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Autologous meniscus fragments embedded in atelocollagen gel enhance meniscus repair in a rabbit model

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Aims

Meniscal injuries are common and often induce knee pain requiring surgical intervention. To develop effective strategies for meniscus regeneration, we hypothesized that a minced meniscus embedded in an atelocollagen gel, a firm gel-like material, may enhance meniscus regeneration through cell migration and proliferation in the gel. Hence, the objective of this study was to investigate cell migration and proliferation in atelocollagen gels seeded with autologous meniscus fragments in vitro and examine the therapeutic potential of this combination in an in vivo rabbit model of massive meniscus defect.

Methods

A total of 34 Japanese white rabbits (divided into defect and atelocollagen groups) were used to produce the massive meniscus defect model through a medial patellar approach. Cell migration and proliferation were evaluated using immunohistochemistry. Furthermore, histological evaluation of the sections was performed, and a modified Pauli's scoring system was used for the quantitative evaluation of the regenerated meniscus.

Results

In vitro immunohistochemistry revealed that the meniscus cells migrated from the minced meniscus and proliferated in the gel. Furthermore, histological analysis suggested that the minced meniscus embedded in the atelocollagen gel produced tissue resembling the native meniscus in vivo. The minced meniscus group also had a higher Pauli's score compared to the defect and atelocollagen groups.

Conclusion

Our data show that cells in minced meniscus can proliferate, and that implantation of the minced meniscus within atelocollagen induces meniscus regeneration, thus suggesting a novel therapeutic alternative for meniscus tears.

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Keywords: Atelocollagen, Meniscus, Rabbit model of massive meniscus defect

Article focus

- Cell migration and proliferation of minced meniscus in atelocollagen scaffold were evaluated.
- Meniscus regeneration following implantation of the minced meniscus into a massive meniscus defect rabbit model was investigated.

Key messages

- Cells from the minced meniscus proliferated in atelocollagen gel.
- Implantation of minced meniscus induced meniscus regeneration.

Strengths and limitations

- Atelocollagen as a scaffold for implantation of the minced meniscus is easy to use and effective.
- Mechanical properties of the regenerated tissue were not evaluated, and results in humans may be different from those in rabbits.

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Introduction

Meniscal injuries are common and often induce knee pain and dysfunction, necessitating surgical treatment.[1] In particular, meniscal tears in avascular regions do not heal completely due to a lack of blood supply; thus, partial meniscectomy is performed to improve symptoms.[2] For large radial or severe degenerative tears, partial meniscectomy often results in massive meniscus defects frequently leading to osteoarthritis of the knee.[3] Therefore, novel techniques for regenerating an injured meniscus are needed to prevent joint degeneration after a massive loss of the meniscus.

To achieve meniscus regeneration, cell therapy and/or tissue engineering techniques have been evaluated in both animal studies and clinical trials, and stem cell therapy has been recognized as a promising strategy.[4,5] Multipotent mesenchymal stem cells (MSCs) have been used for meniscal regeneration. They offer several advantages, such as easy isolation and expansion, and multidifferentiation ability.[6-10] Other cells, including fibrochondrocytes, chondrocytes, and myoblasts, have also been used for meniscus regeneration in animal models.[11-14] Although these cell therapies have produced desirable results, they require cell isolation and culture, and typically involve a two-step surgery. Thus, the cell source and implantation technique should be considered to develop a streamlined one-step surgery approach.

In cartilage repair, the use of cartilage fragments has attracted considerable attention.[15,16] This involves a one-step procedure known as the cartilage autograft implantation system or particulated juvenile allograft cartilage, and good clinical results have been reported.[17] Chondrocytes have been shown to migrate with this method from the cartilage fragments, and subsequently proliferate to produce the cartilage matrix.[18,19] These results demonstrate that cell isolation from tissues and culture for implantation is less advantageous for tissue repair. Similarly, the advantages of minced meniscus over cultured cells for meniscus regeneration have been highlighted in animal models.[20,21] Implantation of juvenile allografts and minced fragments have been reported to promote the healing of an avascular meniscal injury in an organ culture model.[20] Implantation of autologous meniscal fragments wrapped with a fascia sheath into a meniscal defect significantly enhanced fibrocartilage regeneration in rabbits.[21] Therefore, in order to develop a more effective procedure for meniscus regeneration, the combination of the minced meniscus and a scaffold should be examined.

