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An *In Vitro* Study Using Organ Culture and Aggregate Culture

滑膜の軟骨分化の特徴

器官培養、凝集培養を用いたIn Vitro研究

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Introduction

The joint capsules are composed of an external layer consisting of dense fibrous tissue called the *fibrous layer* or *fibrous capsule* and an inner *synovial layer* which is more cellular and is thought to secrete the viscoid, colorless liquid of the joint cavity. The synovial layer, therefore, lines the internal surfaces of diarthrodial joints; all such surfaces, including the ligaments and fat pads but excluding the articular surfaces of cartilage and menisci, are covered by this layer. In this present study, the word "synovium" can be defined as this synovial layer.

In terms of function, the synovium strongly affects the metabolism of tissues within the joint, particularly cartilage which lacks a direct supply of blood, lymph, and nerve. The synovium is critically involved in the joint lubrication and, in an indirect way, in maintaining the mechanical stability of the joint. The influence of the synovium is also reflected in the pathophysiology of various diseases of joints which are often accompanied by marked synovial dysfunction.

Anatomically, the synovium is very variable, being composed of several layers, any of which may be present or absent at a given site (1). By light microscopy it can be seen as a cellular layer, approximately 25 μm deep, covering a relatively acellular subsynovium (2). Accordingly, the synovium can be divided into three layers: a cellular *intimal layer*, a *subintimal layer* and a relatively

acellular *subsynovial layer*. Depending on its anatomic position within the joint, the subsynovial layer comprises an adipose, areolar, or fibrous connective tissue (2). Beyond the subsynovial layer lies the fibrous capsule of the joints. In electron micrographs, the intimal layer consists of at least 2 different types of cells, macrophage-like cells (type A) and fibroblast-like cells (type B) (2, 3). Although there has been controversy over the number of different types of cells and their identity, there is no doubt that these cells are differentiated from mesenchymal stem cells (MSCs), from which originate chondrocytes, osteocytes or osteogenic progenitor cells in the periosteum (2-6).

There is increasing evidence about the role of TGF-ß in the induction and enhancement of chondrogenesis in post-natal mesenchymal cells or tissues (7-11). Several studies have suggested that the periosteum has a chondrogenic potential, especially as shown by recent *in vitro* studies with agarose culture system using TGF-ß (12-14). Such studies have given rise to the following assumption: If the periosteum has a potential for differentiation into chondrogenic lineage, the synovium, one of the post-natal mesenchymal tissues, may also have a chondrogenic potential under particular culture conditions.

For the past decade, several investigators have indicated the possibility of synovial chondrogenesis: Iwata et al. demonstrated that a bone morphogenetic protein-coated monolayer culture of synoviocytes derived from rabbits' synovium induced cartilage (15), Hunzinker et al. reported that, in the treatment for

partial-thickness defects of cartilage, a continuous layer of MSCs extended from the synovial membrane into the defect-containing TGF-ß1 (16), and Allard et al. suggested in their histologic study on the synovium-cartilage junction that the articular cartilage could be differentiated from the overlying marginal zone of tissue by positive immunostaining to keratan sulfate and type II collagen (17).

In clinical situations, we often encounter patients with joint disorders or conditions the pathogenesis of which is unknown but suspected to be due to the chondrogenesis of the synovium. Synovial chondromatosis is one such disorder, and is characterized by the formation of multiple cartilaginous nodules in the synovium. It is thought to be benign reactive metaplasia of the synovium, but the exact pathogenesis is unknown (18-20). Osteophytes in osteoarthritis may also be one of those conditions; they are one of the pathologic responses in the osteoarthritic process and are comprised of a central core of bone capped by layers of hyaline and fibrocartilage (21). Although a number of etiologic explanations for the development of osteophytes have been given, including mechanical instability (22, 23), proliferative responses secondary to synovial inflammation (24), or tissue responses to stretching of the synovium at its insertion, the exact mechanism of formation of the osteophytes is unknown. Recently, van den Berg et al have suggested that the osteophytes, which were differentiated from the chondrocyte-like cells depositing the unaffected margins of the preexisting bone in the joint cavity, were caused by the periosteal chondrogenesis

(25). However, for the reason that the osteophytes in their study were observed around the patella in spite of the absence of the periosteum on the patella, it would seem improbable that the osteophytes originate from the periosteum. In the light of the occurrence of synovial hyperplasia which was consistent with the formation of osteophytes in their study, it is likely that the synovium provided the chondrocyte-like cells.

It will follow from these clinical observations, as well as from the fundamental studies, that the synovium has a chondrogenic potential. Nevertheless, there have been no *in vitro* works that indicated the formation of cartilage in the synovial explant such as the chondrogenesis of periosteal explant in the agarose gel culture system. If the chondrogenesis can be induced in the synovial explant cultured, the key to elucidating the pathogenesis of synovial chondromatosis or osteophytes can easily be obtained by means of studying the conditions of the culture or the characteristics of its chondrogenesis.

The purpose of this paper is to indicate the chondrogenesis of synovial explant and to identify the characteristics of its chondrogenesis. Firstly, based on the study of chondrogenesis of the periosteal explants (12), I examined whether the synovial explants have chondrogenic potential in the same conditions of the periosteal explants. Secondly, based on this synovial-explant culture, I clarified whether the chondrogenic potential of the synovial explant is dependent on the presence of TGF-ß1, the presence of serum, donor site, or donor's age. Finally, I

investigated whether there is any other culture model available for characterization of its potential. Thus, I analyzed the chondrogenesis of synoviocytes derived from the synovium using a pellet culture system, which is an adaptation of the aggregate culture and facilitates the terminal differentiation of growth-plate chondrocytes (26, 27) or the chondrogenic differentiation of MSCs (28), and which is characterized by its effectiveness in the study of each factor involved in the process of chondrogenesis, as well as by a three-dimension cell culture.

Materials and Methods

Procurement of the synovial explants

All explants were obtained aseptically from a total of 13 male New Zealand White rabbits. Of these, 5 rabbits were 1 month, 5 were 4 months, and the others were 9 months old at sacrifice (average weight = 0.4 kg, 2.9 kg, 3.7 kg, respectively). Each rabbit was given a lethal injection of sodium pentobarbital, and all explants were obtained within 45 minutes of death.

As shown in Fig.1, synovial explants were harvested from 5 different sites in the knee: medial inner (S2) and lateral inner (S4), which overlie the medial and lateral condyles of the femur in continuity with the border of the cartilage, and medial outer (S1) and lateral outer (S3), which are reflected from the femur and form the joint capsule with the fibrous layer. Fat pad explants (S5) were obtained from the infrapatellar region. Periosteal explants (P) were also harvested from the anteromedial sides of the proximal tibiae.

