evacuation promotes appropriate differentiation in transplanted MSCs. It is important to observe MSC differentiation in an undamaged nucleus pulposus microenvironment. However, if one were to apply this technique clinically, target intervertebral discs would be damaged to some extent. Furthermore, many obstacles remain to be overcome before application to humans can be considered; these include the appropriate age of patients, extent of disc degeneration, and the efficacy of treatment of degenerative damage caused by aging *versus* postspinal surgery. With regard to the effects of age and extent of disc degeneration, it is suggested that it would be best to apply this technique to mildly to moderately damaged intervertebral discs, as it is impossible to restore highly damaged discs by altered nutrition, and a ruptured anular structure makes cell survival and retention in the closed cavity crucial.

■ Conclusion

We have shown that MSCs can differentiate into cells expressing some of the major phenotypes of nucleus pulposus chondrocyte-like cells. Because it is easier to obtain MSCs from bone marrow than from chondrocytes, there is a good chance that the former may become an alternative source of cells for cell therapy in intervertebral disc regeneration. Although there is a need for further research to define protocols for obtaining the maximum effect of this treatment and to develop patient selection methods, our data indicate that transplantation of autologous MSCs may become an effective option for treating the relentlessly progressive process of intervertebral disc degeneration.

■ Key Points

- Differentiation of mesenchymal stem cells was assessed after transplantation to the nucleus pulposus area of degenerative disc model in rabbits.
- Transplanted mesenchymal stem cells survived and proliferated after transplantation to degenerative discs.
- In phenotype comparison performed before and after transplantation, mesenchymal stem cells expressed apparently different character, suggesting differentiation toward nucleus pulposus cells after transplantation.
- Proteoglycan content and matrix-related gene expression recovered close to normal after mesenchymal stem cell transplantation.
- Despite many obstacles needed to overcome in concerning clinical application, mesenchymal stem cells may become an alternative cell source for cell transplantation therapy for treatment of disc degeneration.

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Effect of Reinsertion of Activated Nucleus Pulposus on Disc Degeneration: An Experimental Study on Various Types of Collagen in Degenerative Discs

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We examined the emergence and sequential changes in type I, II, and VI collagen production in an experimental rabbit model of disc degeneration. Type I collagen was minimally present initially and did not change over 24 weeks. Type I collagen seemed to have no effect on the degenerative process in this model. Staining for type II collagen was positive circumferentially in chondrocytelike cells and was mild in the early phase of disc degeneration, when the chondrocytelike cells began to appear in the inner layers of the annulus fibrosus. The stain became stronger during the middle phase when the chondrocytelike cells arranged themselves in cluster. Compared with type II collagen, the staining for type VI collagen was relatively strong early in the degenerative process. These findings led us to speculate that these chondrocytelike cells play an active role in the degenerative process. The reinsertion of nucleus pulposus cells cocultured with annulus fibrosus delayed disc degeneration and the emergence of chondrocytelike cells. Considering that the emergence of chondrocytelike cells which produce type II and type VI collagen is delayed in discs with the injection of cocultured nucleus pulposus cells by annulus fibrosus cells, we conclude that chondrocytelike cells that produce type VI collagen also seems to accelerate degeneration. Type VI collagen is produced at an earlier phase than type II collagen and may be both active agent and a marker for disc degeneration.

Keywords Low Back Pain, Disc Herniation, Nucleus Pulposus, Annulus Fibrosus.

Lumbar disc herniation is one of the main causes of chronic low back pain and neurologic disorders. Using an animal model, Nishimura et al. [7] found that the reinsertion of autogenous nucleus pulposus can delay further disc degeneration. Okuma et al. [8] reported that the reinsertion of nucleus pulposus cells, which had been activated by coculture with annulus fibrosus cells, delayed the progression of degenerative change in an animal model. The authors also observed the emergence of chondrocytelike cells, which stained positively for type II collagen. On the other hand, while the main types of collagen in normal discs are types I and II, other types of collagen also exist in abundance. The purpose of this article is to document the emergence and record the chronologic change in types I, II, and VI collagen production stimulated by reinsertion of cocultured nucleus pulposus cells.

MATERIALS AND METHODS

This study was conducted under a protocol approved by the Animal Experimentation Committee, Tokai University School of Medicine.

Experimental Study

Twenty Japanese white rabbits weighing 1.3 kg were anesthetized with intravenous pentobarbital sodium (30 mg/kg). The nucleus pulposus from four intervertebral discs (L2/L3, L3/L4, L4/L5, and L5/L6) were aspirated through an anterolateral approach using a 21-gauge needle on a 10-ml syringe to create a model of a degenerative disc under aseptic conditions. After the nucleus pulposus was aspirated, the ventral one-fourth of the annulus fibrosus of the L2/L3 and L3/L4 intervertebral discs also were excised.

Cell Culture

Cell culture was performed according to the method of Shinmei et al. as described previously [14].

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The aspirated nucleus pulposus and the extirpated annulus fibrosus were used for cell culture. The nucleus pulposus was digested in a mixture of 0.2% pronase E (actinase E; Kakenkagaku, Tokyo) and 0.0125% collagenase P (Boehringer Mannheim GmbH, Mannheim, Germany) for 1 h. The annulus fibrosus was digested in 0.025% collagenase P for 6 h after treatment for 1 h with 0.3% actinase. The digested nucleus pulposus and annulus fibrosus cells were washed with Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo) three times. Centrifugation was done at 1500 rpm to collect the cells [13, 14].

A specially designed culture plate (Iwaki Glass, Chiba, Japan) was used for coculture of the two cellular components. A membrane culture insert (Iwaki Glass, Chiba, Japan) was set in each well of the tissue culture plate, dividing the well into two chambers. This membrane was a mixed cellulose ester with 0.45-μm pores at the bottom and prevented any exchange of cellular components. The cells collected by centrifugation were used for coculture of the nucleus pulposus and annulus fibrosus (Figure 1). Coculture was performed for 2 weeks in the medium containing 10% fetal bovine serum (FBS; lot SF10305, Bocknet, Ontario, Canada) and 50 mg/L ascorbic acid (Daiichi Chemical, Tokyo).

Preparation for Reinsertion

After 2 weeks, the cocultured nucleus pulposus cells had increased in number and adhered to the bottom of the culture plate. These cocultured cells was scraped and gathered for reinsertion. The cells were resuspended in 0.02% DMEM. A second procedure was performed on the contralateral side to the first operation under anesthesia induced by intravenous injection of pentobarbital sodium. Cocultured nucleus pulposus cells suspended in 0.02 ml DMEM were injected into the L5/L6 intervertebral disc using a 27-gauge needle with a microinjector.

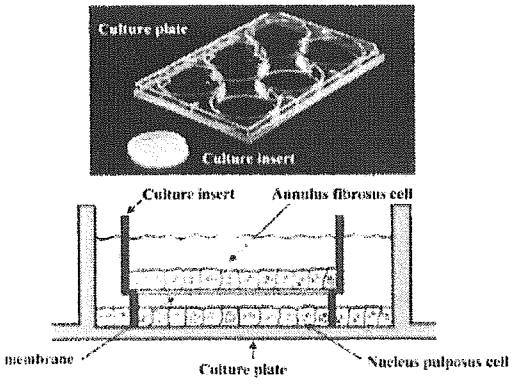


Figure 1. A specially designed culture plate (Iwaki Glass, Chiba, Japan) was used for coculture of the two cellular components. A membrane culture insert (Iwaki Glass, Chiba, Japan) was set in each well of the tissue culture plate, dividing the well into two chambers. This membrane was a mixed cellulose ester with 0.45-μm pores at the bottom and prevented any exchange of cellular components. The cells collected by centrifugation were used for coculture of the nucleus pulposus and annulus fibrosus.

Tissue Preparation

Five rabbits each were killed by intravenous injection of high-dose pentobarbital sodium (120 ml/kg) 2, 4, 8, and 24 weeks after the second procedure, and the L4/L5 disc (negative control group), the L5/L6 disc (injected cell group), and the L6/L7 disc (normal control group) were excised immediately. Frozen sections were made for histologic examination. Samples were fixed with 4% paraformaldehyde in phosphate-buffered saline immediately after their removal, decalcified in Plank–Rychlo solution, dehydrated in a graded alcohol series (70%, 80%, 85%, 90%, 95%, and 99%), and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA). Seven-micrometer-thick sections were stained with hematoxylin and eosin.

Immunohistochemistry

Each section was stained with goat anti-type I, II, or VI collagen (Southern Biotechnology Associates, Birmingham). Data from injected cell (L5/L6), negative control (L4/L5), and normal control (L6/L7) discs were compared.