Atelocollagen, a firm gel-like material, is generated from bovine type I collagen in which the telopeptides on the peptide chains, which are antigenic determinants, are removed. This 3D structure is suitable as a scaffold for cell culture.[22] We hypothesized that minced meniscus embedded in atelocollagen gel would enhance meniscus regeneration through meniscus cell migration and proliferation in the gel. This study aimed to investigate cell migration and proliferation in atelocollagen gel seeded with autologous meniscus fragments in vitro, and examine the therapeutic potential of minced meniscus combined with atelocollagen gel for tissue regeneration in a massive meniscus defect in a rabbit model in vivo.

Methods

Animals. A total of 34 Japanese white rabbits weighing between 2.5 kg and 3.2 kg were used (45 knees for in vivo study (15 knees per group) and 22 knees for in vitro study). Surgery was performed under general anaesthesia by intramuscular injection of xylazine (3 mg/kg) and ketamine (10 mg/kg). Through a medial parapatellar approach, the patella was dislocated laterally, and the medial meniscus was exposed. The anterior half of the medial meniscus was then excised to produce the massive meniscus defect model.[22] Resected meniscus tissue was manually minced to pieces < 1 mm^3 in a petri dish using a scalpel as reported previously.[23] In the minced meniscus group (n = 5), 25 mg of the minced meniscus was mixed with 100 µl of atelocollagen, placed in a culture dish to form a firm gel for 30 minutes, and then implanted in the meniscus defect (Supplementary Figure a). In the atelocollagen group, 100 µl of atelocollagen was implanted in the defect sites (n = 5). Meniscus defects in the defect group remained empty, and the resected meniscus tissues from this group were used for the in vitro study (n = 5). Thereafter, the joint capsule and skin were closed. The rabbits were killed at eight, 12, and 16 weeks using sodium pentobarbital, and the medial meniscus was harvested. The tissues were fixed using 4% paraformaldehyde phosphate-buffered solution (PFA) (Wako Pure Chemical Industries, Japan) at 4°C overnight, embedded in paraffin, and cut into 4 µm sections. All procedures were performed according to the Guidelines for Animal Experimentation at Hiroshima University with the approval of the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University. An ARRIVE checklist is included in the Supplementary Material to show that the ARRIVE guidelines were adhered to in this study.

Cell culture. Meniscus tissues from the anterior half of the medial meniscus in the defect group were manually minced in phosphate-buffered saline (PBS) to pieces < 1 mm^3 with a surgical scalpel, washed in PBS three times, and embedded in atelocollagen gel. To examine the effect of the volume of the minced meniscus on cell migration and proliferation, the tissues were divided into three groups, in which 25 mg (group A), 50 mg (group B), or 125 mg (group C) of the minced meniscus was embedded in 100 µl atelocollagen. The composites were placed on the culture dish and allowed to form a firm gel for 30 minutes, followed by the addition of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, USA) with 10% fetal bovine serum (Sigma-Aldrich, USA) and 1% antibiotics (penicillin, streptomycin, and fungizone) (BioWhittaker, USA). The dishes were incubated in a