The harvesting of the synovial explants and fat pad explants was conducted as follows; The knee was shaved, prepared and draped using a sterile technique. A median longitudinal incision was made through the skin on the level of the knee joint, and the outer joint capsule, the fibrous capsule, was exposed. Medial and lateral parapatellar incisions through the outer joint capsule and the subsynovial

inner capsule was exposed superiorly to the level of the upper pole of the patella, inferiorly to the medial or lateral meniscus, medially to the patellar tendon, and laterally to the medial or lateral collateral ligament. The exposed subsynovial inner capsule was lifted with fine toothed forceps and was excised (S1 and S3; Fig.2). The remaining synovium, which was exposed by removal of subsynovial inner capsule and which overlies the noncartilaginous areas of the medial and lateral condyles of the femur, was excised (S2 and S4; Fig.3). The patella tendon was cut off at the distal end on the tibial tuberosity and was reflected proximally. The exposed infrapatellar fat pad was excised (S5; Fig.4).

The methods of harvesting the periosteal explants were as previously reported in the periosteal organ culture model by O'Driscoll et al (12). Briefly, the periosteal explants were elevated by subperiosteal dissection with a sharp periosteal elevator to ensure that the plane of dissection was between the cambium layer of the periosteum and the underlying bone (Fig.5).

Each explant, measuring approximately 3 x 4 mm, was divided into 2 samples of approximately equal sizes. They were temporarily bathed in Dulbecco Minimum Essential Medium (DMEM) at 4 ° C for no more than 1.5 hours prior to placement into culture wells.

Culture of the synovial explants in agarose gel suspension

The explants obtained were cultured in agarose suspension for 6 weeks.

Two of the four explants obtained from each site of the bilateral knees in a rabbit were given TGF-ß1 (10 ng/ml) for the first 9 days (TGF-ß(+) group), while the remaining two explants were not given TGF-ß1 through the whole period (TGF-ß (–) group).

The tissue culture was conducted as follows. Twenty-four-well flat bottom culture plates (CORNING, Corning,NY) were prepared. Each well was first precoated with 500μl of 1 % high-melting-temperature agarose (Bio-Rad, Richmond, CA); 150μl of a 1:1 mixture of 2.0 % low-melting-temperature agarose (Bio-Rad, Richmond, CA); and 2 times the normal concentration of DMEM was then added to each well and allowed to set. One explant was placed on the agarose base in each well, and 350μl of a 1:1 mixture of 2.0% low-melting-temperature agarose and 2 times the normal concentration of DMEM was added. Finally, each explant was suspended in 500μl of a 1:1 mixture of 2.0% low-melting-temperature agarose and 2 times the normal concentration of DMEM. The agarose gel was then allowed to fully congeal at 4° C for 20 minutes. In the wells of the TGF-ß(+) group, each 1.0 ml gel layer was covered with 0.5 ml DMEM containing 10% fetal bovine serum (FBS: Hyclone), 50μg/ml ascorbic acid and 10 ng/ml TGF-ß1. In the wells of the TGF-ß(–) group, each was covered with the same medium without TGF-ß1.

The cultures were maintained at 37° C in 5% CO_2 and humidified room air for 6 weeks. The medium above the gel layer was replaced every third day. In the

TGF-ß(+) group, TGF-ß1 was added every third day when the medium was changed.

After 6 weeks, the explants were removed from the culture for histologic analysis.

Isolation of synoviocytes

For pellet culture study, the synovium was harvested from the medial outer synovium in the knees of four 4-month-old male New Zealand White rabbits. Isolation of synoviocytes was as reported in the method for isolation of synoviocyte by CH Evans et al (29, 30); the synovium harvested was digested with 0.2% clostridal collagenase grade CLS (Worthington) in Gey's balanced salt solution (GIBCO), and cells were grown as monolayers in Ham's F12 medium containing 10% fetal bovine serum.

Aggregate culture

When the cells on the dish reached confluence, adherent cells were tripsinized, counted and $2x10^5$ cell aloquots were spun down at 500 x g in 15ml polypropylene conical tubes. The FBS containing medium was then replaced with a defined medium, consisting of DMEM with ITS+Premix™(Collaborative Biomedical Products; insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenous acid (6.25 μg/ml) and linoleic acid (5.35 μg/ml) with bovine serum albumin (1.25 μg

/ml). Pyruvate (1 mM) and ascorbate 2-phosphate (37.5 μg/ml) were also added. Aggregates were cultures with or without dexamethasone (10^{-7} M), TGF-ß1 (10 ng/ml), or a combination of these agents.

The pelleted cells were incubated at 37° C, 5% CO_2. Medium changes were carried out at 2 day intervals and duplicate aggregates were harvested at 7, 14 and 21 days after centrifugation.

Histology and Immunohistochemistry

For histological and immunohistochemical analyses, explants and aggregates were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into sections 5 μm thick. For histological evaluation, sections were stained with toluidine blue.

For immunohistochemistry, the paraffin embedded sections were put in xylene and ethanol briefly and rinsed with distilled water. Blocking of non-specific antibody binding sites was done by doting in 5% bovine serum albumin (BSA) for 30 minutes. The sections were incubated with primary antibody for one hour, diluted in 0.5% BSA in phosphate buffered saline (PBS). Monoclonal antibody with epitope in type II collagen was used (II-II6B3: Developmental studies Hybridoma Bant). To facilitate antibody access to the collagen, the sections were pre-digested with chondroitinase ABC (0.1 U/ml in 1% BSA in PBS) for 30 minutes. Reactivity was detected with fluorescence

microscopy after incubation for one hour with an FITC linked secondary antibody (Gout anti-mouse Ig, Cappel) diluted in 1% BSA in PBS.

For histomorphometry, the number of samples that demonstrated cartilage was counted. The cartilage was indicated by the presence of a metachromatic-staining matrix, the chondrocytic appearance of the cells and the detection of type II collagen.

Statistical analysis

Statistical analysis was performed by the chi-square test, with a bilateral significance level of 5% ($p<0.05$).

Results

Explants Cultured in Agarose

A total of 124 synovial explants were cultured in agarose with TGF-ß1 (TGF-ß(+) group), and formation of cartilage, which had metachromatic staining with toluidine blue, was observed in 27 explants of them (21.8%) (Table I, Figs. 6-A, 6-B, 7-A, 7-B, 8-A, 8-B). In immunohistochemistry, positive immunostaining of type II was detected in regions of the explants that had metachromatic staining (Fig. 8-C). On the other hand, of the 127 synovial explants in the TGF-ß(–) group, no formation of cartilage, excluding 1 explant, was observed (0.8%) (Table II, Figs. 9, 10, 11). There was significant difference between the 2 groups ($p<0.001$).

In the periosteal explants, 6 of 26 explants in TGF-ß(+) group and 2 of 26 explants in TGF-ß(–) group exhibited formation of cartilage (23.1%, 7.7% respectively; Figs.12-A, 12-B). There was no significant difference of chondrogenic potential in either TGF-ß(+) or (–) group between synovial and periosteal explants.