RESULTS

Histological Findings

The cultured cells were subconfluent at reinsertion. No evidence of infection was observed. In the normal control group, the structure of the annulus fibrosus and nucleus pulposus tissue had been morphologically preserved in all discs at each stage (Figure 2). Although the nucleus pulposus cells were still present at week 8 in the negative control group, any nucleus pulposus cells disappeared at week 24. In addition the proliferation of fibrous tissue and a serpentine or indistinct appearance of the annulus fibrosus architecture were observed. There were typical degenerative changes in disc structure. Chondrocytelike cells were seen mainly in the inner layer of the annulus fibrosus from week 4 to 8. At the week 24, chondrocytelike cells were present throughout the annulus fibrosus (Figure 3). In the injected cell group, the nucleus pulposus cells were still present at week 24. Chondrocytelike cells in the inner layer of annulus fibrosus first emerged at week 8 and continued to increase in number through week 24. However, the number of chondrocytelike

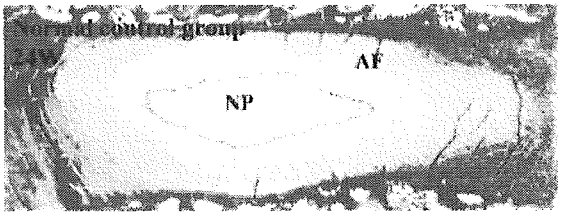
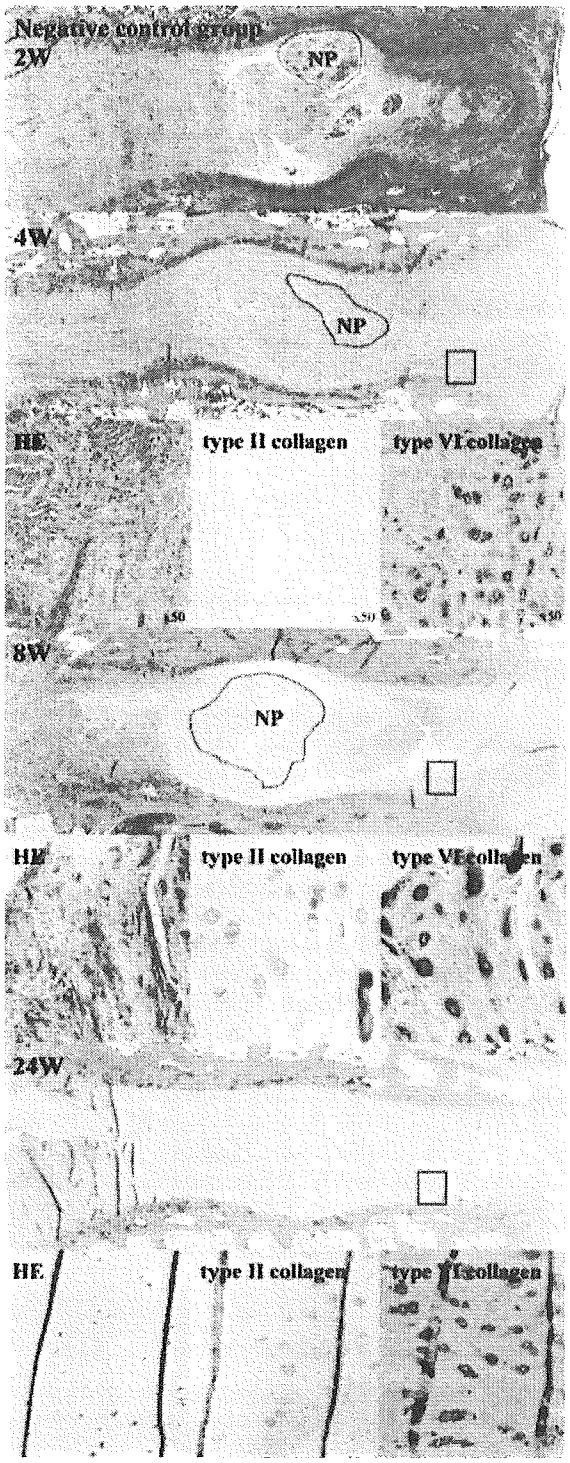


Figure 2. Normal control group. The structure of the annulus fibrosus and nucleus pulposus tissue had been morphologically preserved (week 24, H&E, ×6.6).

cells in this group was smaller than in the negative control group. Degenerative changes in the disc structure were less extensive in the injected cell group than in the negative control group (Figure 4).



Immunohistochemistry

Type I Collagen

Staining for type I collagen tended to occur in the outer layer of the annulus fibrosus in the normal control group (Figure 5). However, the staining was not prominent and did not change over time. No significant differences between normal control group, negative control group, and injected cell group were found.

Type II Collagen

Staining for type II collagen was relatively more prominent in the nucleus pulposus and the inner layer of annulus fibrosus in the normal control group (Figure 5). In the negative control group, faint circumferential staining for type II collagen was detected inside the chondrocytelike cells in the inner layer at week 4, with stronger staining at week 8. By week 24, all layers stained more strongly as the number of chondrocytelike cells increased (Figure 3). In contrast, in the injected cell group, in which the emergence of chondrocytelike cells was delayed, staining for type II collagen inside the chondrocytelike cells did not appear until week 8, and all layers stained positive for type II collagen by week 24 (Figure 4).

Type VI Collagen

Staining for type VI collagen was stronger in the normal control group than staining for type I or II collagen. Staining for type VI collagen was observed throughout the disc, including the nucleus pulposus and the inner and outer layers of the annulus fibrosus (Figure 5). In the negative control group, strong circumferential staining for type VI collagen was detected inside the chondrocytelike cells in the inner layer at week 4. Staining for type VI collagen was stronger than for type II collagen at the week 4. Proliferating fibrous tissues were also stained. Strong circumferential staining inside the chondrocytelike cells also was observed at weeks 8 and 24 (Figure 3).

In the injected cell group, the staining patterns for type VI collagen were similar to that in the negative control group. However, the additional staining for type VI collagen appeared along with the emergence of chondrocytelike cells as it did with type II collagen. In short, the delayed emergence of chondrocytelike cells in the injected cell group correlated with a delay in type VI collagen production. But chondrocytelike cells were present in all layers at week 24, and all layers stained positively for type VI collagen (Figure 4).

Figure 3. Negative control group. Although the nucleus pulposus cells were still present at week 8 in the negative control group, any nucleus pulposus cells disappeared at week 24. In addition, proliferation of fibrous tissue and a serpentine or indistinct appearance of the annulus fibrous architecture were observed. There were typical degenerative changes in disc structure (H&E, ×6.6). Chondrocytelike cells were seen mainly in the inner layer of the annulus fibrosus from week 4 to 8. At week 24, chondrocytelike cells were present throughout the annulus fibrosus. The difference between the staining for type VI collagen and that for type II collagen was detected at week 4. Staining for type VI collagen was stronger than for type II collagen at the week 4 (H&E, type II collagen, type VI collagen, ×50).

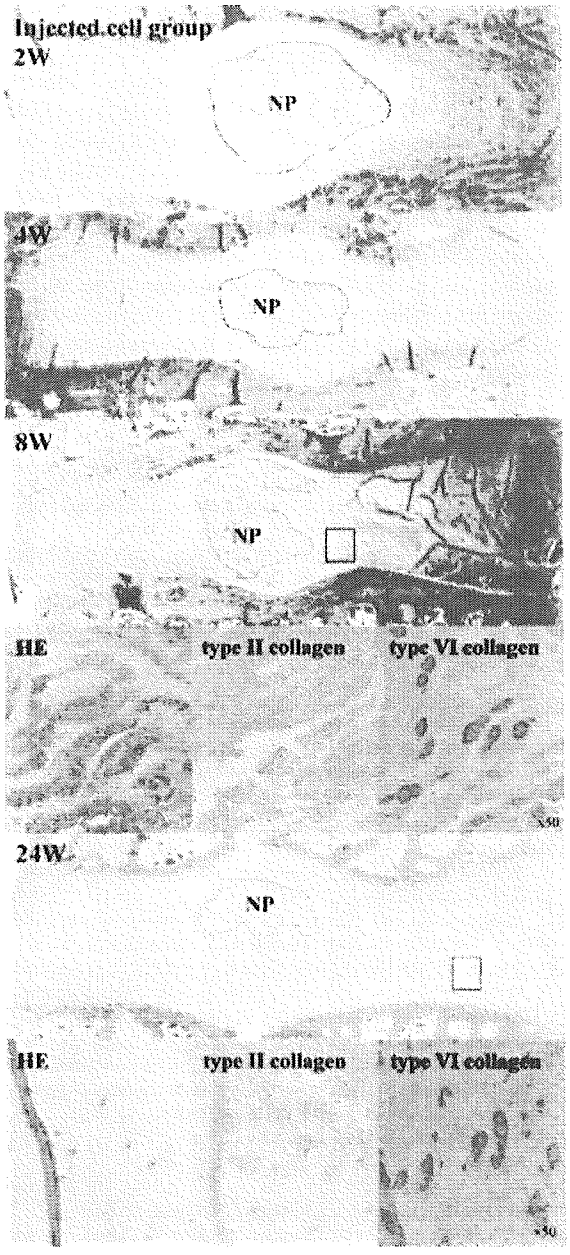


Figure 4. Injected cell group. The nucleus pulposus cells were still present at week 24. Degenerative changes in the disc structure were less extensive in the injected cell group than in the negative control group (H&E, ×6.6). Chondrocytelike cells in the inner layer of annulus fibrosus first emerged at week 8 and continued to increase in number through week 24. However, the number of chondrocytelike cells in this group was smaller than in the negative control group. When the emergence of chondrocytelike cells was delayed, staining for type II and VI collagen inside the chondrocytelike cells did not appear until week 8 (H&E, type II collagen, type VI collagen, ×50).

