demonstrated that differentiation of cartilage cells was stimulated by an osteogenic cytokine, OP-1/ BMP-7, *in vitro*. However, the usefulness of such compounds in repair of osteochondral defects can be compromized by such problems as immunogenic response and concentration-related side effects.

Recent studies have suggested that 3-D culture of cells with chondrocytic potential can be beneficial in terms of promoting and maintaining a characteristic of chondrocytic phenotype [78-82]. Although a number of studies on chondrocytes have been carried out, relatively few other studies have been performed which examine the effects of 3-D culturing conditions on pluripotential cells such as those derived from perichondrium. In one such study, O'Driscoll, et al reported that periosteum explants maintained in agarose gels were capable of generating a cartilage-like matrix as determined by

safranin O staining and type II collagen; they further observed that the effect was enhanced with addition of TGF-β1 [95]. In the present study we have demonstrated that the chondrogenecity of perichondrium-derived cells can similarly be stimulated by culturing in a three dimensional mileau without the addition of specific growth factors to the growth medium. To our knowledge this is the first report of such a unilateral effect on this type of cell.

The present study has clearly shown that rib perichondrium-derived cells cultured in alginate quickly assumed and maintained a rounded morphology typical of a chondrocyte and displayed a chondrocytic phenotype. It has been reported that a rounded shape is fundamentally important for differentiation into a chondrocyte. Although the mechanisms by which this induction of chondrogenity is promoted in 3-D cultures has yet to be determined, a three-dimensional mileau such as alginate may provide an environment similar to that naturally experienced by chondrocytes *in vivo*. It is interesting to note that perichondrial cells cultured in alginate were largely immobile, similar to what is observed with chondrocytes embedded within normal articular cartilage. Alginate-cultured perichondrial cells, however, were capable of thymidine incorporation indicating proliferation.

These findings may have relevance to use the method of 3-D alginate culture in an *in vivo* perichondrial cell transplantation for the repair of osteochondral defects employing a tissue engineered model of transplanted perichondrial cells. Because an alginate gel can be readily dissolved, cells may be easily isolated for analysis or subsequent implantation. [78-80, 83, 96].

Owing to the poor regenerative capacity of cartilage, cartilaginous defects are

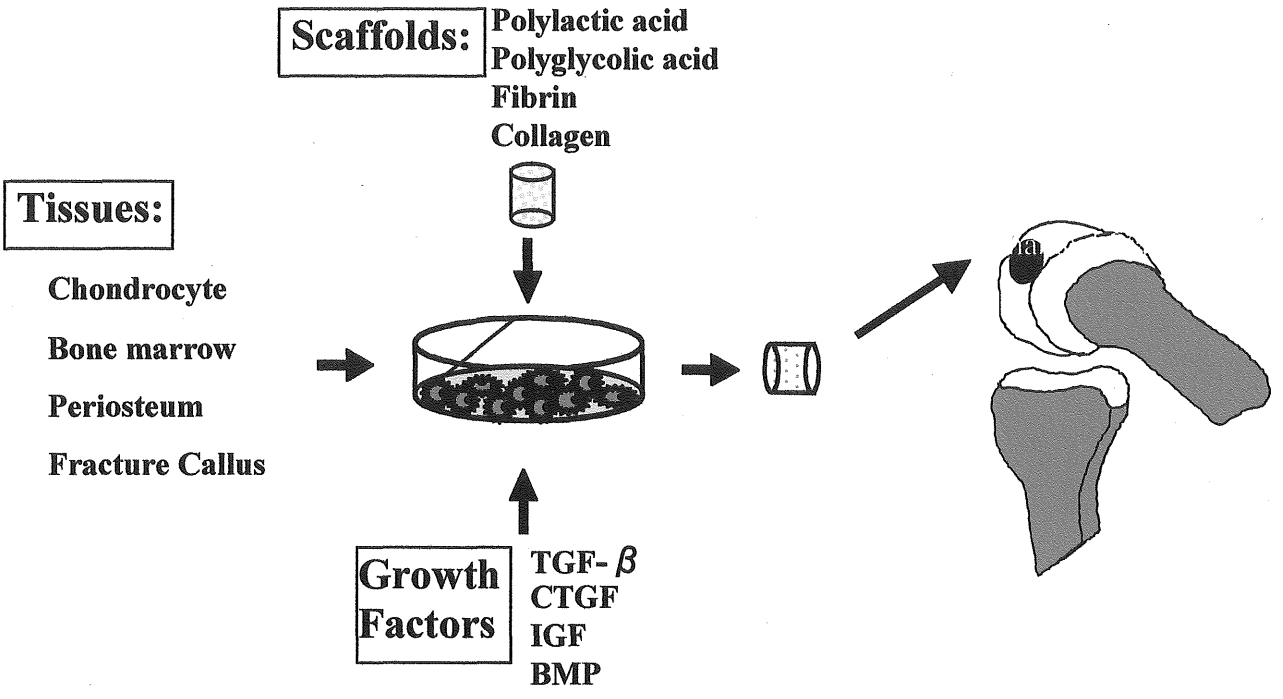
considered to represent pre-arthrotic factors. In addition to autologous and allogenic osteochondral fragments, proliferative tissue, such as periosteum and perichondrium are increasingly being used as a graft material. The aim of treatment is to eliminate the defect and to restore the load-bearing capacity and function of the affected joint. A new, recently introduced, approach aims to stimulate the formation of new cartilage via autologous cultured chondrocyte implantation (ACI) .**(Illustration A)**