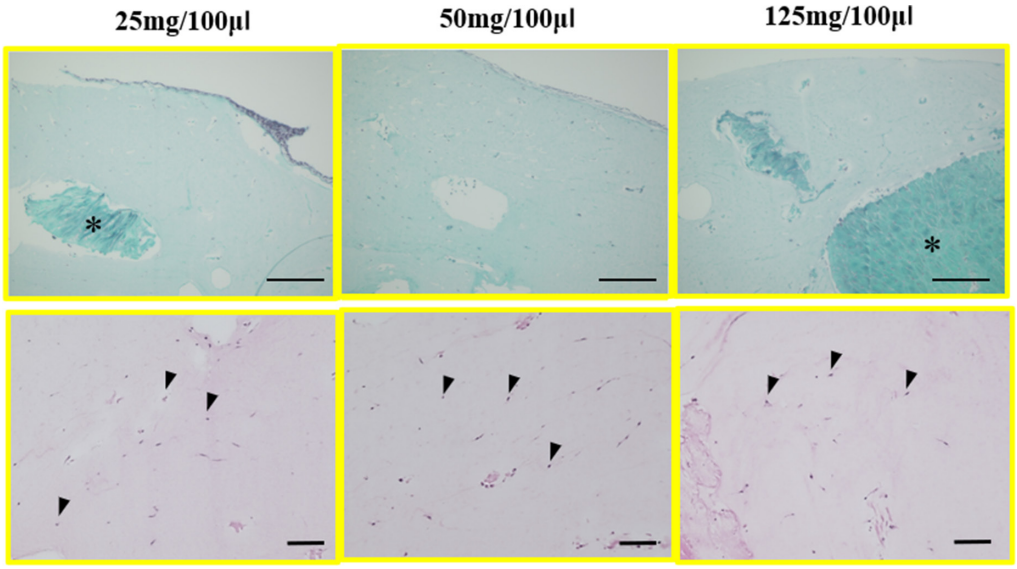

Fig. 1

Upper section: Safranin-O/Fast green staining in each group; asterisks denote minced meniscus. Scale = 300 µm. Lower section: haematoxylin and eosin staining in each group. Arrowheads indicate the cells in the atelocollagen gel, scale = 100 µm. The measurements 25 mg, 50 mg, and 125 mg indicate the volume of minced meniscus embedded in 100 µl of atelocollagen.

humidified atmosphere with 5% CO_2 at 37°C and cultured for three weeks. The medium was changed every three days. At three weeks, the composites were fixed in 4% PFA at 4°C overnight. Samples were embedded in paraffin and cut into 5 µm sections along the axial plane. Sections were stained using Haematoxylin and Eosin (HE) and Safranin-O for histological assessment.

Immunochemistry in vitro. Sections were incubated with antigen retrieval reagent (Immunoactive; Matsunami Glass, Japan) for one hour, followed by 0.3% H_2O_2 for 30 minutes, normal blocking serum for 30 minutes, and primary antibody against Ki67 (Cell Signalling Technology, USA) overnight at 4°C. The next day, the sections were visualized using the avidin-biotin system (Vectastatin Elite ABC Mouse IgG Kit; Vector Laboratories, USA) and 3,3′diaminobenzidine (Peroxidase Substrate Kit; Vector Laboratories) according to the manufacturer's instructions. To assess cell proliferation, the total number of cells, the number of cells stained by the Ki67 antibody, and the ratio of stained cells to total cells were determined in nine randomly selected sections of 300 µm² in atelocollagen areas.

Macroscopic evaluation. The tibial plateaus were photographed, and the sizes of the medial and lateral meniscus were measured using ImageJ software (version 1.43, National Institutes of Health, USA). The ratio of the medial meniscus area to the lateral meniscus area was calculated as previously described.[23]

Histological evaluation. Sections were stained using HE, Safranin-O/Fast green, and Toluidine blue. A modified Pauli's scoring system was used[24,25] for the quantitative evaluation of the regenerated meniscus, performed in a blind manner by two orthopaedic surgeons (TN and MI). The modified scoring system consists of four sections: morphology of the regenerated meniscus, cellularity of the chondrocytes, collagen fibre organization, and matrix staining. The scoring of each section was 6, 3, 3, and 3 points, respectively.

Statistical analysis. Statistical analysis was performed using SPSS v.22.0 (IBM, USA). Scheffe's F-test was used for comparison between groups in the in vitro experiment and comparison between the respective groups in the in vivo experiment at each timepoint. Results with a p-value < 0.05 were considered statistically significant.

Results

To examine meniscus cell migration and proliferation in atelocollagen gel, the cells in the gel seeded with three different doses were quantified. Migrated and proliferated cells were observed in the gel and on the surface of the composites in all groups (Figure 1). Areas (300 µm × 300 µm) were randomly selected, and the mean cell number was calculated. The mean number of cells in groups A, B, and C was 6.7 (standard deviation (SD) 4.5), 6.0 (SD 5.6), and 4.4 (SD 3.2), respectively. Although group A produced the highest number of cells, there was no significant difference in the number of cells among the groups. In the immunological analysis, areas (300 µm × 300 µm) were randomly selected, and the mean positive cell number was calculated. Immunohistochemistry revealed a high number of Ki67-positive cells both in the gel and minced meniscus (Figure 2a and Supplementary Figure b). The mean total number of Ki67-positive cells per field of view in groups A, B, and C was 5.0 (SD 4.3), 4.6 (SD 5.5), and 2.8 (SD 3.2), respectively (group A vs group B, p = 0.731; group B vs group C, p = 0.247; group A vs group C, p = 0.125) (Figure 2b). The mean percentage