Examining the difference among the rabbits of three different ages in the TGF-ß(+) group, 4 of 47 synovial explants obtained from 1-month-old rabbits, 16 of 48 from 4-month-old rabbits and 7 of 29 from 9-month-old rabbits exhibited formation of cartilage (8.5%, 33.3% and 24.1%, respectively) (Table I); namely, the

synovial explants harvested from 4-month-old rabbits tended to have the highest chondrogenic potential, and those from 1-month-old rabbits tended to have the lowest potential among the three. There was a significant difference of the potential between 1- and 4-month-old rabbits ($p<0.01$). On the other hand, the periosteal explants from 1-month-old rabbits tended to present the most cartilage among the three. Further, in 1-month-old rabbits, the periosteal explants demonstrated significantly more cartilage than the synovial explants ($p<0.05$).

In a comparison of chondrogenesis between the outer donor and inner donor sites (Table I), of 50 explants obtained from medial and lateral inner synovium, 15 demonstrated formation of cartilage, and 7 of 48 explants obtained from medial and lateral outer sites presented chondrogenesis. There was no significant difference between the two kinds of donor sites. However, in the 4- and 9-month-old rabbit groups with the exception of the 1-month-old rabbit group, 14 of 30 explants obtained from inner sites showed cartilage, while 5 of 30 from outer sites showed chondrogenesis. Thus, in so far as the 4- and 9-month-old rabbit groups are concerned, the explants obtained from inner donor sites had significantly higher chondrogenic potential than those from outer sites.

On the other hand, there was no significant difference of chondrogenic potential between medial and lateral donor sites, with no relation to whether the 1-month-old rabbit group was excepted or not (Table I).

Subsequently, in order to clarify whether factors other than TGF-β1

contribute to the chondrogenesis of the explants, we compared synovial and periosteal explants cultured in a serum-containing medium with those in a defined medium. Periosteal and fat pad explants harvested from a 1-month-old rabbit were divided into 2 groups of equal numbers. In one group, the explants were cultured in agarose gel suspension with FBS-containing medium for 6 weeks as described earlies (FBS group). In the other, the FBS-containing medium was replaced with a defined medium (Defined group). Duplicate samples in each group were cultured, and all of them were given TGF-ß1 (10 ng/ml) for a whole period.

The results obtained are shown in Table III. Both periosteal and fat pad explants in FBS group showed definite cartilage, while only a part of the fat pad explant showed cartilage in the Defined group.

Synoviocytes in Pellet Culture

No formation of cartilage was seen in aggregates incubated without TGF-ß1; on the other hand, chondrogenesis was seen in aggregates with TGF-ß1 as early as day 7, and, by day 14, all aggregates demonstrated chondrogenesis (Table IV, Figs. 13-A and 13-B). The aggregates formed in the presence of both dexamethasone and TGF-ß1 appeared larger and to have undergone more hypertrophic change than those incubated with TGF-ß1 alone. In order to better define the extent of the chondrogenic differentiation of the synoviocytes, we also immunostained representative sections of aggregates with anti-type II antibody. Positive

immunostaining of type II collagen was observed in the aggregates harvested at day 14 and day 21.

Discussion

Several investigators have recently indicated the possibility of synovial chondrogenesis: Iwata et al. demonstrated that a bone morphogenetic protein-coated monolayer culture of synoviocytes derived from rabbits' synovium induced cartilage (15), and Hunzinker et al. reported that, in the treatment for partial-thickness defects of cartilage, a continuous layer of MSCs extended from the synovial membrane into the defect-containing TGF-ß1 (16). Nevertheless, the formation of cartilage in *in vitro* synovial explants has not been demonstrated, whereas the periosteal chondrogenesis has been generally known since O'Driscoll et al. reported the chondrogenesis in a periosteal organ culture system (12-14). From the standpoint of cell differentiation into chondrogenic lineage, the synovium, one of the post-natal mesenchymal tissues like the periosteum, may also induce cartilage. In this study, we cultured the synovial explants obtained from rabbits' knees in agarose gel with TGF-ß1. Consequently, 27 of 124 synovial explants incubated with TGF-ß1 exhibited cartilage, in contrast with only one of 127 explants without TGF-ß1. The data suggests that the synovium has chondrogenic potential under particular conditions, and that the presence of TGF-ß1 is essential for its chondrogenesis. The effects of TGF-ß on chondrogenesis or cartilage matrix production *in vitro* have varied from stimulatory to inhibitory, but

most investigators have suggested the former (7-11, 31). The result obtained also supported the former, the stimulatory effect of TGF-ß1 on chondrogenesis of the synovium.

Several clinical significances will be attached to the result obtained from the study. Firstly, it will lead to clarification of the pathogenesis of synovial chondromatosis characterized by the formation of multiple cartilaginous nodules in the synovium. Secondly, it may be related to the pathogenesis of osteophytes. Recently, van den Berg et al. described experiments where multiple intra-articular injections of TGF-ß1 induced synovial hyperplasia and osteophyte formations which were differentiated from the chondrocyte-like cells depositing the unaffected margins of the preexisting bone in the joint cavity (23, 25, 32). The origin of these chondrocyte-like cells has been uncertain. From autoradiographic images and the localization of osteophytes, van den Berg et al. suggested that the osteophytes originated from the periosteum. However, the osteophytes which were exhibited around the patella are unlikely to originate from the periosteum because the patella has no periosteum anatomically. In the light of the occurrence of synovial hyperplasia which are consistent with the formation of osteophytes after the injection of TGF-ß1, the synovium is thought to induce the chondrocyte-like cells. Thus, the result that the synovium in the presence of TGF-ß1 exhibited chondrogenesis will support the hypothesis that osteophytes originate from the synovium.

Another clinical significance derived from the result concerns the possibility of the application of synovial chondrogenesis for the treatment of an articular cartilage defect. There are numerous studies demonstrating that cells isolated from bone marrow can be reimplanted *in vivo* and undergo osteochondral differentiation (4, 33-36). Furthermore, a recent study demonstrated that free autogenous periosteal grafts could repair a large full-thickness defect in a joint surface (37). The result obtained from the present study shows the possibility that the synovium as well as bone marrow or the periosteum can provide the chondrogenic potential for a defect in a joint surface.

Of interest is the fact that there was a difference in chondrogenic potential of the synovial explants between inner donor and outer donor sites. Especially in 4- and 9-month-old rabbits, the potential at the inner sites was significantly higher than that at the outer sites. Several investigations of the synovium-cartilage junction have shown that fibroblastic-shaped cells are observed within the wedge-shaped tongue of synovial tissue that overlies the cartilage surface, and, as the articular surface of cartilage is approached, the marginal tissue containing the fibroblastic-shaped cells becomes thinner and merges with the cartilage without a clear line of demarcation (38). Recent study by Allard et al. indicated that, in some young patients, positive staining with monoclonal antibody to synovial lining cells was observed on the articular surface of the cartilage, as well as on the synovial lining layer in the marginal tissue (17). These observations suggest that there may

be uncommitted cells adjacent to cartilage that are capable of synthesizing cartilage components. The present data, indicating that the inner sites have higher chondrogenic potential than the outer sites, will substantiate this possibility.

