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Original article

Autologous living chondrocytes contained in the meniscal matrix play an important role in in vivo meniscus regeneration induced by in situ meniscus fragment implantation

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ABSTRACT

Introduction: Implantation of autogenous meniscal fragments wrapped with a fascia sheath significantly enhances fibrocartilage regeneration in vivo in defect cases at 12 weeks after implantation. The specific effects of the implanted autologous living chondrocytes and meniscal matrix have not been elucidated, however. The aim of this study was to clarify the role of autologous living chondrocytes contained in the meniscal matrix in in vivo meniscus regeneration induced by in situ meniscus fragment implantation.
Hypothesis: Implantation of meniscus fragments containing autologous living chondrocytes may result in significant in vivo meniscus regeneration.
Materials and methods: Seventy-five rabbits were used in this study. A partial meniscectomy of the anterior one-third of the medial meniscus including the part of the anterior horn was performed. The rabbits were divided into 3 groups. In Group I, no treatment was applied to the defect. In Group II, the autogenous meniscal fragments devitalized by freeze-thaw treatment were reimplanted into the defect. In Group III, the autogenous meniscal fragments were reimplanted. In each group, the defect was covered with a fascia. Five rabbits from each group were subjected to morphologic and histologic evaluations at 3, 6, and 12 weeks, and 5 rabbits from each group were subjected to biomechanical evaluations at 6 and 12 weeks.
Results: Histologically, no cells were seen in the grafted meniscal fragments at 3 weeks in Group II, whereas chondrocytes in the grafted meniscal fragments were alive at 3 weeks in Group III. Histologic and biomechanical data for Group II were slightly but significantly better than those of Group I at 12 weeks after implantation ($p = 0.007$ and $p = 0.002$, respectively), whereas the data for Group III were significantly superior to those of Groups I and II at 12 weeks ($p < 0.0014$ and $p < 0.0029$, respectively).
Discussions: Grafted autologous living chondrocytes contained in the meniscal matrix play an important role in in vivo meniscus regeneration induced by in situ meniscus fragment implantation.
Study design: II, Controlled laboratory study.

1. Introduction

Recently, a variety of strategies have been investigated for regenerating meniscus tissue. These strategies include the use of allografts, biologic scaffolds, and cultured tissues [1–5]. However, the usefulness of these strategies has not been fully established. Recently, a in vivo study was conducted using rabbits that was based on a meniscus regeneration strategy [6]. Small pieces of

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meniscal fragments created from the resected meniscus were implanted into the meniscus defect and then covered with a fascia sheath. Fibrocartilage regeneration occurred in vivo in the defect by 12 weeks after implantation, although this experience was conducted with meniscus autografts which does not correspond to any clinical situation. However, the mechanism underlying this phenomenon remains to be elucidated. In meniscus tissue, a chondrocyte and its surrounding extracellular matrix compose the chondron [7].

Recent in vitro studies reported that chondrocytes in the meniscus can be used as a cell source for meniscus regeneration [8–10]. By contrast, other recent studies reported that scaffold materials play an important role in meniscus regeneration [4,11–21]. The natural extracellular matrix of the meniscus contains a variety of proteoglycans and collagens, which strongly suggests that implanted meniscal fragments could function as natural extracellular matrix and induce in situ meniscus regeneration. It is thus important to answer the above-mentioned question to elucidate the mechanism underlying in situ autogenous meniscal fragment implantation. However, this question cannot be answered by studies involving implantation of cultured chondrocytes separated from the meniscus, because the strategy differs from that of meniscus implantation [19].

The following 3 major hypotheses were examined in the present study:

- does implantation of devitalized extracellular matrix have a slight but significant effect on in vivo meniscus regeneration after implantation?
- Does implantation of meniscus fragments containing autologous living chondrocytes resulted in significant in vivo meniscus regeneration after implantation? and;
- is the degree of any observed effect significantly greater in implantation using autologous meniscal matrix fragments containing living chondrocytes than implantation using devitalized meniscal matrix?

2. Methods

2.1. Study design

The study used a total of 75 mature female Japanese White rabbits, each weighing 3.8 ± 0.3 kg. Rabbits are suitable for preliminary studies because they are cost-effective and easy to control. In addition, rabbits are frequently used as model for meniscal defect and treatment [6,22–24]. Animal experiments were carried out at the Institute of Animal Experimentation, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, under the rules and regulations of the Animal Care and Use Committee (08-0068).