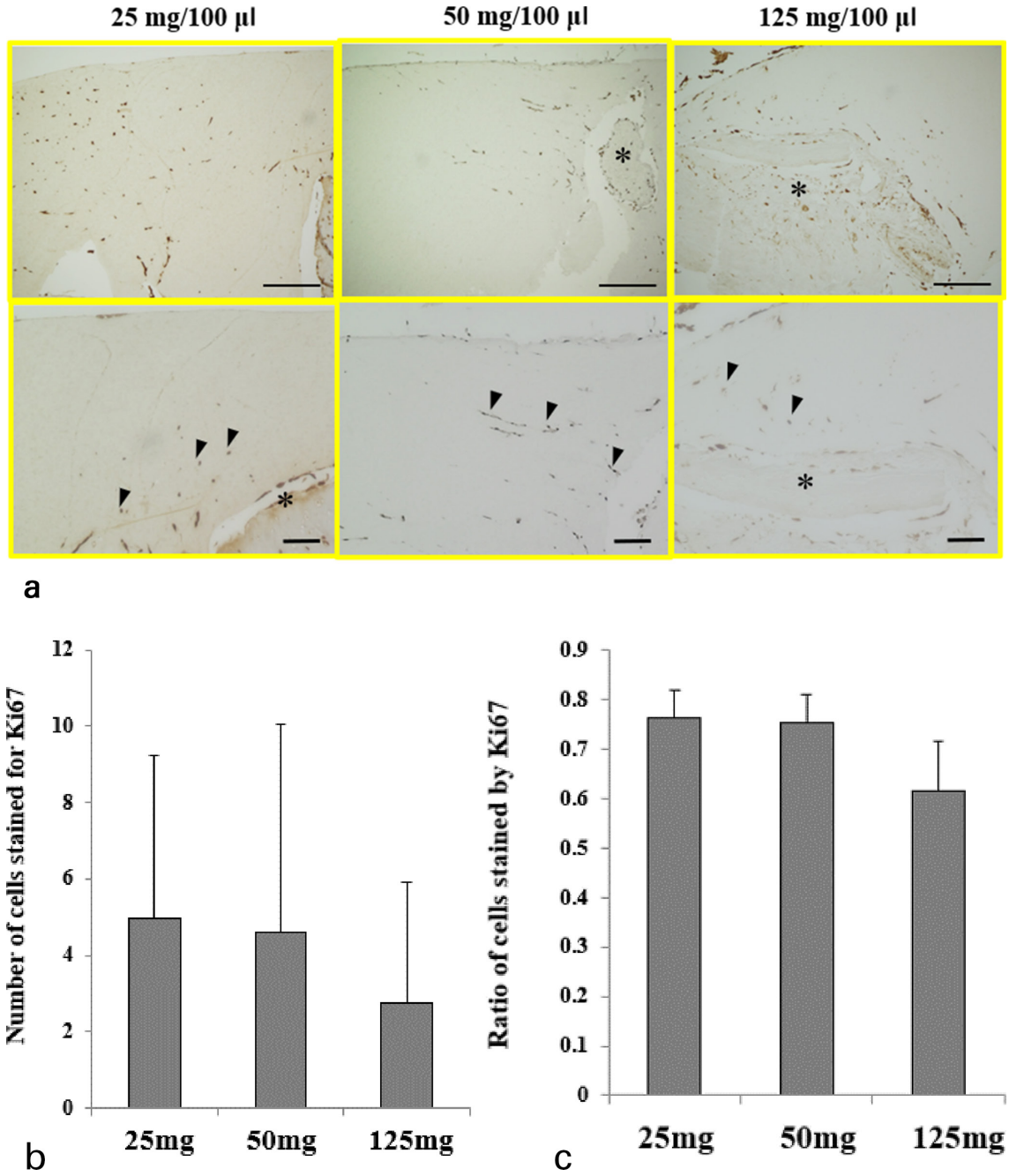

Fig. 2

a) Immunohistochemistry for Ki67 in each group; asterisks denote minced cartilage. Arrowheads indicate the cell stained for Ki67 in the atelocollagen gel; asterisks denote minced cartilage; thin bar = 300 µm, thick bar = 100 µm. The quantities of 25 mg, 50 mg, and 125 mg indicate the volume of minced meniscus embedded in 100 µl of atelocollagen. b) Number of cells stained by Ki67 for each group. There is no significant difference between groups. The quantities of 25 mg, 50 mg, and 125 mg indicate the volume of minced meniscus embedded in 100 µl of atelocollagen. c) The ratio of cells stained by Ki67 for each group. There is no significant difference between groups. The quantities of 25 mg, 50 mg, and 125 mg indicate the volume of minced meniscus embedded in 100 µl of atelocollagen.

of Ki67-positive cells out of the total cells was 76.0% (SD 5.4%), 75.0% (SD 5.7%), and 62.0% (SD 9.9%), respectively (group A vs group B, p = 0.783; group B vs group C, p = 0.271; group A vs group C, p = 0.190, all Kruskal-Wallis test) (Figure 2c). The mean meniscal cell count from one fragment of minced meniscus per 10 mg was 11.3×10^4 cells (SD 5.4×10^4) in 3D culture and 7.5×10^4 cells (SD 8.7×10^4) in 2D culture (Supplementary Figure c). Using a three-lineage differentiation assay, it was confirmed that the meniscus cells did not differentiate into osteoblasts and adipocytes. However, they formed a pellet with positive staining with Safranin O. Chondrocytes were also observed in the pellet, suggesting