The result obtained also indicates that some factors other than TGF-ß1 may contribute to the chondrogenesis of synovial explants. In the experiment on the comparison between the serum-containing medium and the defined medium, the result showed the serum-containing medium induced more chondrogenesis than the defined medium. Because of the small number of samples, the findings obtained do not enable me to reach any firm conclusions; nevertheless, the possibility exists that some factors in the serum other than TGF-ß1 also contribute to inducing cartilage in the synovium. It is uncertain what factors in the serum are involved. Detailed studies on other factors than TGF-ß1 are necessary to clarify the exact mechanisms of synovial chondrogenesis.

The stimulatory effects of TGF-ß1 on chondrogenesis of the synovium were proposed from the result obtained, as mentioned before. However, the result also suggested this stimulating effect of TGF-ß1 on synovial chondrogenesis seemed to vary with age. In a paper on periosteal explants reported by O'Driscoll et al., formation of cartilage was seen in all periosteal explants obtained from 2-month-old rabbits in the presence of 10 ng/ml TGF-ß1 in the first 2 weeks, although they did not specify the difference in the chondrogenic potential with age (12). A more recent study suggested that the stimulating effect of TGF-ß1 on

periosteal chondrogenesis occurred at a relatively early stage in chondrogenic differentiation since even transient exposure for 30 minutes to TGF-ß1 was effective (39). Based on these observations, the current study was undertaken with administration of 10 ng/ml TGF-ß1 in the first 9 days. The results obtained showed that the synovial explants harvested from 1-month-old rabbits presented less chondrogenesis compared to those from 4- and 9-month-old rabbits, while, consistent with aging, the periosteal chondrogenesis tended to decrease. Further, our data also showed that fat pad explants, harvested from a 1-month-old rabbit and exposed to TGF-ß1 for 6 weeks, demonstrated chondrogenesis. These results suggest that there may be a difference of the optimum duration of exposure to TGF-ß1 in synovial explants between 1-month-old rabbits, and the 4- and 9-month-old rabbits. Consequently, further studies are required on differing periods of exposure to TGF-ß1 and variable dosages of TGF-ß1 in variable aged rabbits.

The important point to notice in this study is that there is no doubt that the synovial explant obtained was heterogeneous tissue: the explant was composed of the intimal layer, subintimal layer and subsynovial layer. Thus, there remain major questions: which layer in the explant and which type of cells in the corresponding layer contributed to chondrogenesis. In the periosteal explant, recent studies have suggested that the cambium layer, which contains undifferentiated cells that are believed to be capable of differentiating into

chondrocytes, contributes to formation of cartilage (12). It is unknown whether there are any histomorphometric similarities between the periosteum and synovium because such analysis was not undertaken on the grounds that it was beyond my present purpose. Based on the induction of chondrogenesis in the pellets made by synoviocytes, of which the majority are fibroblasts, it is likely that the fibroblast-like cells in the intimal layer contribute to the chondrogenesis. In subsequent experiments, full-thickness explants, including the fascia, fibrous joint capsule and synovium, but excluding the skin, cartilage and bone, obtained from rabbits' knees were cultured in the same conditions as synovial explant culture in order to identify which layer of the explant presented the formation of cartilage. But no formation of cartilage was observed (unpublished data). There is a possibility that the condition of the culture for the full-thickness explants is different from that for the synovial explants. Accordingly, it will be necessary to investigate the optimum conditions of agarose suspension culture for the full-thickness explants in order to clarify the chondrogenic sites in the explants anatomically.

From the standpoint of pathogenesis of osteophytes or synovial chondromatosis, it is noteworthy that such diseases are frequently observed at the inner sites, close to the border of articular cartilage, which presented high chondrogenic potential of the synovium (20, 21). However, this "interesting" relationship between the chondrogenic potential of the synovium and the diseases

raises the next question: why relatively young rabbits, 1-, 4-, and 9-month-old, were used in the present study which is related to pathogenesis of osteophytes and synovial chondromatosis. Radiographically, evidence of closure of growth plates in the distal femur occurred at 20-23 weeks; in the proximal tibia, at 22-27 weeks; and in the proximal fibula, at 23-31 weeks (40). Based on the radiographic evidence and their weight, it is thought that each age in the rabbits corresponds to babyhood, teenage, and twenties in human life, respectively; on the other hand, formation of osteophytes is seen at old age in humans, and synovial chondromatosis also occurs during the third to fifth decades of life (20, 21). There were two major reasons why I did not use old rabbits that corresponded to the osteophytes- or synovial chondromatosis-observed age in humans but, rather, I selected relatively young rabbits. Firstly, in the periosteal explant studies by O'Driscoll et al, the rabbits which they used were less than 6 months old, and the majority of them were 2 months old. Secondly, in previous experiments at my laboratory on treatment for the cartilage defect using bone marrow-derived MSCs, chondrogenic potential in young rabbits was much higher than that in old rabbits; 9 months was the age limit for the investigation of chondrogenesis of MSCs (unpublished data). Further detailed studies on the chondrogenic potential of the synovium obtained from old rabbits are necessary.

In this study, a pellet culture experiment was also carried out because the pellet culture system provides a means for studying the process of chondrogenesis,

including TGF-ß1 and other particular factors that regulate the progression of cells through the entire chondrogenic lineage (26-28). Several papers have described the chondrogenic potential of bone marrow-derived MSCs in the pellet culture using TGF-ß1, or a combination of TGF-ß1 and dexamethasone (28), but there have been no reports on chondrogenesis of synoviocytes derived from the synovium using this system. Based on the previous studies, the aggregate culture of synoviocytes was performed under the same conditions as the previous experiments. As a consequence, the aggregate culture provided a useful means for an investigation on the regulation of chondrogenesis of synoviocytes: all rabbit synovium-derived cell aggregates incubated with TGF-ß1, or a combination of TGF-ß1 and dexamethasone, progressed to form chondrogenesis. The results, therefore, support the idea that TGF-ß1 is essential for inducing cartilage in aggregates formed by synoviocytes as well as synovial explants. However, the effect of adding dexamethasone is unclear, as demonstrated by its ability to induce multiple end-phenotypes when added to cultured fetal rat calvarial cells with differentiation potential (41, 42). In the current study, impairment of chondrogenesis was seen in the pellets incubated with dexamethasone alone, but, the aggregates formed in the presence of both dexamethasone and TGF-ß1 appeared larger and to have undergone more hypertrophic change than those incubated with TGF-ß1 alone. Sufficient concentration of TGF-ß1 may, therefore, be necessary for the dexamethasone-induced changes to facilitate

chondrogenesis.