Surgery was carried out under intravenous anesthesia (pentobarbital, 25 mg/kg). A medial arthrotomy was performed on the right knee. A partial meniscectomy was performed on the anterior one-third portion of the medial meniscus including the part of the anterior horn according to our previous study [6] (Fig. 1A). Following surgery, the Japanese White rabbits were divided into 3 groups of 25 animals each. In Group I (No treatment group), nothing was implanted into the meniscal defect, but the defect was covered with a rectangular fascia membrane (Fig. 1B) harvested from the left thigh and trimmed to 12×15 mm (Fig. 1C). In Group II, devitalized meniscus fragments were reimplanted into the defect and then covered with a rectangular fascia membrane (Fig. 1F), as performed in Group I. Chondrocytes buried in the meniscal matrix were devitalized using freeze-thaw treatment, which killed the chondrocytes but retained the biological properties of the meniscal matrix [25,26]. Namely, the resected meniscus was fragmented into small pieces (approximately $0.5 \times 0.5 \times 0.5$ mm each) using a sharp blade (Fig. 1D). The fragments were immersed in liquid nitrogen for 1 min (Fig. 1E) and then thawed by placing them in saline solution (37 °C) for 1 min. This procedure was repeated three times. In Group III, same size of the above-described meniscus fragments containing living chondrocytes were reimplanted into the defect and covered with a rectangular fascia membrane in the same manner as used in Group II. The animals were not immobilized after surgery. In each group, 15 of the 25 rabbits were randomly selected for histologic examinations, and 5 rabbits were sacrificed at 3, 6, and 12 weeks after surgery. The remaining 10 rabbits were used for biomechanical evaluations, with 5 rabbits sacrificed at 6 and 12 weeks after surgery. The opposite knee was used to obtain normal meniscus data.

2.2. Evaluation methods

2.2.1. Gross and histologic observations

The volume and quality of tissues regenerated at the meniscal defect were then scored according to the semi-quantitative criteria [6]. The total score was defined as the gross observation score of the regenerated tissue. Harvested specimens were fixed in 10% neutral-buffered formalin solution for 3 days and then cast in paraffin blocks. The specimens were sectioned in the transverse plane of the meniscus, which passed through the center of the meniscal defect. Sections (5 μm thick) were then stained with hematoxylin and eosin, safranin O, and toluisin blue. The cross-sectional area of the meniscus was calculated using the following method [27]. Namely, the height and width of the meniscus on each triangular cross-section was measured; the cross-sectional area of the meniscus was then calculated using the formula for an isosceles triangle: (height × width)/2. Histologic findings of light microscopy analyses were quantified using the scoring criteria [6]. The cross-section of the regenerated tissue was divided into 3 zones: outer-rim zone, middle zone, and inner-rim zone. The scores from the 3 zones were then summed, and the total score for each animal was defined as the histologic score.

2.3. Biomechanical evaluation

Each prepared tibia-medial meniscus-tibia complex specimen was mounted onto a tensile tester using a set of specially designed grips [6], so that the tensile force was applied longitudinally to the tissue regenerated in the meniscal defect. Two parallel lines were drawn axially on the meniscus surface using nigrosine stain, just posterior and anterior to the previously created defect to serve as gauge-length markers for elongation measurements. Before the tensile test, each specimen was preconditioned with a static preload of 0.5 N for 5 min, followed by 10 cycles of loading and unloading (3% strain) at a cross-head speed of 5 mm/min. Each specimen was then stretched to failure at a cross-head speed of 20 mm/min. Thus, Quasi-hoop stress was subsequently applied to the previously created defect. Elongation of the regenerated tissue was determined by measuring the distance between the 2 gauge-length markers using a video dimension analyzer.

2.4. Statistical analysis

Statistical analyses were conducted using one-way analysis of variance (Anova) with Fisher's protected least significant difference test for multiple comparisons. The significance level was set at $p = 0.05$.