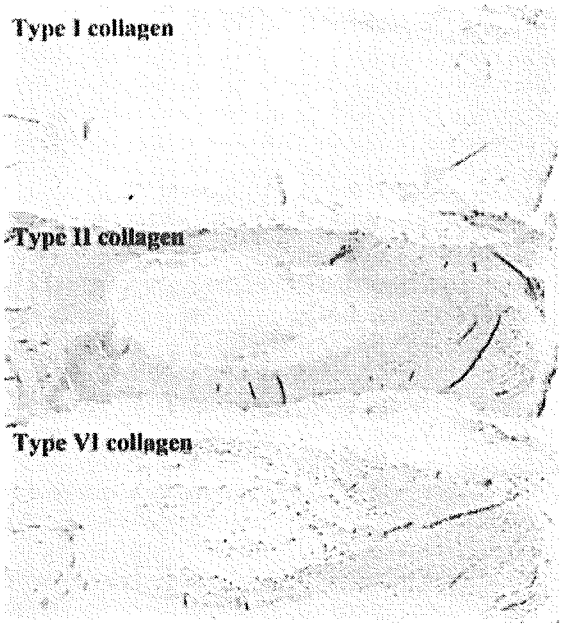


Figure 5. Staining for various types of collagen in normal disc. Staining for type I collagen was not prominent, but this staining tended to occur in the outer layer of the annulus fibrosus. Staining for type II collagen was relatively more prominent in the nucleus pulposus and the inner layer of the annulus fibrosus. Staining for type VI collagen was stronger than that for type I or II collagen. Staining for type VI collagen was observed throughout the disc, including the nucleus pulposus and the inner and outer layers of the annulus fibrosus. Type I collagen, type II collagen, type VI collagen, ×6.6.

DISCUSSION

Structural degeneration and mechanical failure of the intervertebral disc is a major clinical problem, and some corrective surgical procedures, such as herniotomy via the posterior approach, percutaneous nucleotomy, and laser disc decompression, can actually accelerate disc degeneration under some circumstances [9].

To better understand the degenerative process of the disc components, it is helpful to have a better appreciation of the changes in the amount and distribution of the different types of collagen. The collagen framework of the intervertebral disc is composed primarily type I and II collagen. Takaishi [12] reported that the gene expression of type II collagen increases early in the process of disc degeneration. In addition, biochemical and immunohistologic analyses have demonstrated other types of collagen in the intervertebral disc [1–6, 10, 11]. Type VI collagen is surprisingly abundant in the bovine disc [10], and staining intensity for type VI collagen shows a tendency to be reduced in disc disease [1]. Andreas [6] reported immunolocalization of major interstitial collagen (types I–III, V, VI, and IX) in human lumbar intervertebral discs in patients of various ages, but the reason for this finding remains unclear.

In this experimental rabbit model of disc degeneration, staining for type I collagen tended to be limited to the outer layer of the annulus fibrosus in the normal control group. However, staining was rather mild. In addition, the staining pattern did not change over time, and there was no significant difference between normal control group, negative control group, and injected cell group. Therefore type I collagen does not seem to figure prominently in the degenerative process in this experimental model. The staining for type II collagen was observed in a circumferential pattern within the chondrocytelike cells, which signaled the beginning of the degenerative process, as reported by Nishimura and by Okuma [7, 8]. The intensity of staining within the chondrocytelike cells for type II collagen was mild at week 4, when the chondrocytelike cells first began to appear in the inner layers of the annulus fibrosus, and became stronger by the week 8, when the chondrocytelike cells began to arrange themselves in clusters. Staining remained strong through week 24, when the chondrocytelike cells were observed in all layers of the annulus fibrosus.

The staining pattern for type VI collagen resembled that of type II collagen. Strong circumferential staining for type VI collagen was detected inside the chondrocytelike cells. The difference between the staining for type VI collagen and that for type II collagen was detected at week 4. Staining for type VI collagen was stronger than for type II collagen at the week 4. Additionally, strong staining was observed in fibrous tissue that proliferated to fill the void in the nucleus pulposus. Many reports suggest that the deposition of type IV collagen is beneficial to healing. Although a similar increased deposition of type IV collagen was observed during the early phase of disc degeneration in our experimental model, the degeneration actually appeared to progress rather than regress as a result. Further, many chondrocytelike cells were observed at the week 24 in this experimental model. We hypothesize that these cells produce type VI collagen early in disc degeneration, but subsequently change to produce both type II and type VI collagen. It was not possible to determined whether the chondrocytelike cells that produced type VI collagen during the early phase of degeneration were the same cells that produced type II collagen subsequently.

Nishimura et al. [7] have developed an innovative procedure in which autogenous nucleus pulposus is reinserted into a degenerated disc. Using an experimental model, the authors showed that this intervention retards further degeneration of the disc. Furthermore, Okuma et al. [8] reported that reinsertion of nucleus pulposus cells activated by coculture with annulus fibrosus cells delays disc degeneration in an animal model similar to the one we used in the current study. Reinsertion of nucleus pulposus cells retarded disc degeneration and fewer chondrocytelike cells were observed. Okuma et al. [8] also have reported that the presence of type II collagen correlated with the appearance of chondrocytelike cells in the inner layer of annulus fibrosus during progression of the degenerative process. The authors suggested that the presence of type II collagen appears to accelerate degeneration. We identified chondrocytelike cells that produced type VI collagen prior to the production of type II collagen. Considering that the emergence of chondrocytelike cells that produce type II and type VI collagen is delayed in the discs in which cocultured nucleus pulposus cells had been reinserted, we conclude that chondrocytelike cells that produce type VI collagen also seems to accelerate degeneration. Type VI collagen emerges at an earlier phase than type II collagen, and therefore may be an important marker for disc degeneration in addition to the role it plays in the actual pathophysiology.

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Transplantation of mesenchymal stem cells embedded in Atelocollagen® gel to the intervertebral disc: a potential therapeutic model for disc degeneration

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Abstract

Intervertebral disc degeneration is considered to be one of the major causes of low back pain. Despite this irreversible phenomenon, attempts to decelerate disc degeneration using various techniques have been reported. However, to date there has been no proven technique effective for broad clinical application. Based on previous studies, we hypothesize that maintenance of proteoglycan content in the disc is achieved by avoiding the depletion of nucleus pulposus and preserving the structure of the annulus is a primary factor in decelerating disc degeneration.

One novel approach to solve the dilemma of intervertebral disc degeneration is found at the stem cell level. Mesenchymal stem cells (MSCs) are known to possess the ability to differentiate into various kinds of cells from mesenchymal origin. Although the majority of cells that contribute to disc formation are known to obtain chondrocyte-like phenotypes, no reported study has emphasized the correlation with mesenchymal stem cells.

To evaluate the possible potential of MSCs in disc cell research and treatment of degenerative disc disease, autologous MSCs embedded in Atelocollagen® gel were transplanted into the discs of rabbits which had undergone a procedure proven to induce degeneration.

The results suggest that MSC transplantation is effective in decelerating disc degeneration in experimental models and provided new hopes for treatment of degenerative disc disease in humans. Atelocollagen® gel served as an important carrier of MSCs in transplantation, permitting proliferation, matrix synthesis and differentiation of MSCs. This study strengthens the viable efficacy of practical application of MSCs in treatment of intervertebral disc disease.

Keywords: Intervertebral disc; Mesenchymal stem cells; Cell transplantation; Tissue engineering; Atelocollagen®

1. Introduction

Low back pain is a common medical and social problem in the adult population in the world today [1–3]. Intervertebral disc (IVD) degeneration is an irreversible phenomenon, which is often associated with low back pain [1–2]. Multiple elements contribute to disc degeneration including genetic factors, environmental factors and aging [2]. Chronic weight loading, which occurs in the context of heavy work, intense physical activity, and obesity, are conditions, which are found to accelerate the process of disc degeneration [2,3]. Moreover, degeneration secondary to certain corrective surgical approaches such as long fusion, posterior herniotomy, and percutaneous nucleotomy or laser disc decompression sometimes elicit lumbago and neurological dysfunction [3–5]. Recent experimental studies focusing on decelerating disc degeneration have provided insight into the future management of degenerative disc diseases [3,6–12]. However, adequate methods

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for broad clinical application have not yet been available.

The etiology and pathophysiology of disc degeneration is still unknown [2,3]. However, disc degeneration can be described clinically as a loss of proper stability and mobility, which are the two major roles of the disc [13,14]. Morphologically and histopathologically, disc degeneration can be described as a decrease in water content associated with proteoglycan diminution of the nucleus pulposus and inner annulus. This effect results in destruction of annular structure and flattening of the disc [15–17]. Disc tissues have a limited ability to regenerate, since they are avascular and nutritionally supported only by passive diffusion at the end plates [3,18,19]. Consequently, once the degenerative process is activated, it is difficult to decelerate, and is ultimately considered to be an irreversible condition [3].