One unique characteristic of the aggregate culture of synoviocytes is that the majority of cells involved in pellets are fibroblasts. Although type B cells, fibroblast-like cells, appear to divide very slowly in the normal synovium *in vivo*, they are capable of rapid division *in vitro*, whereas it is generally agreed that type A cells, macrophage-like cells, do not divide. Consequently, at the step of monolayer culture, the majority of the culture-expanded synovial-derived cells were fibroblast. This observation will support the idea described before that the fibroblast-like cells in the intimal layer have chondrogenic potential and contribute to chondrogenesis of synovial explants. If synoviocytes undergo several passages before making pellets, the cells on the monolayer culture can be purified into fibroblasts. The homogeneity of cells involved in pellets, in contrast to heterogeneity of the synovial explants in the agarose gel, will provide a useful means for studying synovial chondrogenesis. It is necessary to study the chondrogenesis of the pellets made by the cells which undergo several passages.

The question which we must consider next is that, from the standpoint of the role of essential factors on chondrogenesis, the observation obtained from the pellet culture experiment is not in complete agreement with that from the synovial explant experiment. Namely, the chondrogenesis in the pellet could be induced by only TGF-ß1, whereas the explant cultures in agarose gel needed both TGF-ß1 and certain factors in the serum for the chondrogenesis. The key point for answering

this question may be characteristics of this "pellet"; that is, an aggregate form, which allows cell-cell interactions analogous to those that occur in precartilage condensation during embryonic development (28). There is a possibility that cell-cell interactions are essential in any culture system for differentiation of cartilage, and that some unknown factors in the serum-containing medium work on these cell-cell interactions to facilitate the chondrogenesis in the explant culture model.

Ceramic cube assay should also be included in this discussion because of the usefulness of aggregate culture in studying the synovial chondrogenesis. For subsequent experiments, I analyzed the chondrogenesis of synoviocytes in *in vivo* ceramic cube assay (43-45), in which the development of bone and cartilage is observed when MSCs are loaded into porous calcium phosphate ceramic cubes and implanted subcutaneously into immunocompromised hosts. The use of ceramic cube assay is a critical component of the testing format, since it has been established as a verification of the stem cell status or the maintenance of the undifferentiated state of the marrow-derived MSCs exposed to serum-containing medium. Nevertheless, the results showed that implanted synoviocyte-loaded cubes demonstrated impaired chondrogenesis or osteogenesis (unpublished data).The reason for this impairment is uncertain, but there is a possibility that endogenous TGF-ß1 in the subcutaneously implanted cubes was not sufficient to induce cartilage.

In this study, I clarified that synovial explants had chondrogenic potential, which was influenced not only by TGF-ß1 but also by several other factors: age, donor sites and serum. Moreover, I revealed that the pellet culture is effective for studying synovial chondrogenesis. These results obtained will not only give a clue to elucidating the pathogenesis of synovial chondromatosis or osteophytes, but also mark a new step in the treatment of cartilage defects such as the use of the periosteal flap for the treatment of full thickness defect. Further studies will be carried out to identify the exact mechanisms of chondrogenesis of the synovium.

Summary

I analyzed the chondrogenic potential of the synovium using the organ culture system and the pellet culture system in order to identify the characteristics of the chondrogenesis of synovium.

The results obtained were as follows:

1. Synovial explants showed chondrogenic potential in the agarose suspension culture with TGF-ß1.
2. The chondrogenic potential varied with age and donor sites: one-month-old rabbits showed less chondrogenesis than 4-month-old rabbits; and, in the case of 4- and 9-month-old rabbits, the inner donor sites which were close to the border of cartilage showed more chondrogenesis than the outer donor sites.
3. The explants cultured with serum-containing medium showed more chondrogenesis than those cultured with serum-free medium.
4. Synoviocytes in the aggregate culture system also showed chondrogenesis in the presence of TGF-ß1.

These results lead to certain assumptions: the presence of TGF-ß1 is important for synovial chondrogenesis, but factors other than TGF-ß1 also contribute to inducing cartilage; the optimum period of exposure to TGF-ß1 in 1-month-old rabbits is different from that in 4- and 9-month-old rabbits; and the

fibroblast-like cells in the intimal layer correspond to chondrogenesis of the synovial explant.

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Figure legends

Figure 1.

Synovial and periosteal donor sites. S1: Medial Outer, S2: Medial Inner, S3: Lateral Outer, S4: Lateral Inner, S5: Infrapatellar Fat Pad, P: Periosteum

Figure 2.

A synovial explant at day 0 that was harvested from S3, lateral outer, in a 9-month-old rabbit (toluidine blue staining, x120)

Figure 3.

A synovial explant at day 0 that was harvested from S4, lateral inner, in a 9-month-old rabbit (toluidine blue staining, x120)

Figure 4.

A synovial explant at day 0 that was harvested from S5, infrapatellar fat pad, in a 9-month-old rabbit (toluidine blue staining, x120)

Figure 5.

A periosteal explant at day 0 that was harvested from the proximal tibia in a 9-month-old rabbit (toluidine blue staining, x120)

Figures 6-A and 6-B.

A synovial explant that was harvested from S1, medial outer, in a 9-month-old rabbit and cultured in agarose for six weeks in the presence of TGF-ß1 (10 ng/ml) for the first 9 days: [Figure 6-A] low-power photomicrograph (toluidine blue staining, x30), [Figure 6-B] higher-power photomicrograph of the same section (toluidine blue staining, x240)

Figures 7-A and 7-B.

A synovial explant that was harvested from S4, lateral inner, in a 9-month-old rabbit and cultured in agarose for six weeks in the presence of TGF-ß1 (10 ng/ml) for the first 9 days: [Figure 7-A] toluidine blue staining (x48), [Figure 7-B] toluidine blue staining (x240)

Figures 8-A , 8-B and 8-C.

A synovial explant that was harvested from S4, lateral inner, in a 4-month-old rabbit and cultured in agarose for six weeks in the presence of TGF-ß1 (10 ng/ml) for the first 9 days: [Figure 8-A] toluidine blue staining (x48), [Figure 8-B] toluidine blue staining (x120), [Figure 8-C] immunostaining with anti-type II antibody (x120)

Figure 9.

A synovial explant that was harvested from S1, medial outer, in a 9-month-old rabbit and cultured in agarose for six weeks without TGF-ß1 (toluidine blue staining, x48)

Figure 10.

A synovial explant that was harvested from S4, lateral inner, in a 9-month-old rabbit and cultured in agarose for six weeks without TGF-ß1 (toluidine blue staining, x48)

Figure 11.

A synovial explant that was harvested from S4, lateral inner, in a 4-month-old rabbit and cultured in agarose for six weeks without TGF-ß1 (toluidine blue staining, x48)

Figures 12-A and 12-B.

A periosteal explant that was harvested from proximal tibia in a 4-month-old rabbit and cultured in agarose for six weeks without TGF-ß1: [Figure 12-A] toluidine blue staining (x240), [Figure 12-B] immunostaining with anti-type II antibody (x240)

Figures 13-A and 13-B.

Synovium-derived cells cultured as aggregates for 21 days in the defined medium with 10 ng/ml TGF-ß1 : [Figure 13-A] toluidine blue staining (x48), [Figure 13-B] toluidine blue staining (x240).