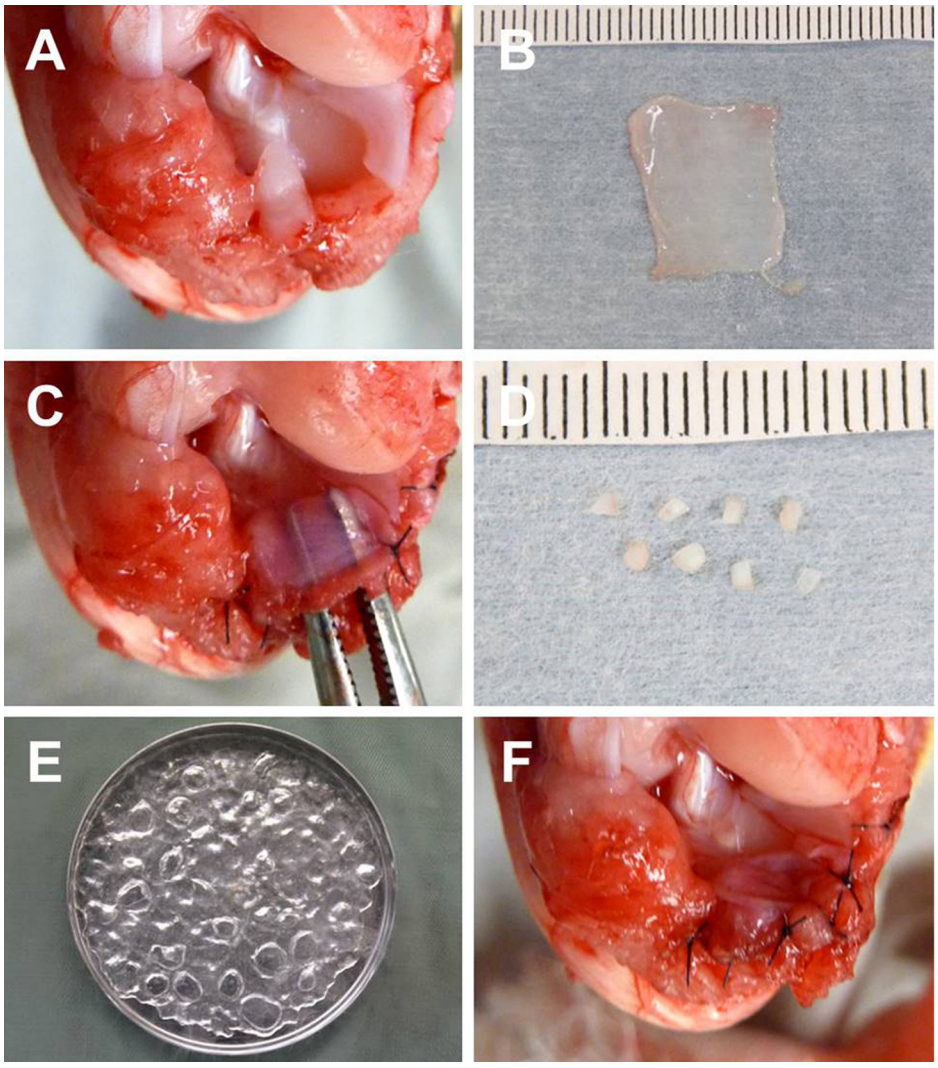

Fig. 1. A. Partial meniscectomy of the anterior one-third portion of the medial meniscus. B. The rectangular fascia membrane (12 × 15 mm). C. The defect is covered with the fascia membrane. D. Fragmentation of the resected meniscus. E. Immersion of the meniscus fragments in nitrogen liquid. F. Implantation into the defect and coverage with the fascia membrane.

3. Results

3.1. Gross observations

In Group I, the meniscal defect was filled with soft fibrous tissue at 3 weeks (Fig. 2A), whereas the width of the fibrous tissue gradually decreased by 6 and 12 weeks (Fig. 2B and C). In Groups II and III (Fig. 2D–I), the defect was filled with fibrous tissue at 3 and 6 weeks and with meniscus-like elastic tissue at 12 weeks. These tissues in Group II appeared to be thinner than those in Group III (Fig. 2F and I). The meniscus-like tissue appeared firmly attached to the remaining meniscus. The surface and radial width of the meniscus-like tissue were rougher and narrower, respectively, than normal tissues.

Concerning the gross observation score at 12 weeks (Table 1), one-way Anova showed a significant difference between the groups ($p = 0.0055$). The post hoc test indicated that scores for Groups II and III were significantly greater than the score for Group I ($p = 0.0205$ and $p = 0.0018$, respectively), whereas there was no significant difference between Groups II and III.