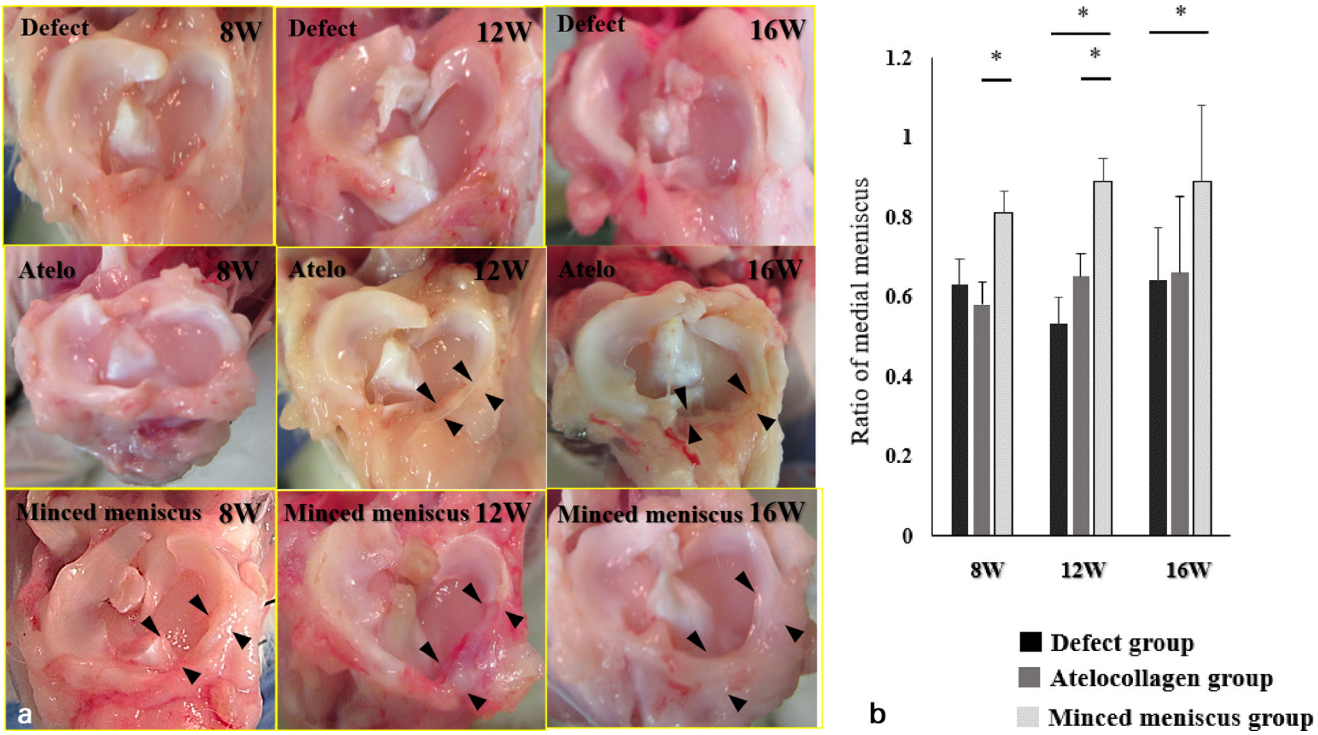

Fig. 3

a) Upper sections (defect groups). The scar tissue can be seen at eight weeks, although the tissues are incomplete. Middle sections represent the atelocollagen groups. Steady but thin tissues can be seen at 12 weeks. Lower sections represent minced meniscus groups. Steady and complete tissues can be seen at 16 weeks. Arrowheads indicate regenerated tissues. b) The percentage of the regenerated medial meniscus for both groups was defined as that of the area of the regenerated medial meniscus to the lateral meniscus. The percentages of medial minced meniscus are better in the minced meniscus groups than in the other groups ($p = 0.011$, $p = 0.036$, $p = 0.043$, and $p = 0.026$, respectively). *$p < 0.05$.

that the meniscus cells were capable of chondrogenesis (Supplementary Figure d).

No adverse effects were seen after implantation of the minced meniscus. In the defect group, the meniscus defect remained unchanged at 12 weeks. In the minced meniscus group, the presence of the transplanted minced meniscus in the meniscus defect was first confirmed by MRI at 12 weeks (Supplementary Figure e). Based on gross appearance, the defect group exhibited an empty meniscus defect at eight weeks. A small amount of scar tissue appeared at 12 weeks, and these scars expanded to link the meniscus defect. The atelocollagen group showed bridging formation, however the defect area was not filled with a regenerative tissue wall at 16 weeks. By contrast, the minced meniscus group showed continuity of the meniscus defect and soft tissue at eight weeks, and this soft tissue formed a solid meniscus-like tissue (Figure 3a). The mean percentages of regenerated tissue in the defect, atelocollagen, and minced meniscus groups were: 64.6% (SD 6.5%), 57.7% (SD 