Table 1: Chondrogenesis in TGF-ß1 (+) group

Number of explants containing cartilage / total number of explants

	S1	S2	S3	S4	S5	Total	P
1m	0/9	1/9	2/9	0/10	1/10	4/47 4m**	4/10
4m	2/9	4/10	1/9	5/10	4/10	16/48	2/10
9m	2/6	1/5	0/6	4/6	0/6	7/29	0/6
1m+4m+9m	4/24	6/24	3/24	9/26	5/26	27/124	6/26
(S1+S3) : (S2+S4)			7/48 : 15/50 ns				
4m+9m	4/15	5/15	1/15	9/16	4/16	23/77	2/16
(S1+S3) : (S2+S4)			5/30 : 14/30 *				

χ^2 test: ns: not significant, *: $p<0.05$, **: $p<0.01$

S1: medial outer synovium; S2: medial inner synovium; S3: lateral outer synovium;
S4: lateral inner synovium; S5: infrapatellar fat pad; P: periosteum;
1m: 1-month-old rabbits; 4m: 4-month-old rabbits; 9m: 9-month-old rabbits

Table 2: Chondrogenesis in TGF-ß1 (–) group

No. of explants containing cartilage / total no. of explants

	S1	S2	S3	S4	S5	Total	P
1m	0/10	0/9	0/10	0/10	0/10	0/49	1/10
4m	0/10	0/10	0/8	1/10	0/10	1/48	1/10
9m	0/6	0/6	0/6	0/6	0/6	0/30	0/6
1m+4m+9m	0/26	0/25	0/24	1/26	0/26	1/127	2/26

χ^2 test: ns: not significant, *: $p<0.05$, **: $p<0.01$

S1: medial outer synovium; S2: medial inner synovium; S3: lateral outer synovium;

S4: lateral inner synovium; S5: infrapatellar fat pad; P: periosteum;

1m: 1-month-old rabbits; 4m: 4-month-old rabbits; 9m: 9-month-old rabbits

Table 3: Comparison between FBS medium and Defined medium

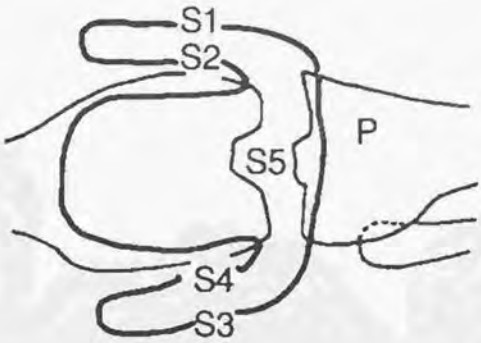
	No. of explants containing cartilage / Total no. of explants	
	FBS group	Defined group
Periosteum	2 / 2	0 / 0
Fat pad	2 / 2	1 / 2

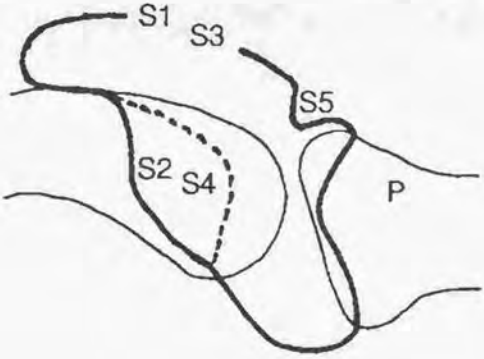
Table 4: Pellet Culture of Synoviocytes:

	No. of pellets containing cartilage / Total no. of pellets			
	Defined	+Dex	+TGFß1	+Dex, TGFß1
1 week	0 / 8	0 / 8	2 / 6	4 / 7
2 weeks	0 / 7	0 / 6	4 / 4	5 / 5
3 weeks	0 / 6	0 / 8	6 / 8	7 / 7

Defined: defined medium; +Dex: defined medium with dexamethasone;
+TGFß1: defined medium with TGF-ß1;
+Dex,TGFß1: defined medium with dexamethasone and TGF-ß1

Figure 1





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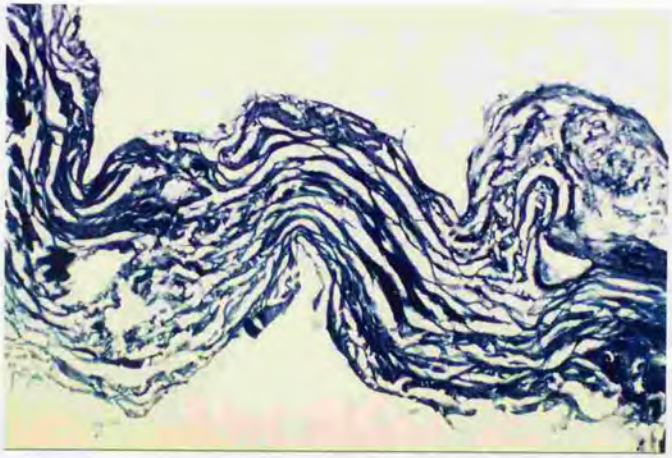
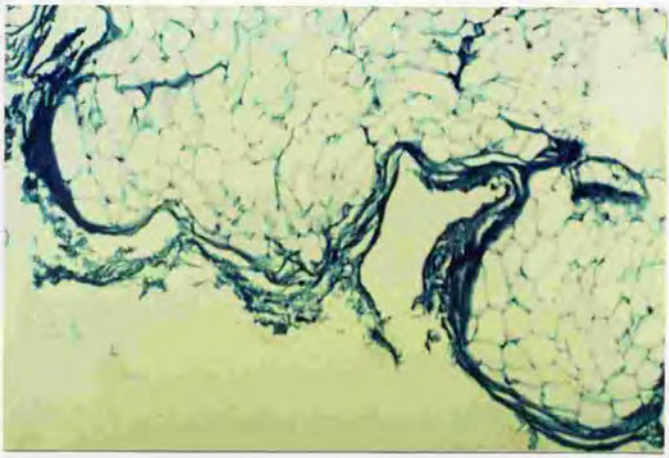


Figure 3

Figure 4



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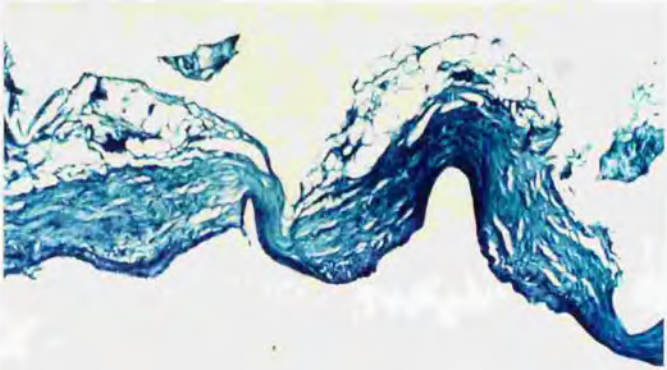