Nishimura and Mochida, coworkers in this study, developed an innovative procedure in which autogenic nucleus pulposus is reinserted into a degenerated disc, resulting in retardation of degeneration [6]. Okuma advanced the efficacy of this procedure by activating the biochemical properties of reinserted nucleus pulposus cells using a nucleus pulposus/annulus fibrosus coculture method, since cell cultures of nucleus pulpous cells alone expresses poor viability [7]. From these studies, we established the concept of utilizing the contained structure of the disc for cell transplantation in attempt to treat disc degeneration.

To date, in vitro studies of the IVD cells have shown that most IVD cells have the capacity to synthesize proteoglycan epitopes similar to chondrocytes and cellular morphology expressing chondrocyte-like behavior [20–25]. However, notochordal cells, which are only found in the nucleus pulposus of animals or in humans of young age, do not resemble chondrocyte-like behavior, since they are said to be the remnants of the notochord [26]. From these facts, it can be assumed that the majority of disc cells originate from the mesenchyme.

Mesenchymal stem cells (MSCs) are undifferentiated cells found in small numbers in the periosteum or in the bone marrow. They possess the unique ability to differentiate into varieties of cells found in connective tissues of mesenchymal origin [27]. Their ability to differentiate is governed mainly by the environment in which they are placed. Conditions favorable for inducing osteogenesis, chondrogenesis and adipogenesis have been well established [28,29]. Recent studies of MSCs have reported successful applications of MSC transplantation in various fields [30–33]. Several studies have reported repair of articular cartilage defects by transplanting MSCs embedded in collagen gels or gelatin sponge [31–33].

Despite the fact that IVD cells possess chondrocyte-like phenotypes as mentioned previously, the association between disc cells and MSCs has never been addressed in the literature. In this study, we describe the first application of MSCs in IVD experimental model by transplantion of autologous MSCs to the degenerated disc.

Atelocollagen® gel was chosen as a cell carrier because of its immunogenic and safety advantages over other carriers. In Atelocollagen®, the antigenic telopeptide region is removed by pepsin digestion and differential salt precipitation during purification. This immunogenic advantage has enabled Atelocollagen® to be used in various clinical settings. It is most widely used as a wrinkle eraser by injecting Atelocollagen® into the skin. Atelocollagen® is also used as a scaffold for keratinocytes and fibroblasts in artificial skin. Basic science research on culturing chondrocytes in Atelocollagen® gel has shown that cultures using Atelocollagen® gel result in greater matrix synthesis when compared to cells cultured in monolayer [34,35].

2. Materials and methods

2.1. Mesenchymal stem cell isolation and culture

After approval by the Animal Experimentation Committee at the author's institution, 15 Japanese white rabbits weighing an average of 1.3 kg were divided equally into five groups (normal controls; degeneration-induced models; MSC transplantation models evaluated at 2 weeks; 4 weeks; 8 weeks). Under inhalation anesthesia with 2.5% isoflurane (Abbott Laboratories, North Chicago, IL, USA), 5 ml of bone marrow blood was aspirated from the iliac crests of MSC transplantation models into 3000 units of heparin using 18-gauge needles and 10 ml syringes. Collected marrow was added to 0.7 ml Ficoll-Hypaque (Sigma, St Louis, MI, USA) and centrifuged at 150 times gravity for 15 min. The cells obtained, following buffy coat isolation and RBC destruction by RBC lysis buffer (Sigma), were washed three times with phosphate-buffered saline (PBS). Mono-nucleated cells were then cultured with 25 cm^2 culture flask in low-glucose Dulbecco's modified eagles medium (DMEM, Gibco, Green Island, NY, USA) containing 10% fetal bovine serum (Gibco) and antibiotics (penicillin G, 100 U/ml; streptomycin, 0.1 mg/ml; amphotericin B, 0.25 μg/ml) at 37°C, 5% CO_2. Medium was changed every 48 h with removal of non-adherent cells and cultured 12–15 days until reaching subconfluency.

2.2. Labeling of MSCs with recombinant adenovirus vector expressing E. coli lacZ Gene (Ad-lacZ)

To confirm the viability of MSCs after transplantation, the cultured MSCs were infected with Ad-lacZ

expressing *E. coli* lacZ gene under the control of CAG promoter consisting of cytomegalovirus IE enhancer, chicken beta-actin promoter, and rabbit beta-globulin polyadenylation signal (kindly provided by Dr. Izumu Saito, Tokyo University, Tokyo, Japan) [36,37]. The MSCs were infected overnight with 1×10^4 PFU/ml of Ad-lacZ at multiplicity of infection (MOI) 1, 5, 10 and 25. To examine the potential of Ad-lacZ infection, 2×10^4 infected MSCs were sampled and stained with 0.1% 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-gal; Takara, Ohtsu, Shiga, Japan) following PBS wash. Nearly 100% of the cells were stained by infection with Ad-lacZ at MOI $\geqslant 5$.

2.3. Confirmation of multilineage differentiation in the MSCs

Osteogenic, chondrogenic and adipogenic induction were assessed to prove multilineage differentiation in MSCs used for this study. Osteogenic induction was performed in monolayered MSCs grown in medium containing 100 nM dexamethasone (Sigma), 10 mM beta-glycerol phosphate (Sigma), and 50 μM ascorbic acid 2-phosphate (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Alkaline phosphatase activity and calcium deposition shown by cresophthalein complexone (Sigma) staining were evaluated.

In chondrogenic induction, MSCs were pelleted by 1500 times gravity for 5 min centrifugation, and cultured in induction medium containing high-glucose DMEM, 1% ITS+ (Beckton Dickinson, Franklin Lakes, NJ, USA), 100 nM dexamethasone, 50 μM ascorbic acid 2-phosphate, 35 μg/ml proline (Sigma) and 5 ng/ml TGF-beta1 (Sigma). The pellet was cultured for 21 days, then cut into frozen sections, and evaluated by histology for Safranin O and immunohistochemistry for type II collagen and proteoglycan.

Adipogenesis was induced by an induction using a medium containing high-glucose DMEM, 100 nM dexamethasone, 200 μM indomethacin (Wako), 10 μg/ml insulin (Sigma), 500 μM 3-isobutyl-1-metylxanthin (Wako). Induction was confirmed by oil red staining.

2.4. Embedding of MSCs into Atelocollagen® gel

Since type II collagen is the main collagen component of IVD, Atelocollagen® gel solution (KOKENCELLGEN; Koken Co., Ltd., Tokyo, Japan) composed of 0.3% type II collagen (pH 3.0) was used as a carrier. Atelocollagen® solution is in liquid form when cooled to 4°C but gelatinizes as the temperature rises to 37°C. Eight milliliter of cooled Atelocollagen® solution was quickly prepared for cell culture by adding 1 ml of 10 × concentrated low-glucose DMEM, 0.1 ml HEPES (pH 7.4) (Sigma) and 0.1 ml 2.2% $NaHCO_3$ (Sigma). All MSCs were infected with Ad-lacZ and transplanted no later than 21 days after isolation. Gene labeled MSCs were embedded in this solution with a final cell density of 1×10^6 cells/ml. Viability of MSCs checked after embedding showed that more than 95% were viable.

2.5. Disc degeneration induction

Disc degeneration in rabbits was performed under inhalation anesthesia to degeneration-induced and MSC transplantation models by aspirating the nucleus pulposus from L2-L3, L3-L4, L4-L5 IVDs through an anterolateral approach using a 21-gauge needle and a 10 ml syringe, as described by Okuma and Nomura [7,8]. Aspirated disc fragments were carefully examined under dissecting microscopy to confirm that only nucleus pulposus was aspirated. The weight of nucleus pulposus inside the needle ranged from 0.005 to 0.008 g.

2.6. Autologus transplantation of MSCs in Atelocollagen®

Autogenous MSCs embedded in Atelocollagen® were transplanted to degeneration-induced L2-L3, L3-L4, L4-L5 discs in anesthesized MSC transplantation models at 2 weeks after second operation in the following manner. Atelocollagen® gel-medium solution (0.04 ml), in which autogenous MSCs were embedded, was injected through a 27-gauge insulin injector to each discs with careful manipulation.

2.7. Evaluations

2.7.1. Histology

At 2, 4, and 8 weeks after the operation, five rabbits including one degeneration-induced model and one normal control were euthanized by intravenous injection of high-dose pentobarbital sodium (120 mg/kg) (Abbott). The L2-L3, L3-L4, L4-L5 discs were excised immediately in continuity with the cranial and caudal vertebral bodies. Femurs with thigh muscles were also harvested. The vertebral body–disc–vertebral body units were then fixed in 10% formalin neutral buffer solution (Wako), decalcified in Plant-Rychlo solution (Decalcifying Solution A; Wako), dehydrated in a graded series of ethanol (70%, 90%, 99%, Wako), and processed individually with paraffin wax embedding. The paraffin blocks were sectioned longitudinally using a microtome into 4-μm sections. The sections were stained with hematoxylin and eosin, and Safranin O for evaluation. Grading system for disc degeneration by Nishimura and Mochida [6] focusing on the morphological changes in the annular structure was used for evaluation: Grade 0, normal structure; Grade 1, mildly serpentine appearance of the annulus fibrosus with rupture; Grade 2, moderately serpentine appearance of the annulus fibrosus with rupture; Grade 3, severely serpentine appearance of the

annulus fibrosus with mildly reversed contour; Grade 4, severely reversed contour; and Grade 5, indistinct.