The rationale for this treatment is the restoration of loadable hyaline or hyaline-like articular cartilage. To date, only lesions on the femoral chondyle and trochlear groove have been effectively treated. Long-term results are unknown yet.

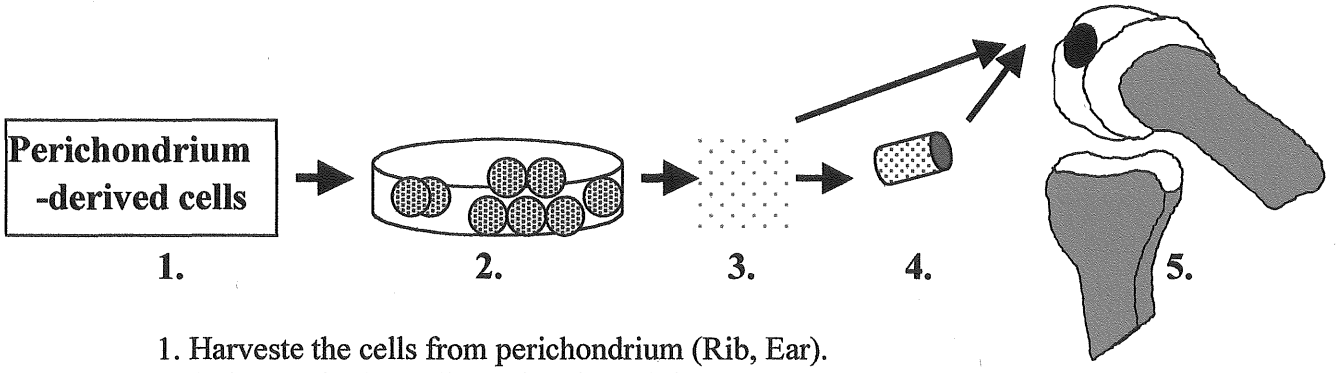
Although this "ACI" technique is useful for treatment of focal osteochondral defect, it has been needed to isolate chondrocyte from normal lesion. Chondrocyte have limitation of proliferation and lose chondrocytic phenotype in several passage cells.

Perichondrium has a chondrogenic capacity and is therefore a candidate tissue for engineering of cartilage. We conclude that perichondrium-derived cells were a useful source of cells for tissue engineering of cartilage when cultured in alginate beads. Although further research is required, we produce new methods of autogenous cartilage implantation by using of perichondrium-derived cells with 3-D culture condition. **(Illustration B)**

A: Various experimental methods for repair of osteochondral defect



B: Practical application for repair of osteochondral defects by alginate culture



1. Harveste the cells from perichondrium (Rib, Ear).
2. Culturing in three-dimensional condition.
3. Recover cells with rich extracelluler matrix and chondrocyte-like phenotype.
4. Seeding the cells into scaffold. Or cell transplantaion directly.
5. Implantation / Transplantation.

3-2. The Limits and Possibility of This Study

Further research is required identifying the cell behavior and production of proteogrycan in scaffold, such as PLA (Polylactic Acid), seeded after freshly isolated from alginate. But the i*n vivo* application of the chondroprogenitor fraction of cells isolated from alginate culture may result in enhanced tissue repair that resembles to normal articular cartilage.

3-3. Conclusion

In conclusion, the present study reports for the first time the effects of alginate 3-D culture conditions on the behavior of perichondrium-derived cells *in vitro*. Our results have demonstrated 3-D culture modulation of perichondrial cell behavior accompanied by upregulation of chondrocytic molecular markers, i.e. type II collagen and increased proteoglycan synthesis. These results are encouraging with regard to enhancing the repair of osteochondral defects in using of perichondrium-derived cells.

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Figure

Figure Legends

Figure 1. Perichondrium (from rabbit rib) with three typical layer

Perichondrial tissue was taken from the lower ribs in rabbits.
a) Safranin O/Fast green stained, b) Immunostained with anti-type II collagen antibody, and c) with anti-type I collagen antibody.

Histochemical and immunohistochemical stain indicating mucopolysaccharides and type II collagen in the transition zone (T) with a decreasing intensity of immunostained with anti-type II collagen towards the proliferarion zone (P).