3.2. Cross-sectional area of the regenerated tissue

The percentage of the cross-sectional area (CSA) of the regenerated tissue was calculated relative to that of the normal meniscus harvested from the contralateral knee (Table 1). At 6 weeks, Anova demonstrated no significant difference between groups, whereas at 12 weeks, there was a significant difference between groups ($p = 0.0348$). The post hoc test showed that the percentage for Group III was significantly greater than that for Group I ($p = 0.0186$), whereas there was no difference between Groups I and II.

3.3. Histologic observations

At 3 weeks (Fig. 3A–C), the grafted fascia was necrotized and appeared swollen in Group I, whereas the proximal surface of the grafted fascia was enveloped with a relatively thick synovium-like tissue. Also at 3 weeks, the grafted fascia was necrotized in Groups II and III. The fascia sheath was filled with the grafted meniscal pieces and loose connective tissue. However, there were obvious

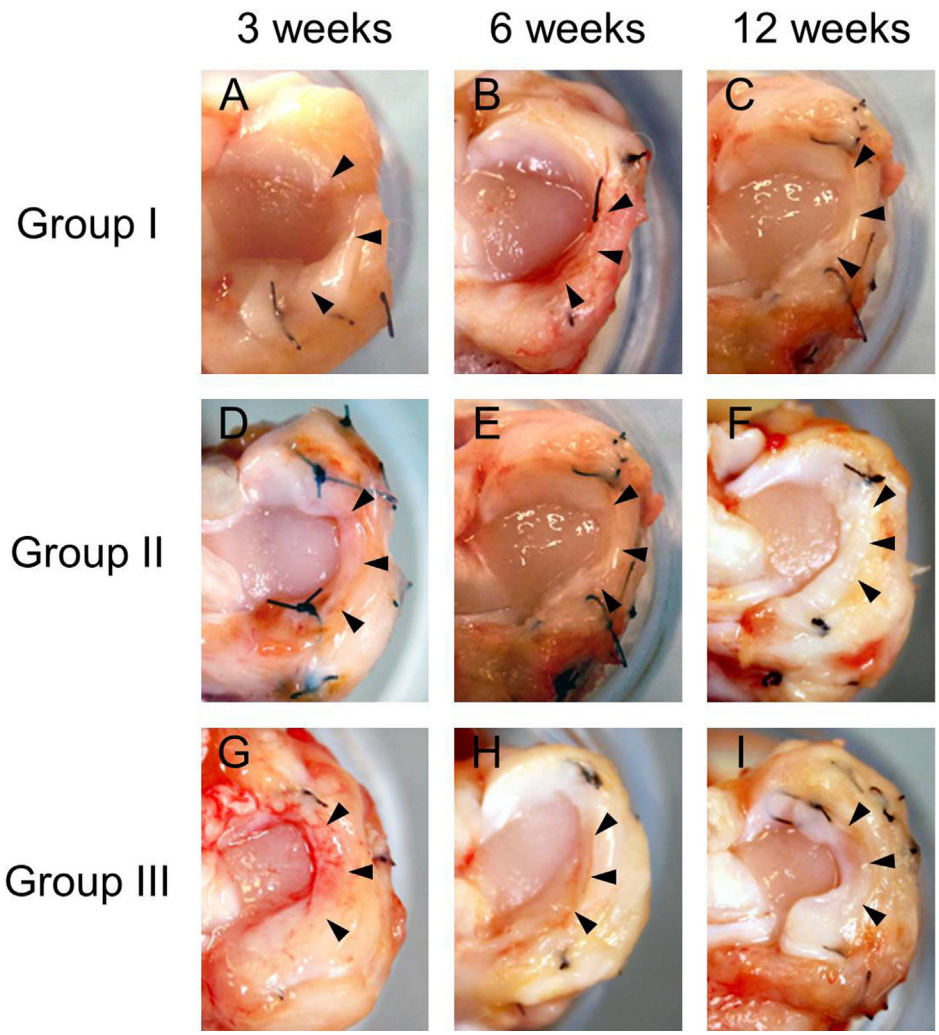

Fig. 2. Gross observations of the regenerated tissues. In Group I, the meniscal defect is filled with a small amount of soft tissue (A, B, and C). In Groups II (D, E, and F) and III (G, H, and I), the defect is filled with fibrous tissue at 3 and 6 weeks and with meniscus-like elastic tissue at 12 weeks.