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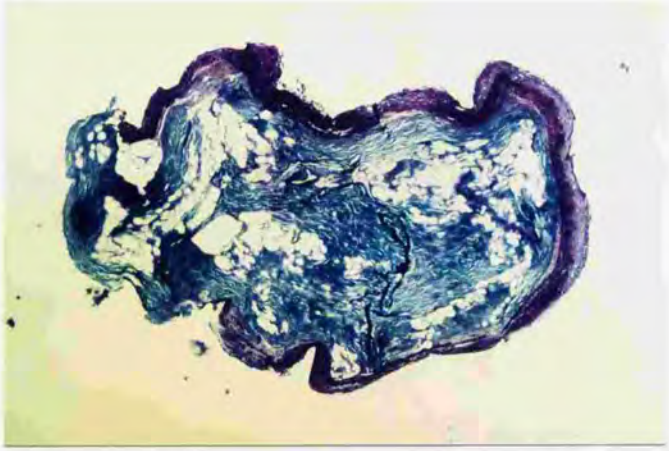

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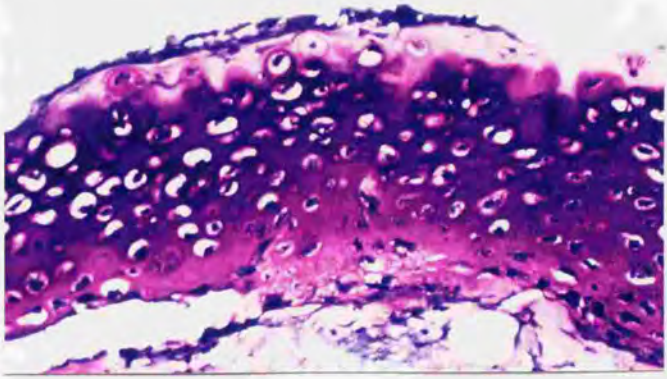

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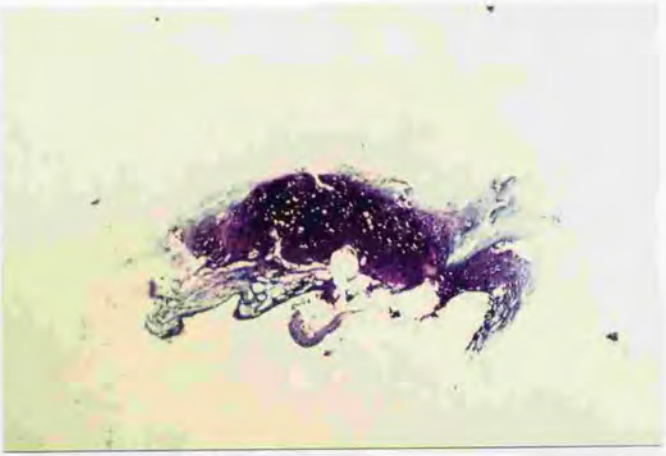

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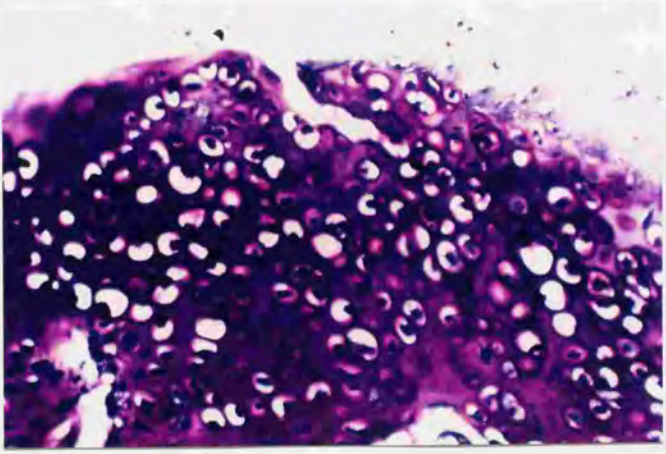

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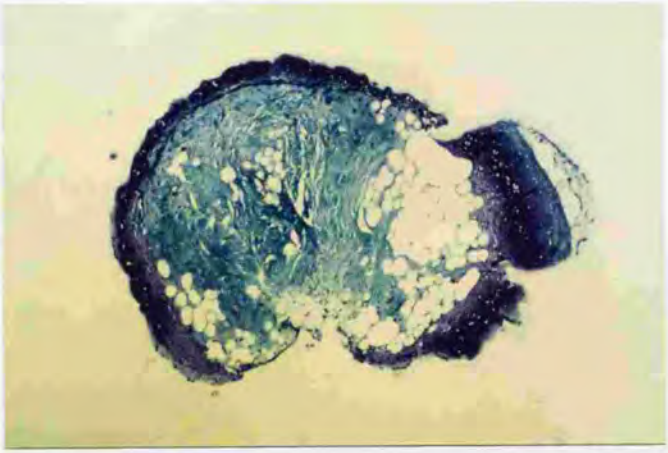

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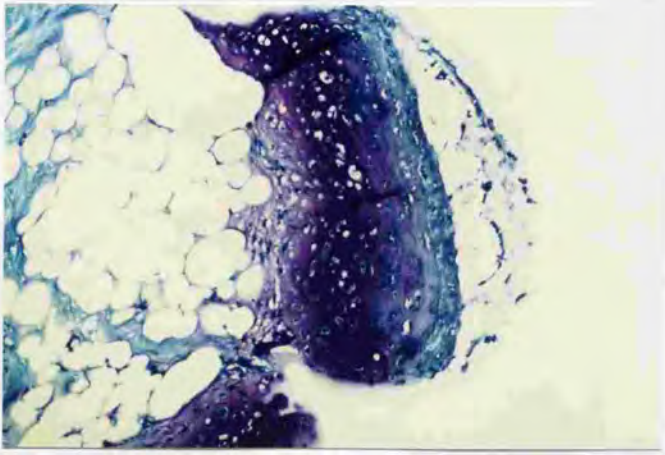

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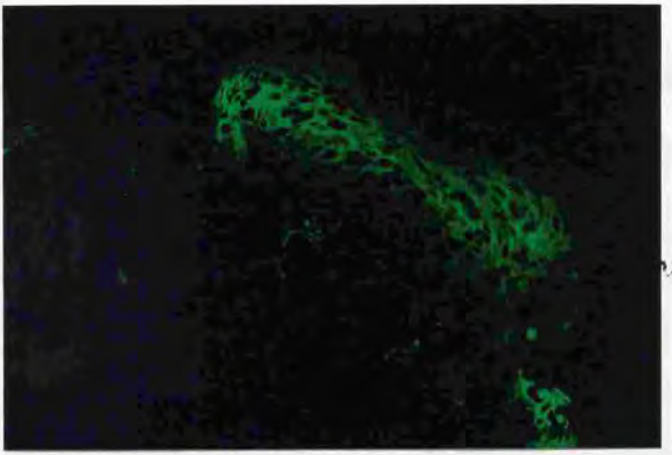