2.7.2. Immunohistochemistry

Proteoglycan accumulation changes in the discs were studied to evaluate disc degeneration. The sections were labeled overnight at 4°C with anti-rabbit proteoglycan monoclonal antibody (Chemicon, Temecula, CA, USA), prepared at a dilution of 1:200 in PBS. This antibody recognizes short peptides substituted with keratan sulfate side chains and core proteins of proteoglycan in the articular cartilage of mouse. The samples were washed with PBS three times and reacted with anti-mouse HRP (Dako A/S, Profuktionsvej, Denmark) at a dilution of 1:100 in PBS for 30 min at 4°C. Finally, the sections were counter-stained with hematoxylin for histologic examination. Cartilage of the femoral head served as a positive control and muscle as a negative control.

2.7.3. Confocal laser microscopy

In order to visualize changes in proteoglycan accumulation more clearly, the sections labeled with proteoglycan antibodies were stained with anti-goat Alexa fluor® 488 (Molecular Probes, Eugene, OR, USA) a fluorescent second antibody, and evaluated under confocal laser scanning microscopy. Statistical analyses were performed between the groups by Wilcoxon's paired signed rank test using Stat View™ software. Significance in all cases were set at $p<0.05$.

2.7.4. Viability of transplanted MSCs

The sections were labeled with monoclonal anti beta-galactosidase antibody (GAL-40, Sigma) and stained with Alexa flour® 488 using the same technique. Viability of the transplanted cells in the discs was examined under fluorescent microscopy.

3. Results

3.1. Confirmation of multilineage differentiation in rabbit MSCs

All of the inductions performed on the obtained MSCs were successful, confirming multilineage differentiation of obtained MSCs (Fig. 1). MSCs grown in osteogenic induction medium demonstrated mineralized bone matrix after 10 days. Alkaline phosphatase was positively stained and cresophthalein complexone staining identified calcium depositions. Immunohistochemistry demonstrated positive staining for type I collagen. In chondrogenic induction, MSCs cultured in pellet showed slight growth over a 21-day period. Frozen sections of the pellet demonstrated cartilage-like matrix, which were stained positive for Safranin O, type II collagen and proteoglycan. MSCs grown in adipogenic induction medium started to show fat deposits after 7 days of culture. These deposits were stained positive by oil red staining.

3.2. Histological analysis

In normal control discs, nucleus pulposus and inner annulus with no major structural changes were seen after 2, 4, and 8 weeks; grade 0 (Fig. 2). The extra cellular matrix of nucleus pulposus was rich in gelatinous structure clearly distinguishable from the inner annulus with cells containing cytoplasm with large vacuoles.

In degeneration-induced models, progressive degenerative changes in both nucleus pulposus and inner annulus fibrosus were apparent over time. Two weeks after operation, the number of vacuolated cells in the nucleus pulposus had markedly decreased with mild rupture of the annulus; grade 1 (Fig. 3a). At 4 weeks, the curved structure of inner annulus fibrosus had collapsed; grade 2–3, with appearance of cells with large nucleus around the nucleus-annulus boarder (Fig. 3b). At 8 weeks post-operation, nucleus pulposus cell were rarely observed and lamellae collapse of the inner annulus had accelerated with invasion of proliferative connective tissue; grade 4–5 (Fig. 3c). Disc height flattening began to appear at this point and Safranin O staining became less intense.

In models receiving MSC transplantation, degenerative changes such as collapse of annular structure and invasion of proliferative connective tissue was normal to minimal through out all time periods; grade 0–1 (Fig. 4a–c). Instead, newly formed tissue had replaced nucleus pulposus cells in areas where it had disappeared (Fig. 4d). These tissues were formed mostly by spindle like cells with fairly loose extra cellular matrix in longitudinal layers with high intensity staining of Safranin O (Fig. 4e). These discs did not show apparent flattening. There was no obvious difference among disc levels in overall grading results in all groups. No apparent changes were noted in the adjacent non-operated sites in all groups.

3.3. Immunohistochemical analysis

Positive staining for proteoglycan was strongly identified in the extra cellular matrix of nucleus pulposus and inner annulus fibrosus in normal controls (Fig. 5a). In the degeneration-induced models, the staining intensity decreased or did not show significant intensity in areas where nucleus pulposus cells were depleted, or in areas where proliferative connective tissues had invaded according to the time course (Fig. 5b). Extra cellular matrix of tissue composed of transplanted cells showed

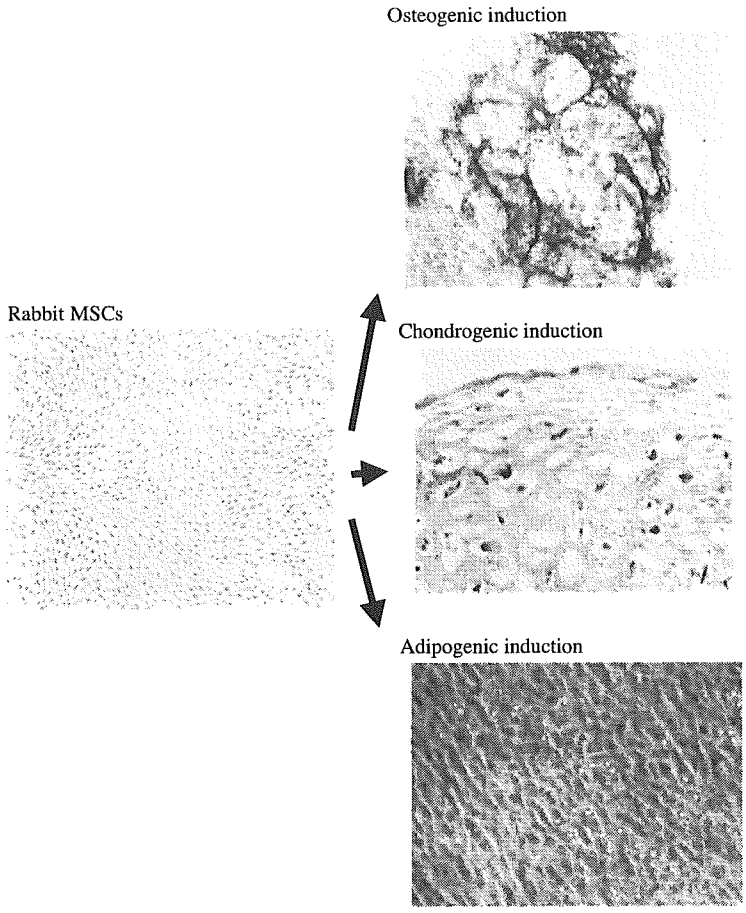

Fig. 1. Confirmation of multilineage differentiation of rabbit MSCs used in the study. (a) Rabbit MSCs cultured for 15 days after isolation. Magnification 4×. (b) MSCs cultured for 14 days in osteogenic induction medium. Mineralized bone matrix formation is observed with positive histological staining for alkaline phosphatase and calcium deposition shown by cresophthalein complexone staining. Magnification 4×. (c) Immunohistochemistry of type II collagen showing positive staining in frozen section of MSCs after 21 days of pellet culture by chondrogenic induction. Magnification 10×. (d) MSCs cultured for 10 days in adipogenic induction medium showing oil red positive staining. Magnification 10×.

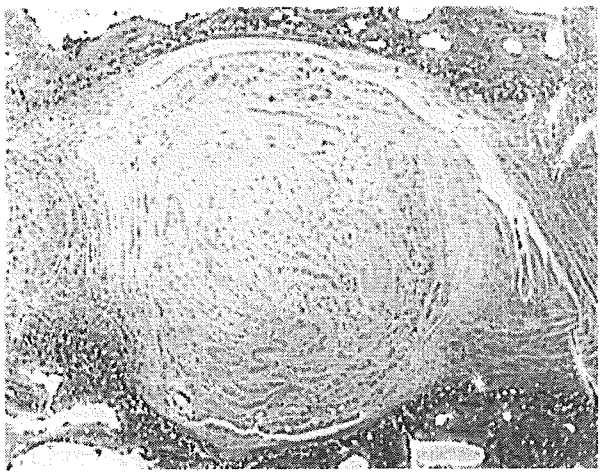

Fig. 2. Disc of a normal control group in week 8 showing no apparent abnormality; annulus degeneration grade 0. Magnification 4×.

a high staining intensity in the extra cellular matrix of the spindle like cells (Fig. 5c).

3.4. Proteoglycan accumulations evaluated by confocal laser scanning microscopy

In evaluation of sections using confocal laser scanning microscopy, results were compatible with that of the non-fluorescent staining but clearer (Fig. 5). Results were significantly different ($p<0.005$) in average peak intensity levels of intensity measured in the center of the disc at 8 weeks post-operation were 3.3 ± 0.5 in normal controls, 2.8 ± 0.4 in MSC-transplanted models and 1.1 ± 0.3 in degeneration-induced models.