Figure 2. 3D-culture by alginate beads

This picture shows some typical alginate beads. One bead include approximately 2×10^5 cells. The advantage of alginate culture on chondrocyte was summarized.

Figure 3. Process of 3-D alginate culture

This is a schema of alginate culture system on perichondrium-derived cells.
1. Perichondrial tissue was taken from the lower ribs cartilage.
2. The perichondrium.was peel off from rib cartilage.
3. That perichondrium was digested by enzyme.
4. Cells were cultured in monolayer.
5. Cells were embedded in alginate beads.
6. We observed that the shape of the perichondrial cells grown under the 3-D alginate condition were rounded in appearance.

Figure 4-A: Photomicrographs of perichondrium-derived cells with alcian blue stain under 3-D culture condition.

A : 7 days ,B: 30 days, C and D: 60 days
The cells developed aggregates consisting of two and more cells during the first 7 of the culture period. The cell aggregates became larger and formed capsules on the surface at 60 days. At 7 days, some cells were stained strongly on cell-associate matrix. At 60 days, the cell aggregates with outer capsule were strongly stained with alcian blue.

Figure 4-B:Immunohistochemistry for type I,II collagens and chondroitin-6 sulfate

A,B,C : 7 days(×375); alginate culture

D,E,F : 30 days(×375); alginate culture

G,H, I : 60 days(×375); alginate culture

L : 60 days(×150); alginate culture

A,D,G : Type I collagen,

B,E,H,L : Type II collagen,

C,F,I : Chondroitin-6-sulfate;

J : 60days control(×375); alginate culture

K : 60 days; monolayer cultured cells stained with anti-type II collagen

At 7 days, type I,II collagens and C-6-S(chondroitin-6-sulfate) were stained on cell-associate matrix. At 30 days, cells divided and formed much extracellular matrix. Type II and C-6-S were well stained on cell-associate matrix. Type I collagen was not detectable in cells under 3-D culture condition.

At 60 days, cells formed cluster and large amount of extracellular matrix. Type II collagen and C-6-S were stained well, but type I collagen was weak stained. Inter-cellular space also stained on type II collagen and C-6-S. Cells under monolayer culture were spindle shape and few cells were stained by type II collagen.

Figure 5. ^{3}H-Thymidine Incorporation

^{3}H-thymidine incorporation demonstrated a statistical difference ($p < 0.05$) in the proliferation of cells grown in 3-D alginate culture vs. 2-D monolayer culture. Proliferation of the perichondrial cells was greater in the 2-D monolayer culture, up to 7 days. At 23 days, however, cell proliferation was greater in 3-D alginate culture when compared to the 2-D monolayer culture environment.

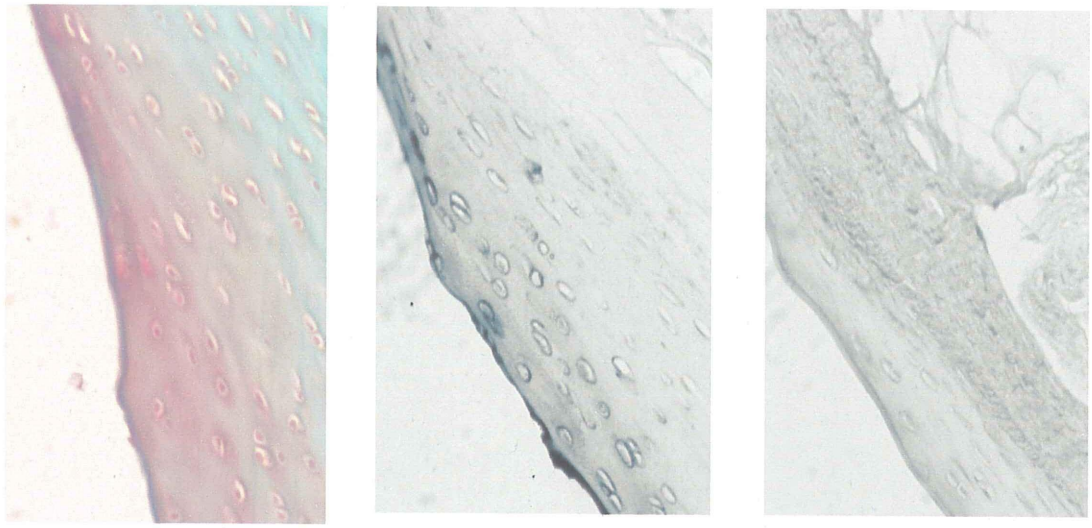
Figure 6. ^{35}S-Sulfate Incorporation

The ^{35}S-Sulfate uptake measuring proteoglycan synthesis was statistically greater for the 3-D alginate culture ($p < 0.05$) when compared to the ^{35}S-sulfate incorporation of the cells grown in a 2-D monolayer culture environment .