Table 1
Comparisons by group of gross observation, cross-sectional area, and histologic score at 6 and 12 weeks for the regenerated tissues.

	Group I	Group II	Group III	Comparisons[a]
Gross observation score, (points)				
6 weeks	6.8 ± 1.1	8.0 ± 0.7	8.6 ± 0.5	I vs. II: $p = 0.0385$ I vs. III: $p = 0.0045$
12 weeks	6.8 ± 1.3	8.4 ± 0.5	9.2 ± 0.8	I vs. II: $p = 0.0205$ I vs. III: $p = 0.0018$
Cross-sectional area, (%)[b]				
6 weeks	81.1 ± 26.2	121.5 ± 10.8	137.1 ± 11.6	I vs. II: $p = 0.0035$ I vs. III: $p = 0.0003$
12 weeks	90.1 ± 14.8	100.7 ± 8.0	108.3 ± 7.3	I vs. III: $p = 0.0186$
Histologic score, (points)				
6 weeks	0.4 ± 0.5	2.6 ± 0.5	4.0 ± 1.6	I vs. II: $p = 0.0051$ I vs. III: $p = 0.0001$
12 weeks	0.8 ± 0.8	3.2 ± 0.8	5.8 ± 1.6	I vs. II: $p = 0.0070$ I vs. III: $p < 0.0001$ II vs. III: $p = 0.0043$

[a] Indicates only between-group comparisons that were significantly different. Comparisons with non-significant differences are not listed.
[b] Percentage of the cross-sectional area of the regenerated tissue relative to that of the normal meniscus harvested from the contralateral knee.

differences in the meniscal pieces between the 2 groups. In Group II, no cells were seen in the grafted meniscal pieces (Fig. 3D–F). By contrast, in Group III, fibrochondrocytes in the grafted meniscal pieces were alive (Fig. 3G–I).

At 6 weeks (Fig. 4A–C), the grafted fascia tissue in Group I had slightly shrunk. A number of small fibrocyte-like cells with a spindle-shaped nucleus were scattered throughout the tissue. In Group II (Fig. 4D–F), a meniscus-shaped homogeneous fibrous tissue had formed. This fibrous tissue was enveloped by relatively thick synovial tissue, and cells with an ovoid or rod-like nucleus were scattered sparsely throughout this tissue. In Group III (Fig. 4G–I), the outline of the grafted meniscal pieces had

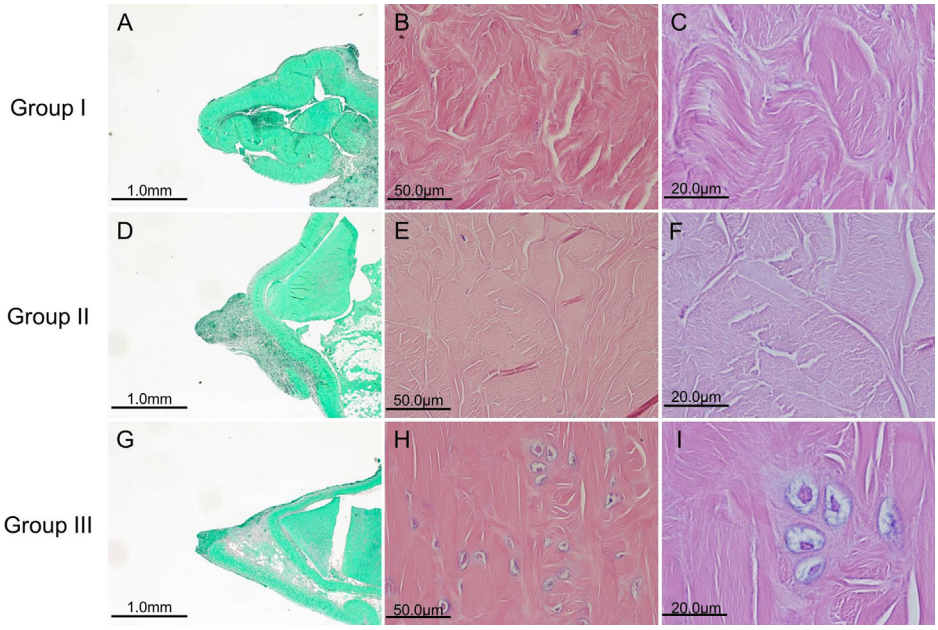

Fig. 3. Histologic findings at 3 weeks: whole cross-sections of Groups I, II, and III are stained with safranin O (A, D, and G. Original magnification × 2). The core portion is stained with hematoxylin and eosin (B, E, and H. Original magnification × 40; C, F, and I. Original magnification × 100). No cells are seen in the grafted meniscal pieces in Group II (E and F), whereas in Group III, fibrochondrocytes are alive (H and I).