3.5. Viability of the transplanted cells

X-gal staining of the MSCs done prior to embedding into Atelocollagen® gel showed good positive staining

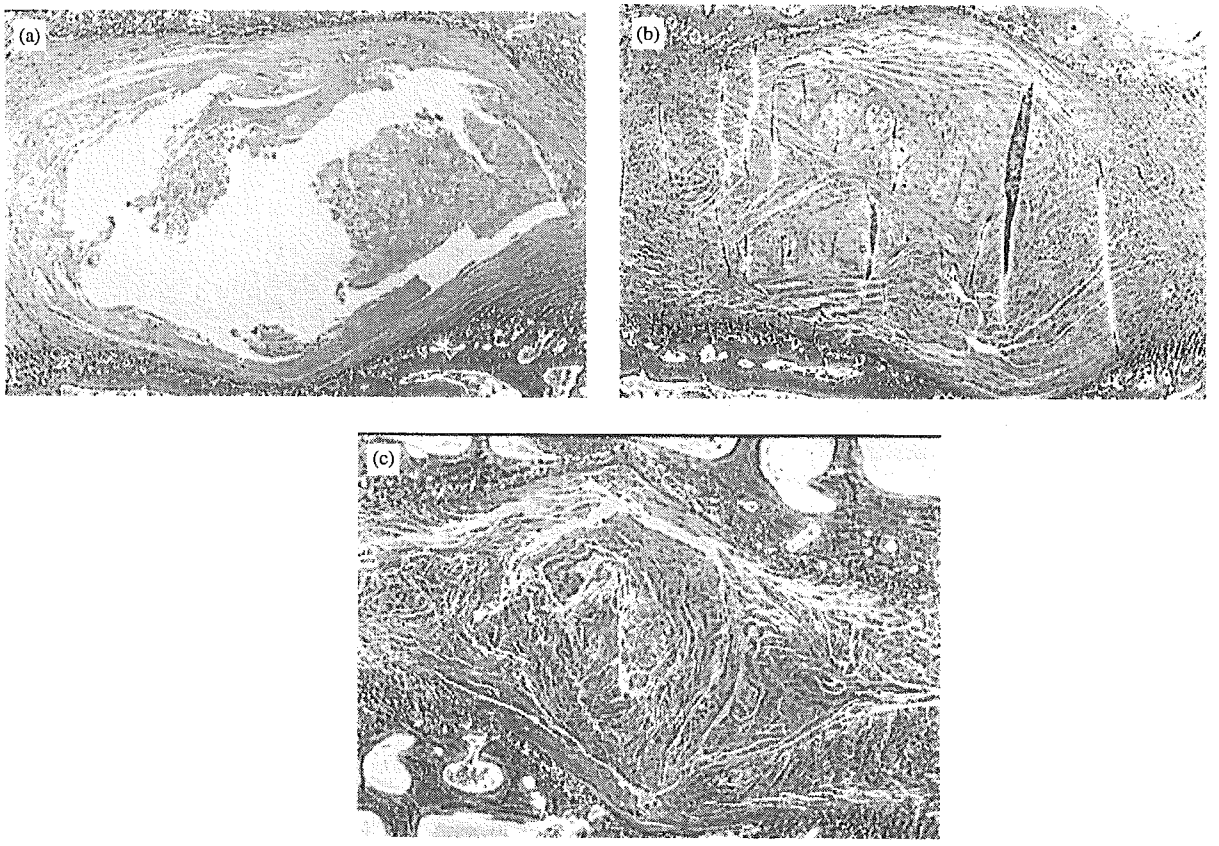

Fig. 3. (a) Week 2 degeneration-induced model. Annular structure is mildly affected although majority of the nucleus pulposus is depleted; annulus degeneration grade 1. Magnification 4×. (b) Week 4 degeneration-induced model. Annular structure is severely serpentine with few nucleus pulposus left; grade 3. Magnification 4×. (c) Week 8 degeneration-induced model. Most of the annular structure is collapsed with severe invasion of proliferative connective tissue; grade 4. Magnification 4×.

in nearly all of the cells infected at each MOI. Fluorescent immunohistochemical staining of the sections harvested from MSC-transplanted models demonstrated positive staining of beta-galactosidase in sections at 2 and 4 weeks after operation (Fig. 6). However, the number of positive cells had decreased over time.

4. Discussion

In this study, partial aspiration of nucleus pulposus tissue was used in order to induce degeneration. Disc annular lesion models and partial nucleus pulposus aspiration models are an established method for creating disc degeneration experimentally [7–9,17,19]. It is obvious from the results of histology and immunohistochemistry in the degeneration-induced models of our experiment that degeneration was successfully induced. This evidence confirms the fact that preserving viability of the nucleus pulposus and annular structure is the primary factor in decelerating disc degeneration.

The approach to delivering cells into the degenerated discs of rabbits was established by Okuma et al. [7]. In that study, decelerating disc degeneration was successful by reinserting nucleus pulposus cells that were activated by coculture with annulus fibrosus cells. Activated nucleus pulposus was shown to have proliferated inside the disc and possibly helped to maintain proteoglycan synthesis and morphological structure. However, in Okuma's experimental model, reinserted cells were suspended in medium, in spite the fact that nucleus pulposus cells retain their character, shared prominent matrix production when cultured in a three-dimensional environment. In addition, no appropriate markers were used to check the viability of the cells after reinsertion.

Chondrocytes when cultured in monolayer are known to express a fibroblastic differentiated morphology and lose their ability to synthesize matrix predominantly of type I collagen distribution [35,38]. Human IVD cells are known to possess chondrocyte phenotypes [20–25]. Like chondrocytes, they also express fibroblastic morphology in monolayer but regain their features when cultured in a three-dimensional environment [40–43]. In

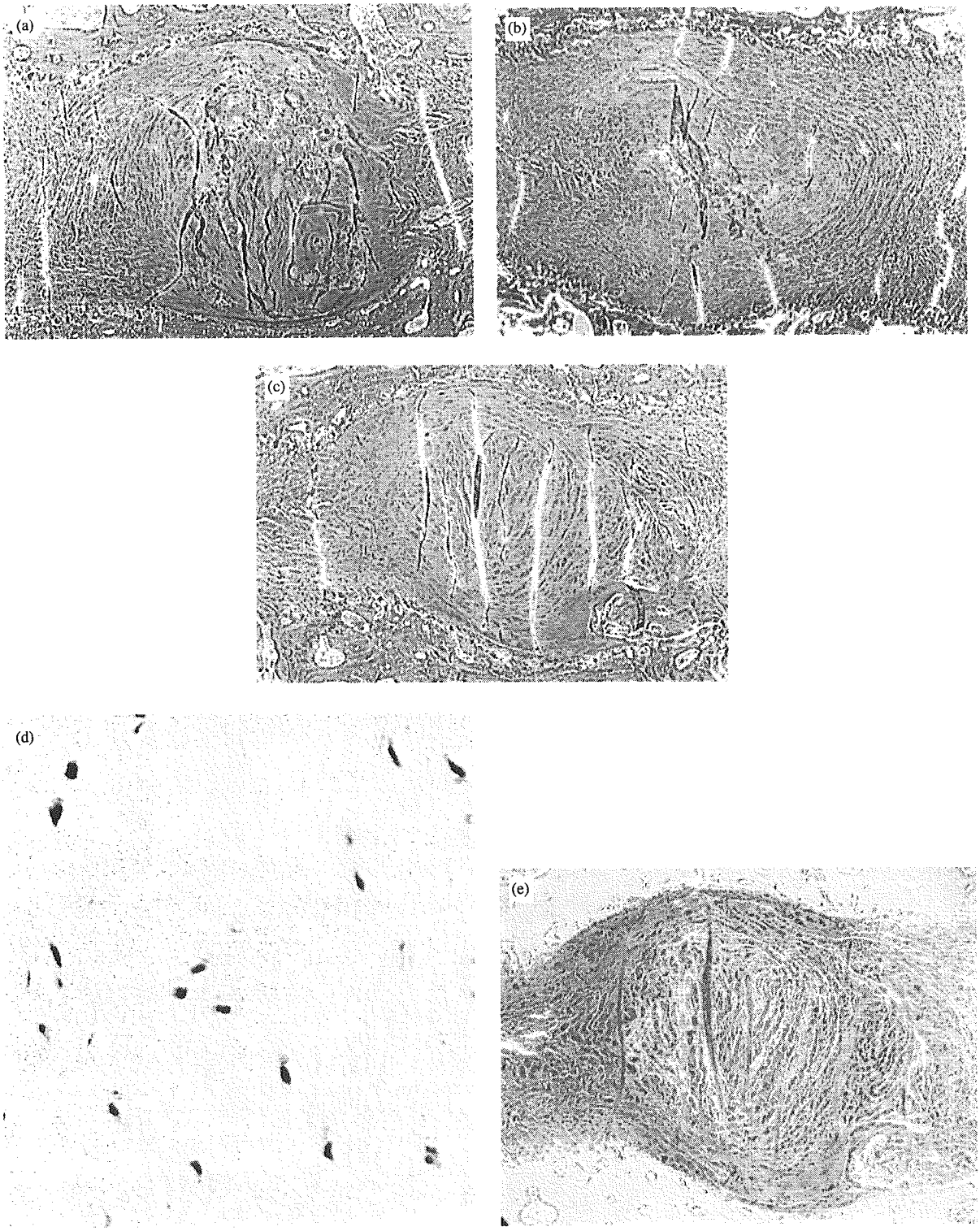

Fig. 4. Week 2 (a), 4 (b) and 8 (c) MSC transplantation models. Transplanted cells are fulfilling the disc space. Magnification 4 ×. The rupture of the annular structure is mild; grade 0–1. (d) Newly formed tissue in the disc 8 weeks after MSC transplantation with rich extra cellular matrix. Magnification 100 ×. (e) Safranin O staining of the tissue. Magnification 4 ×.