Figure 7-A, B : Collagen gene expression by RT-PCR

The ratio of type II to type I collagen gene expression in the perichondrial cells grown in alginate culture at 14, 30 and 60 days demonstrated an up-regulation of type II collagen ($p < 0.05$) when compared to the cells grown in 2-D monolayer culture conditions at the same time period.

Figure 1. Perichondrium (from rabbit rib) with three typical layers



a. Safranin O/fast green **b. Type II collagen** **c. Type I collagen**

F:fibrous layer P: proliferation zone T: transition zone

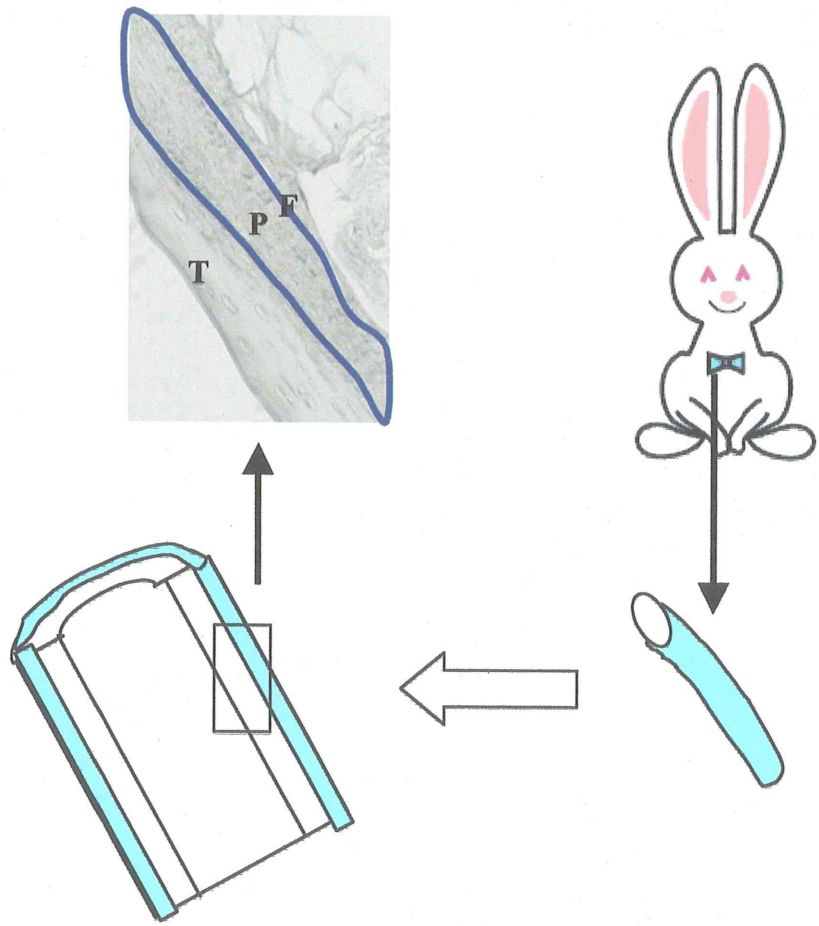
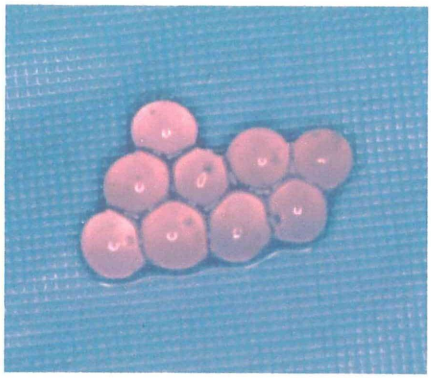


Figure 2. 3D-culture by Alginate beads

Advantage (chondrocyte)



1.Phenotype stability
2.Production of large size of agrecan
3.Possibility of delivery (drug and gene)
4.Easily dissolved by EDTA or phosphates

Alginate = linear polysaccharide consisting of
ß-D-mannuronic acid and α-L-guluronic acid