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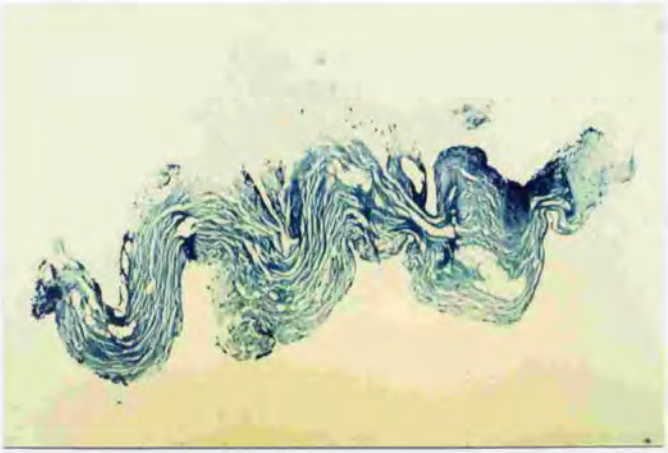


Figure 10

Figure 11

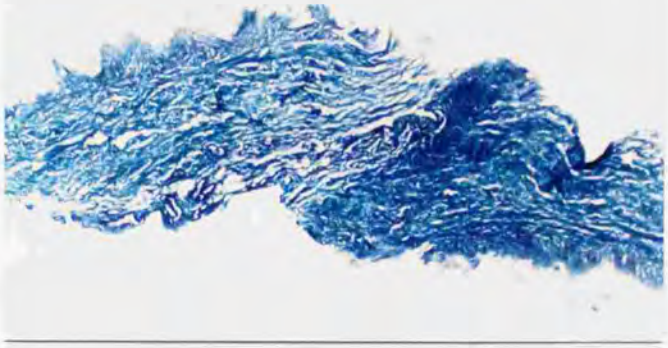


Figure 12-A

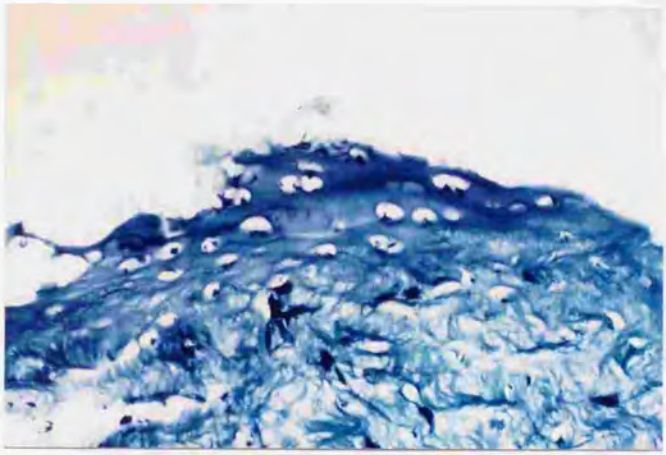


Figure 12-B

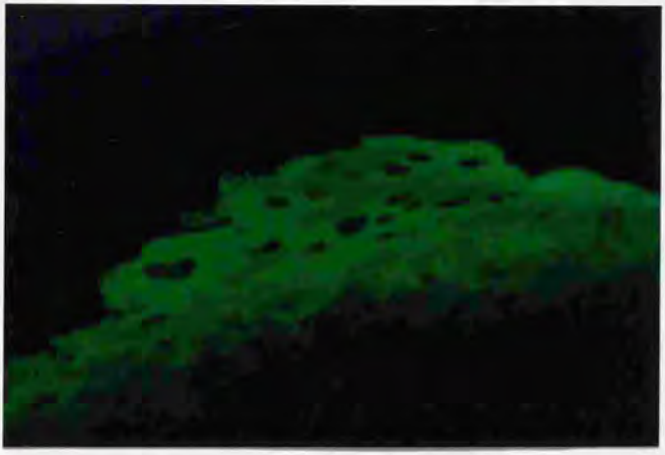
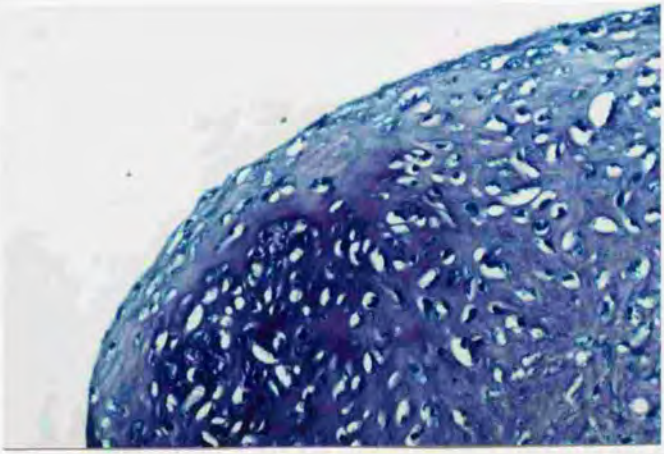
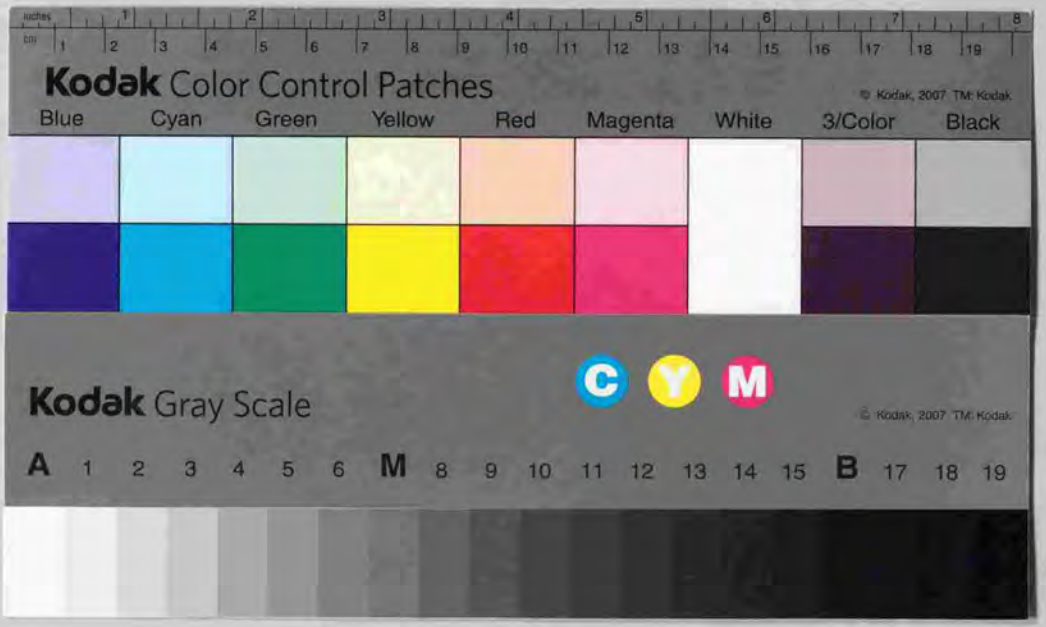


Figure 13-A

Figure 13-B

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