studies of MSC transplantation in cartilage defects, Atelocollagen® gels and gelatin sponges have been used as a successful carrier for cell delivery [39]. Based upon these elements, Type II collagen-based Atelocollagen® gel was used as a cell carrier, making it close to disc component. The type II Atelocollagen solution used in our study was prepared from bovine cartilage, which was decomposed by pepsin digestion with incubation and isolated by differential salt precipitation into acidic solution. This solution is in liquid form when cooled to temperatures near 4°C but gelatinizes when heated to about 37°C. In order to use in cell cultures, Atelocollagen® solution was neutralized by adding HEPES buffer (pH 7.4) and $NaHCO_3$.

The results of cell delivery in our transplant models shared successful construction of tissues without any defects. Proliferation of cells after transplantation was found to be successful with minimal disorientation of annular structure. Using beta -glycosidase staining, it was confirmed that the transplanted MSCs were viable, and it can be assumed that cells in the tissue of transplanted models had differentiated from these MSCs. It can be speculated that expression of beta -glycosidase lasted only 4 weeks at the most, because delivery of genes by recombinant adenovirus vectors are not considered permanent. However, use of Ad-lacZ for marking was considered appropriate in this study due to success in confirming viability and ease of handling.

Histologically, MSC-transplanted models had preserved disc structure with minimal degeneration in all time periods compared to degeneration-induced models. We found that primary morphological features of disc

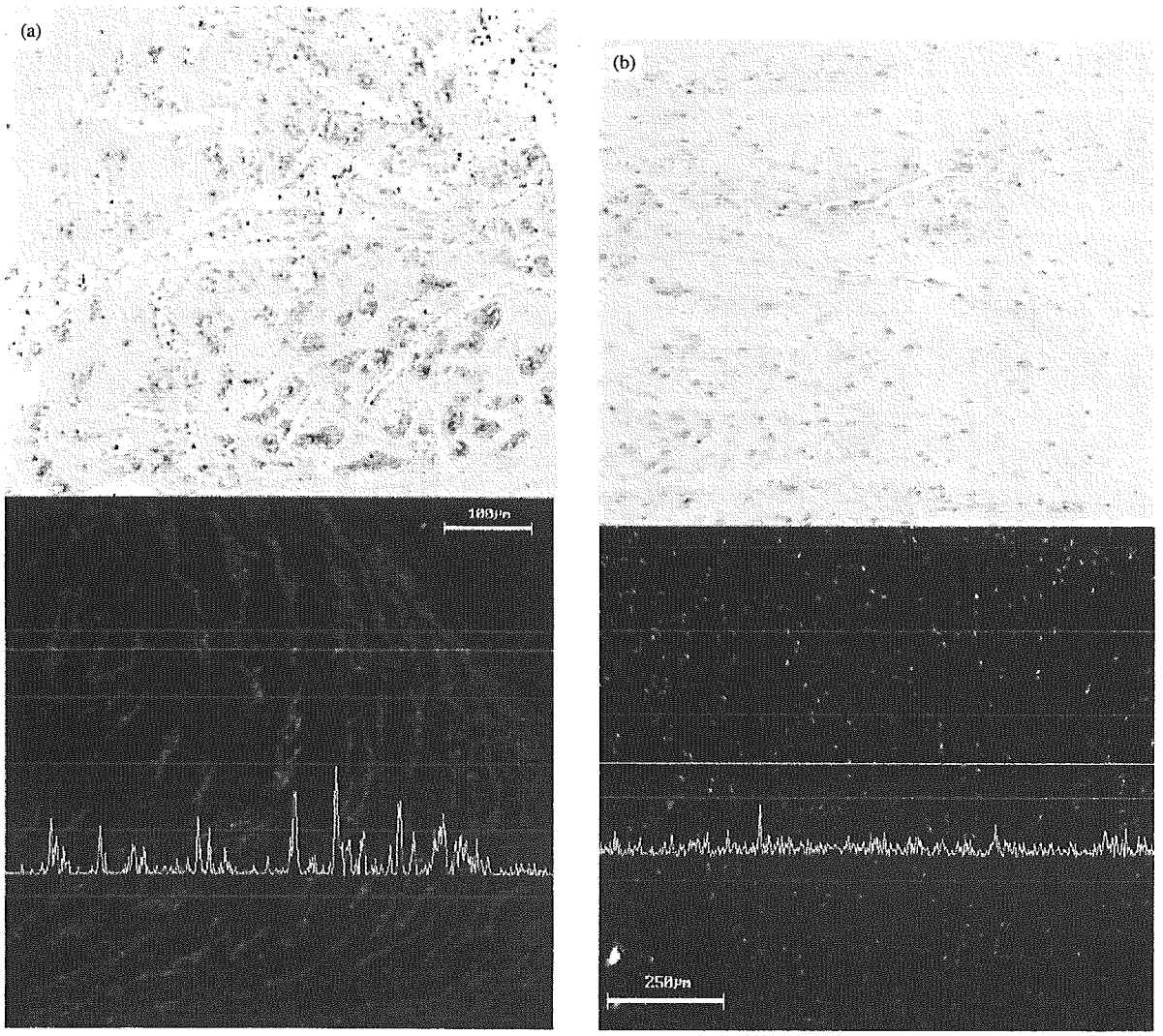

Fig. 5. (a) Proteoglycan staining of a normal disc tissue. Magnification 20 ×. Staining intensity by confocal laser scanning microscopy is shown below. (b) Week 8 degeneration-induced model. Staining intensity of proteoglycan is low. Magnification 20 ×. (c) Week 8 MSC transplantation model shows high intensity peak levels similar to normal control group. Magnification 20 ×.

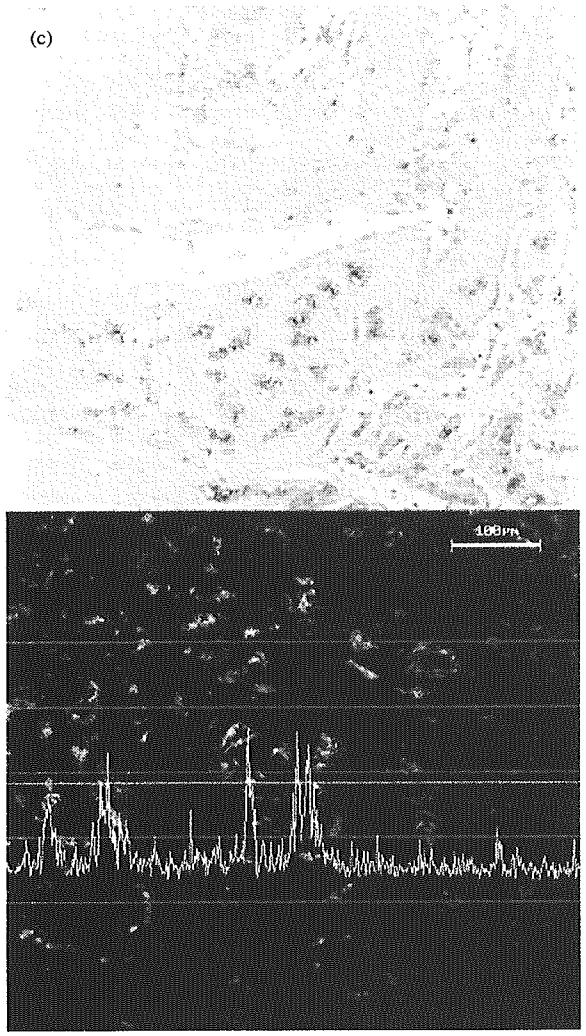

Fig. 5 (*continued*).

degeneration, cell depletion in the nucleus pulposus and disorientation of oval annular structure were prevented by injection of Atelocollagen® gel embedded with MSCs. This unique growth-enhancing process served to maintain cell volume with high viability and matrix gel formation. Surprisingly, transplanted MSCs survived in the environment of the disc, differentiating into spindle-shaped cells arranged in longitudinal layers, which resembled original disc cells. Extra cellular matrix, which was suspected to originate from the MSCs, was observed in interlineating layers. Safranin O stained with markedly high intensity in this matrix and immunohistochemical staining for proteoglycan also showed high intensity, suggesting the potential for rich matrix production, including proteoglycan that these differentiated cells possessed. Proteoglycan staining intensity levels with confocal laser scanning microscopy demonstrated that the intensity patterns of the MSC-

Fig. 6. Cells in disc tissue of a week 4 MSC-transplanted model shows positive staining for beta-galactosidase, which confirms the survival of MSCs at harvest. Magnification 63 ×.

transplanted models were significantly higher than the degeneration-induced models ($p<0.005$) and similar to controls at all time points. This proves that deceleration of degeneration and maintaining proteoglycan preservation was possible.