Figure 3. Process of three-dimensional alginate culture

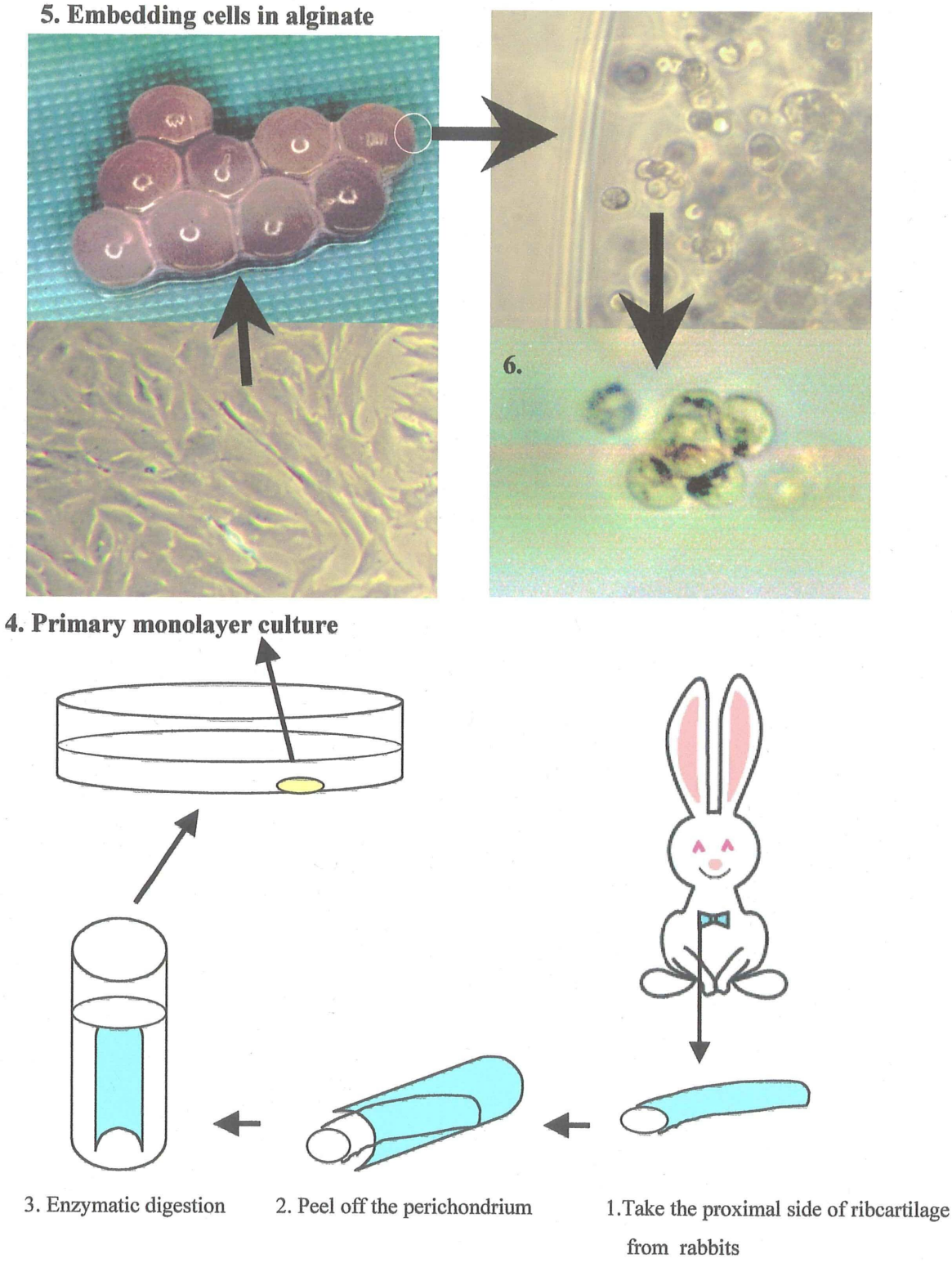


Figure 4-A

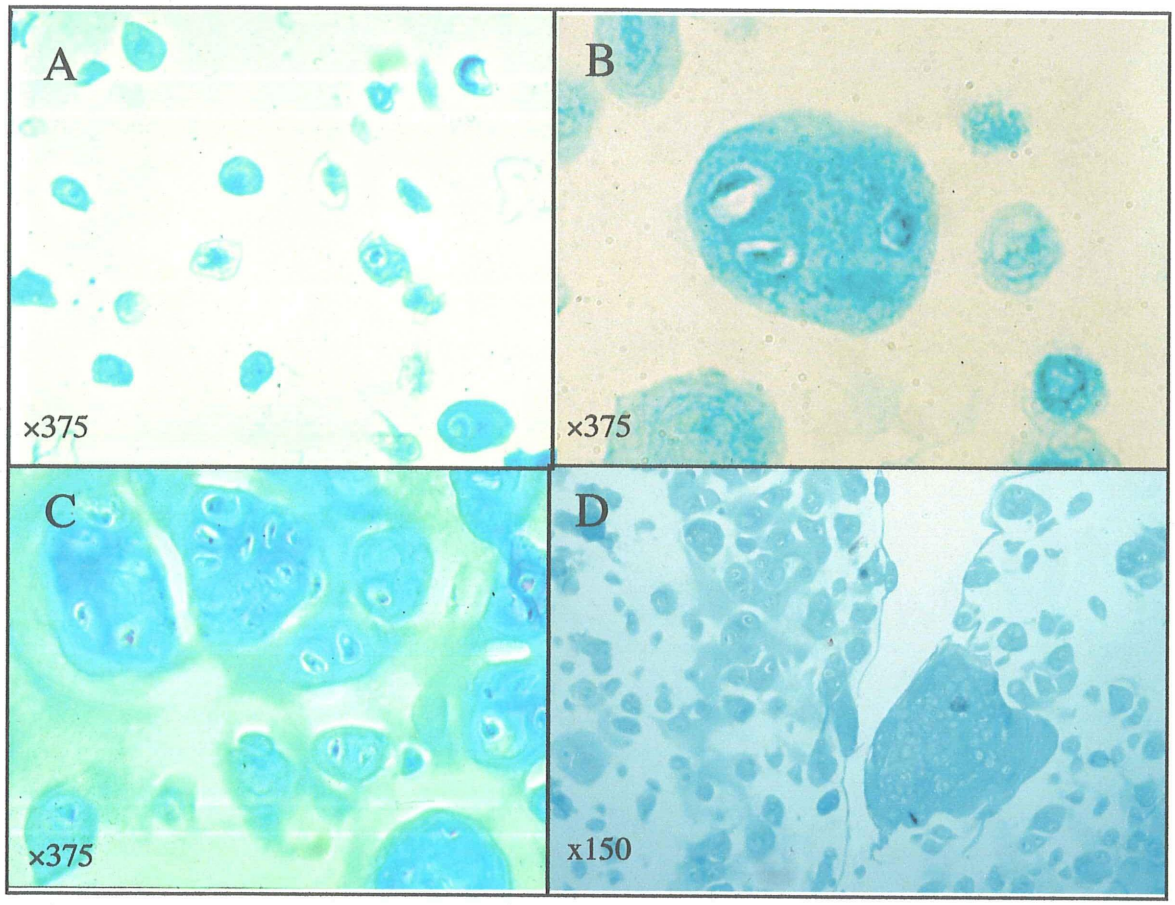


Figure 4-A; Alcian blue staining to cells in alginte culture

A : 7 days
B : 30 days
C , D: 60 days

Figure 4-B

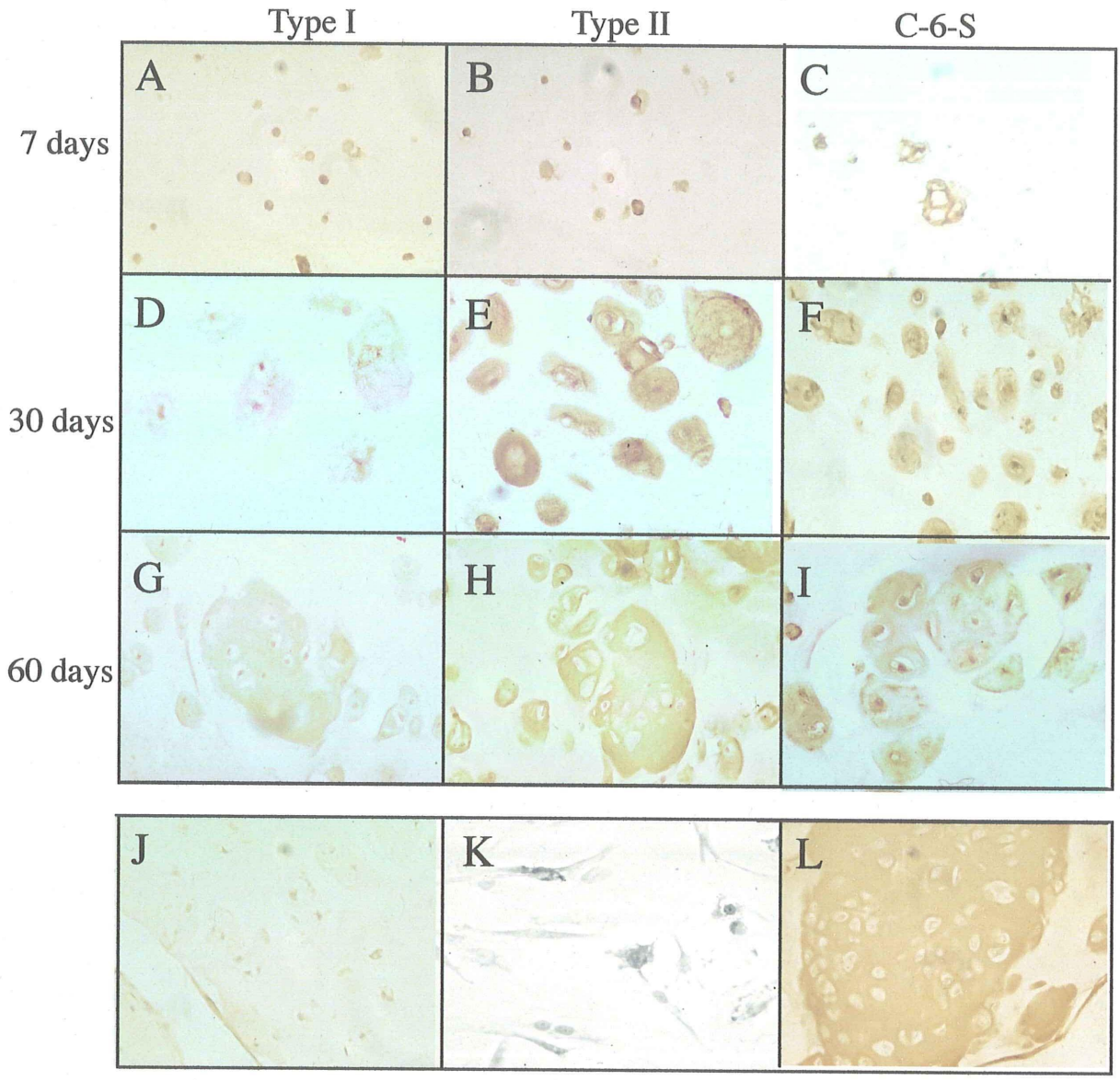


Figure 4-B: Immunohistochemistry for type I,II collagens and chondroitin-6 sulfate

A,B,C : 7 days(×375),
D,E,F : 30 days(×375),
G,H, I : 60 days(×375),
L : 60 days(×150)

A,D,G : Type I collagen,
B,E,H,L : Type II collagen,
C,F,I : Chondroitin-6-sulfate
J : 60days control(×375),
K : Type II staining for 7 passage monolayer cultured cells