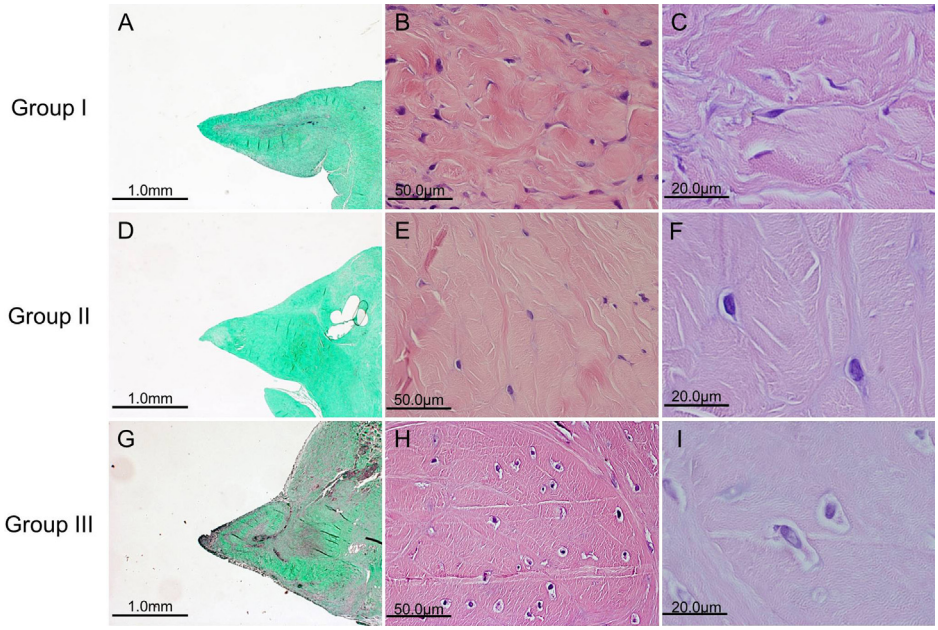

Fig. 4. Histologic findings at 6 weeks: whole cross-sections of Groups I, II, and III are stained with safranin O (A, D, and G. Original magnification × 2). The core portion is stained with hematoxylin and eosin (B, E, and H: original magnification × 40; C, F, and I. Original magnification × 100). In Group II, meniscus-shaped homogeneous fibrous tissue is formed (E and F). In Group III, cells with a relatively large nucleus are sparsely scattered throughout the dense fibrous tissue (H and I).

disappeared by 6 weeks, and meniscus-shaped, homogeneous, dense fibrous tissue had formed. The surface was enveloped by thin synovial tissue. Cells with a relatively large round or ovoid nucleus were sparsely scattered throughout the dense fibrous tissue. The meniscus-shaped fibrous tissue was not positively stained with safranin O.

In Group I (Fig. 5A–C), the fibrous tissue volume had shrunk markedly at 12 weeks. In Group II (Fig. 5D–F), relatively large round cells were scattered in the core portion of the meniscus-shaped tissue at 12 weeks, and the homogeneous fibrous tissue was not positively stained with safranin O. In Group III (Fig. 5G–I), large round cells rich in cytoplasm with a round or ovoid nucleus were scattered in the core portion of the meniscus-shaped tissue at 12 weeks, and the matrix around these cells was positively stained with safranin O.

Concerning the histologic score at 12 weeks (Table 1), one-way Anova demonstrated a significant difference between groups ($p < 0.0001$). The post hoc test showed that the score for Group II was significantly greater than that for Group I ($p = 0.0070$), whereas the score for Group III was significantly greater than that of both Group I ($p < 0.0001$) and Group II ($p = 0.0043$).