We conclude that in attempt to decelerate IVD degeneration, MSC transplantation was effective in preserving annular structure by the use of MSC/Atelocollagen® conjugates. These amalgamates served to fill depleted nucleus pulposus, and preventing proteoglycan decrease by increasing production from the differentiated cells of transplanted MSCs. Results from our study provide initial evidence for the potential of MSCs to differentiate into IVD cells, which provides new information in MSC research. However, further long-term studies with detailed analysis of the newly formed tissue by transplanted MSCs will be needed in the future.

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Low-intensity pulsed ultrasound stimulates cell proliferation and proteoglycan production in rabbit intervertebral disc cells cultured in alginate

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Abstract

Intervertebral disc degeneration, one of the major causes of low-back pain, is known to result from alteration in biosynthesis of proteoglycan in the disc. Therefore, upregulating the synthesis of proteoglycan in intervertebral disc cells may be one approach in treating disc degeneration. Based on the finding that low-intensity pulsed ultrasound stimulates proteoglycan synthesis in rat chondrocytes, we investigated whether low-intensity pulsed ultrasound stimulates biological properties of rabbit intervertebral disc cells in vitro. Nucleus pulposus cells and annulus fibrosus cells isolated from rabbits were cultured in alginate beads. Cells were stimulated for 20 min each day for 5–12 days, starting on the third day after seeding. An ultrasound signal consisting of a 200 μs burst sine wave of 0.5 MHz repeating at 1 kHz, with an intensity of 0, 7.5, 15, 30, 60, 120 mW/cm^2 spatial and temporal average, was applied. DNA and proteoglycan synthesis were evaluated by measuring [^{3}H]-thymidine and [^{35}S]-sulfate incorporation. DNA and proteoglycan content in beads were measured by Hoechst 33258 dye method and dimethylmethylene blue assay. Results demonstrated positive effects on DNA synthesis and content, following low-intensity pulsed ultrasound stimulation with intensities of 7.5 and 15 mW/cm^2. Furthermore, ultrasound stimulation significantly upregulated [^{35}S]-sulfate incorporation and proteoglycan content compared to the control group, following 5 days of stimulation in both nucleus pulposus and annulus fibrosus cells. These findings suggest the possible application of low-intensity pulsed ultrasound in biological repair of intervertebral disc degeneration.

Keywords: Intervertebral disc; Disc degeneration; Ultrasound; Low-intensity pulsed ultrasound (LIPUS); Nucleus pulposus; Annulus fibrosus

1. Introduction

Low-back pain is caused by various factors such as fasciitis, instability of the vertebral joints, cauda equina compression, or organic disease in the abdomen/pelvis [1]. Among these factors, attention has been focused on intervertebral disc (IVD) degeneration in recent years, and many studies have been performed [2–4].

Although the etiology of IVD degeneration is still unclear, it is well known that a decrease in the water content of the nucleus pulposus (NP) is accompanied by loss of its shock absorber function, and this causes non-uniform distribution of pressure on the vertebrae in response to external forces in the early stage of disc degeneration [2]. Proteoglycans (PG) are important for maintenance of the water content of the NP in the IVD,

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and have been shown histologically to become concentrated at the center of the nucleus [2]. In addition, impairment of nutrition due to damage to the cartilage end-plate, and loss of cells due to apoptosis have been reported as factors related to IVD degeneration [2,3]. There are many reports claiming that a decrease in the viability of NP cells and annulus fibrosus (AF) cells, decreased cell proliferation, and a decrease of matrix synthesis are the initial triggers. Thus, the factors underlying IVD degeneration are gradually becoming clear.

Based on such studies, injection of growth factors into IVD [5–7], introduction of genes [6], and transfer of activated NP cells or stem cells [8,9] are being investigated as new treatments for IVD degeneration.

Low-intensity pulsed ultrasound (LIPUS) is effective for promoting bone union [10], and is being used clinically to treat fractures with non-union, making it a popular therapy in the orthopedic field. Ultrasound (US) also stimulates aggrecan synthesis by chondrocytes and has been reported to influence their differentiation in vitro [11,12].

However, there has been no documentation of the effect of LIPUS on NP cells and AF cells, which are similar (but not the same) as chondrocytes and which form the IVD. Accordingly, we performed three-dimensional culture of rabbit NP and AF cells stimulated with LIPUS, and then examined cell proliferation and PG synthesis in order to investigate whether LIPUS has the potential to become a new treatment for IVD degeneration.

2. Materials and methods

The current study was conducted under the protocol approved by the animal experimentation committee of Tokai University School of Medicine.

2.1. Cell isolation and culture

Twenty New Zealand white rabbits (average weight; 1.5 kg, female) were purchased for this study. Next, they were euthanized by intravenous injection of high-dose pentobarbital sodium (120 mg/kg, Abbott Laboratories). Thoracolumbar spines including T5 to L7 levels were removed under aseptic condition. Each disc was cut transversely, and NP and AF were separated from the disc using a spatula and a scalpel. The obtained NP tissue was digested in a mixture of 0.4% pronase (Kakenkagaku, Tokyo, Japan) and 0.0125% collagenase P (Boehringer Mannheim, Mannheim, Germany) for thirty minutes. Also AF tissue was first digested with 0.4% pronase for 1 h and then with 0.025% collagenase P for 3 h. The digested tissue was passed through a cell strainer (BD falcon, USA) with a pore size of 100 μm and washed two times with phosphate-buffered saline (PBS, Gibco). The isolated cells were seeded in six-well culture plates (Primaria, BD) at cell densities of 3.2×10^4cell/cm^2 in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Green Island, NY) with 10% fetal bovine serum (FBS, Gibco), penicillin (100 μg/ml), and streptomycin (250 ng/ml) at 37°C, 5% CO_2 atmosphere.

2.2. Culture of IVD cells in alginate

After three passages, the cultured cells were detached with trypsin–EDTA solution (0.05% trypsin, Gibco BRL, Grand Island, NY) and counted using a hemocytometer. The cells were collected by centrifugation and were resuspended in 1.2% low-viscosity alginate (Clonetics, USA) in 0.15 M sodium chloride at a concentration of 1 million cell/ml. The cell suspension was gently expressed through a 18-gauge needle attached to a 1 ml syringe into a 102 mM calcium chloride solution (Clonetics, USA) to form drops of semisolid beads. After 10 min of polymerization, beads were washed three times with normal saline, and then three more times with DMEM. Ten beads were placed in each well of a six-well plate (non-treated, IWAKI, Japan) and were incubated in DMEM (4.5 ml/well) supplemented with 10% FBS and penicillin (100 μg/ml) and streptomycin (250ng/ml) at 37°C, 5% CO_2 atmosphere.

2.3. Stimulation of ultrasound

A US apparatus, SAFHS (Sonic Accelerated Fracture Healing System; TEJIN Pharma, Tokyo, Japan) (Fig. 1), was used to deliver an US signal with spatial and temporal average intensity of 7.5, 15, 30, 60, 120 mW/cm^2. The frequency was 1.5 MHz with a 200 μs tone burst repeated at 1.0 kHz. The plate cover was removed and the absorption chamber was placed above this, with care taken to exclude air bubbles or air layer between the culture medium and the bottom of absorption chamber (Fig. 2). Coupling gel (Sono Jelly, TOSHIBA, Tokyo, Japan) was dripped on all transducers and the output was confirmed by using an output checker. Then the culture plate and chamber unit were set on the transducer, and the stimulation of LIPUS started. The cells in US group were stimulated for 20 min each day for 5–12 days, starting on the third day after seeding in alginate.

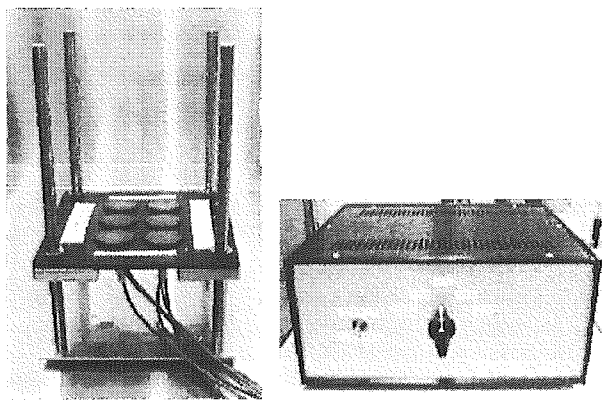

Fig. 1. This unit consists of sonic accelerated fracture healing system (SAFHS) device and transducers to which a six-well culture plate can be connected.