Figure 5

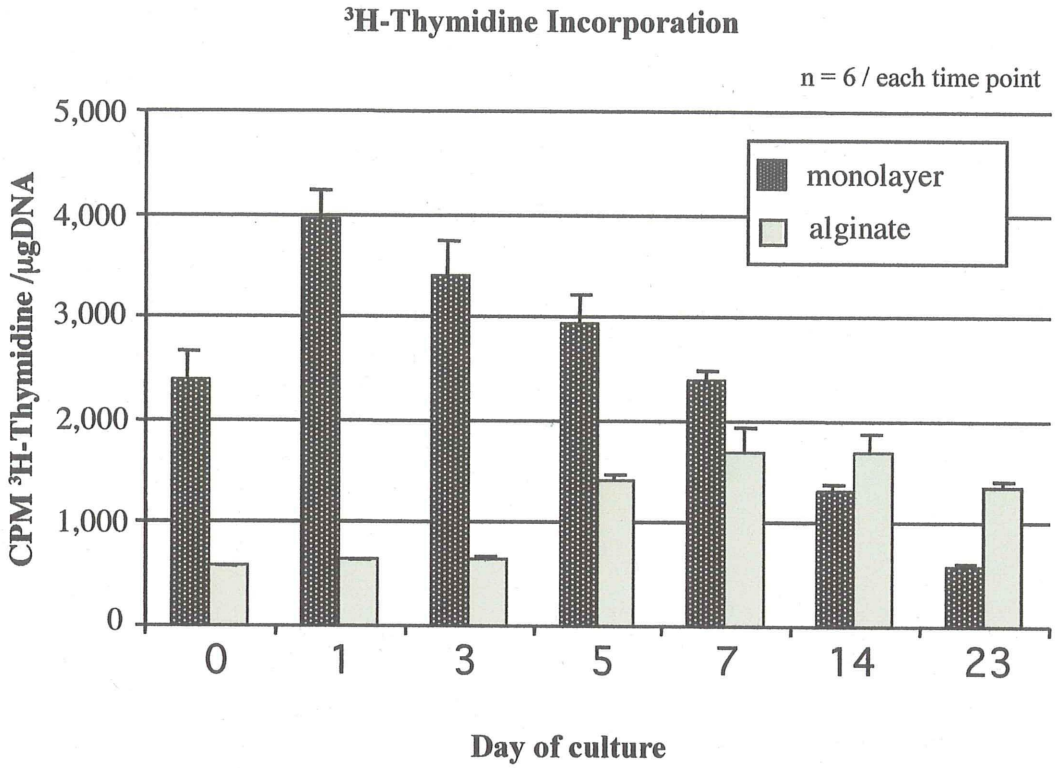


Figure 6

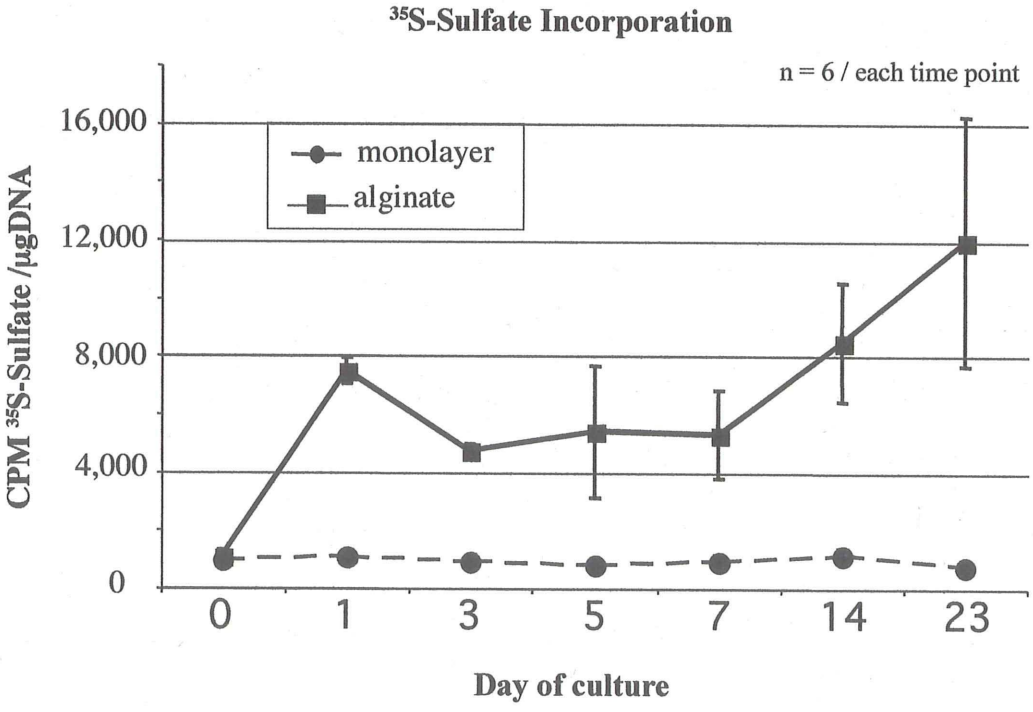
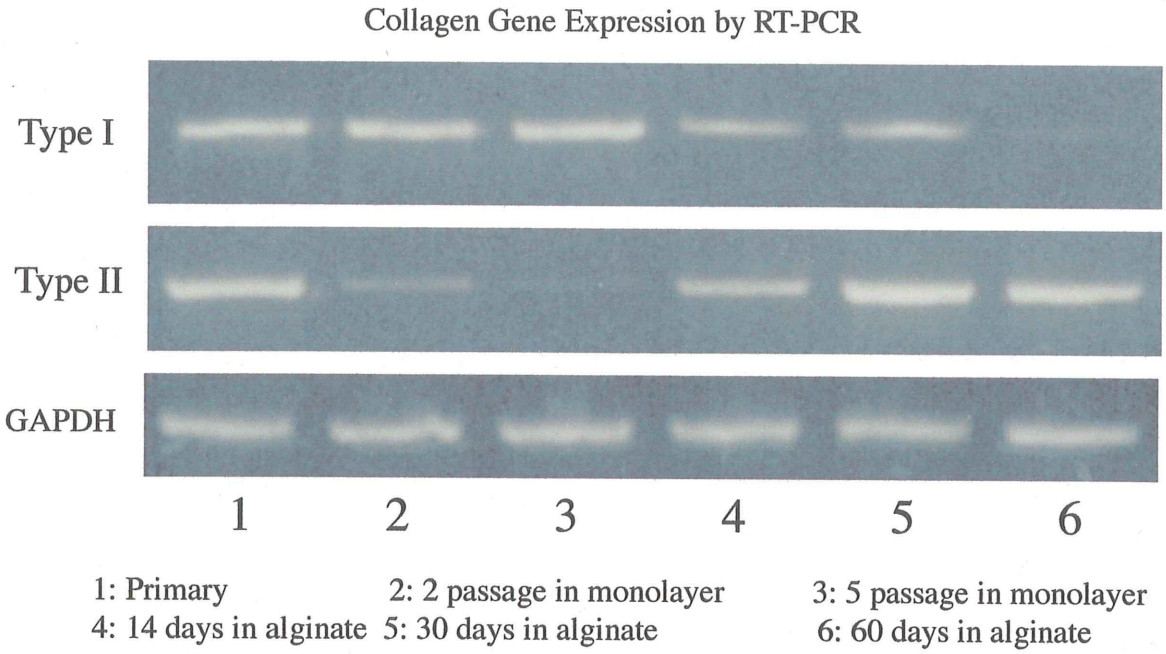


Figure 7-A



1: Primary 2: 2 passage in monolayer 3: 5 passage in monolayer
4: 14 days in alginate 5: 30 days in alginate 6: 60 days in alginate

Figure 7-B