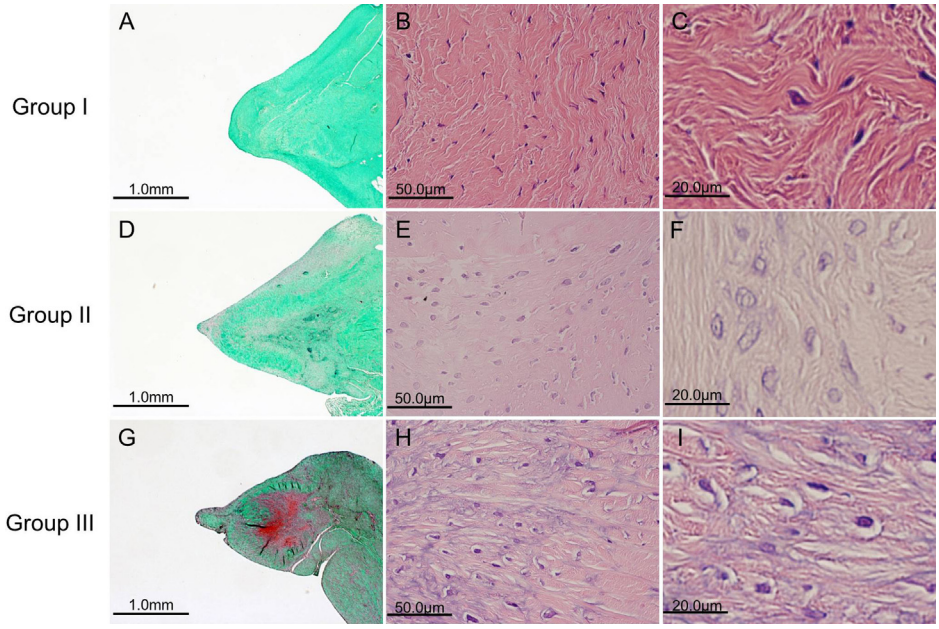

Fig. 5. Histologic findings at 12 weeks: whole cross-sections of Groups I, II, and III are stained with safranin O (A, D, and G: original magnification × 2). The core portion is stained with hematoxylin and eosin (B, E, and H: original magnification × 100; C, F, and I: original magnification × 100). In Group II, relatively large round cells are scattered in the core portion (F), whereas the homogeneous fibrous tissue is not positively stained with safranin O (D). In Group III, large round cells rich in cytoplasm (I) are scattered in the core portion, and the matrix around these cells is positively stained with safranin O (G).

Table 2
Biomechanical comparisons of the regenerated tissues at 6 and 12 weeks between the 3 groups.

	Group I	Group II	Group III	Comparisons[a]
Maximal load, (N)				
6 weeks	12.1 ± 1.4	15.3 ± 1.6	16.8 ± 2.5	I vs. II: $p = 0.0218$ I vs. III: $p = 0.0022$
12 weeks	17.5 ± 6.1	24.5 ± 2.2	30.8 ± 2.3	I vs. II: $p = 0.0164$ I vs. III: $p = 0.0002$ II vs. III: $p = 0.0293$
Linear stiffness, (N/mm)				
6 weeks	3.7 ± 0.7	5.6 ± 1.0	4.8 ± 1.0	I vs. II: $p = 0.0067$
12 weeks	5.4 ± 2.1	7.9 ± 1.6	12.5 ± 3.8	I vs. III: $p = 0.0014$ II vs. III: $p = 0.0205$

[a] Indicates only between-group comparisons that were significantly different. Comparisons with non-significant differences are not listed.

3.4. Biomechanical evaluation

Concerning the maximal load and the linear stiffness at 12 weeks (Table 2), Anova showed significant differences between groups in each parameter ($p = 0.0008$ and $p = 0.0044$, respectively). The post hoc test demonstrated that the maximum load of Group II was significantly greater than that of Group I ($p = 0.0164$), whereas there was no difference in linear stiffness. The maximum load and linear stiffness of Group III were significantly greater than those of both Group I ($p = 0.0002$ and $p = 0.0014$, respectively) and Group II ($p = 0.0293$ and $p = 0.0205$, respectively). However, these structural parameters of Group III were significantly lower ($p < 0.0001$ and $p < 0.0001$, respectively) than those of normal meniscus.

4. Discussion

In the present study, first, implantation of devitalized extracellular matrix had a slight but significant effect on in vivo meniscus regeneration after implantation. Second, implantation of meniscus fragments containing autogenous live chondrocytes significantly affected in vivo meniscus regeneration after implantation. Third, the degree of the effect on meniscus regeneration was significantly greater with implantation of fragments containing autogenous living chondrocytes than implantation of devitalized meniscal matrix. Thus, autogenous living chondrocytes contained in the meniscal matrix play a critical role in in vivo meniscus regeneration induced by in situ meniscus fragment implantation. Thus, our hypotheses have been confirmed.

A strength of the present study was that it assessed biomechanical parameters in addition to morphologic and histologic endpoints. In the present study, a uniaxial tensile test was carried out to compare regeneration of the circumferentially orientated fibers among the three groups, as these fibers play an essential role in normal meniscus function as load transmitters [28]. The regenerated circumferentially oriented fibers in Group III were significantly stronger that those in Groups I and II, in agreement with the results of morphologic and histologic analyses. These observations suggest that the structural properties of the regenerated meniscus in Group III were better than that in Groups I and II.

The present study also demonstrated that implantation of autologous chondrocytes contained in the meniscal matrix enhance meniscus regeneration. Live chondrocytes were observed in the grafted meniscus fragments at 3 weeks after implantation in Group III, whereas no cells were observed in the fragments in Group II at the same time point. This suggests that the live chondrocytes observed at 3 weeks in Group III originated from the implanted native chondrocytes and that the live cells observed at 6 and 12 weeks in the grafted meniscus fragments of Group II had infiltrated

from the surrounding tissues. Previous in vitro studies showed that human chondrocytes can expand from small meniscal specimens and surgical meniscal debris and that such cells are well-suited for use in engineering meniscus constructs [8,9]. In addition, Tumia and Johnstone [10] reported that meniscal chondrocytes can generate new extracellular matrix ex vivo following exposure to various growth factors. The present study suggested that autologous chondrocytes in meniscus fragments can expand in vivo and serve as potent promoters of meniscus regeneration. Thus, differences in the function between autologous chondrocytes and infiltrated cells from the surrounding tissues could have caused the significant differences in the quality and quantity of regenerated meniscus observed at 6 and 12 weeks in the present study. However, some extrinsic cells could have infiltrated into the grafted meniscus fragments at 6 and 12 weeks in Group III. The present study was thus limited because we could not distinguish the effects of extrinsic cells from those of native chondrocyte origin. Further studies are needed to distinguish the effects of these cells.

The present study has some limitations. First, the study used a rabbit model. Therefore, the reparative ability of the meniscal fragment implantation in rabbits, relative to that in humans, might have been underestimated [24,29]. Second, the anterior one-third of the medial meniscus including a part of the anterior horn was resected. Therefore, we cannot refer to another type of meniscal injury model. Third, fresh meniscal fragments were reimplanted. In patients, the torn meniscal tissue is usually degenerated. Forth, the authors did not determine the compressive or viscoelastic properties of the regenerated meniscus-like tissue in the biomechanical evaluation. The maximum load and stiffness of the reparative tissue was evaluated as biomechanical evaluation. However, these stresses are not in any way reflecting the in vivo stress that is put on the normal meniscus. Fifth, this study did not perform a biological evaluation of the regenerated meniscus-like tissues. In the next step, the quality and quantity of the regenerated tissue including collagen type, proteoglycan should be assessed. Beyond these limitations, however, the present study provided important information that clarifies details of the mechanism of meniscus regeneration using in situ meniscal fragment implantation. Further studies using different experimental models and methods are needed to address the limitations of the present study.

Regarding clinical relevance, the present study suggests that the in situ meniscal fragment re-implantation strategy [6] is of potential value for regeneration of the meniscus and should therefore be verified by further studies using allogenic living chondrocyte in the near future.

5. Conclusion

Implantation of autologous meniscus fragments containing live chondrocytes has a significant effect on in vivo meniscus regeneration at 6 and 12 weeks after implantation. The degree of the effect on meniscus regeneration is significantly greater with implantation of autologous meniscal fragments containing living chondrocytes than with implantation of devitalized meniscal fragments. This study demonstrates that grafted living chondrocytes contained in the meniscal fragments play a critical role in in vivo meniscus regeneration induced by in situ meniscus fragment implantation.

Disclosure of interest

The authors declare that they have no competing interest.

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Contribution

Each author certifies that he participated sufficiently in the intellectual content, the analysis of data and the writing of the manuscript to take public responsibility for it. Each author has reviewed the final version of the manuscript, believes it represents valid work, and approves it for publication.

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