11.1%), and 81.4% (SD 12.5%) at eight weeks; 54.0% (SD 3.0%), 64.5% (SD 10.3%), and 88.6% (SD 5.7%) at 12 weeks; and 63.7% (SD 13.4%), 65.9% (SD 7.8%), and 88.9% (SD 19.1%) at 16 weeks, respectively. Furthermore, the percentage in the minced meniscus group was significantly higher than that in the atelocollagen group at eight weeks ($p = 0.011$), in both the groups at 12 weeks ($p = 0.036$), and in the defect group at 16 weeks ($p = 0.026$) (Figure 3b).

Histological analysis revealed that the surfaces of the defect and atelocollagen groups were rough, and the cells were not well distributed including the lamellar layer of both the femoral and tibial sides; however, the surfaces of the minced meniscus group were almost smooth, and the cells were well distributed at eight weeks. The regenerated tissues of the defect and atelocollagen group were less filled with chondrocyte-like cells and less stained by Safranin-O than those of the minced meniscus group. (Figure 4a). The regenerated tissues were not strongly stained with Toluidine blue in the defect and atelocollagen model, but they were strongly stained at 12 weeks in the minced meniscus group. (Figure 4b). The regenerated tissue of the minced meniscus group, but not of the defect and atelocollagen groups was strongly stained by Safranin-O (Figure 4c). The modified Pauli's score based on Toluidine blue staining was significantly higher in the minced meniscus group than that in the defect group at all timepoints (eight weeks: 9.0 (SD 3.9) vs 2.0 (SD 1.6), $p = 0.006$; 12 weeks: 9.0 (SD 4.0) vs 2.6 (SD 3.0), $p = 0.021$; 16 weeks: 12.0 (SD 0.4) vs 2.6 (SD 1.2), $p = 0.001$, all Kruskal-Wallis test) (Figure 4d). Further, the modified Pauli's score was significantly higher in the minced meniscus group than that in the atelocollagen group at

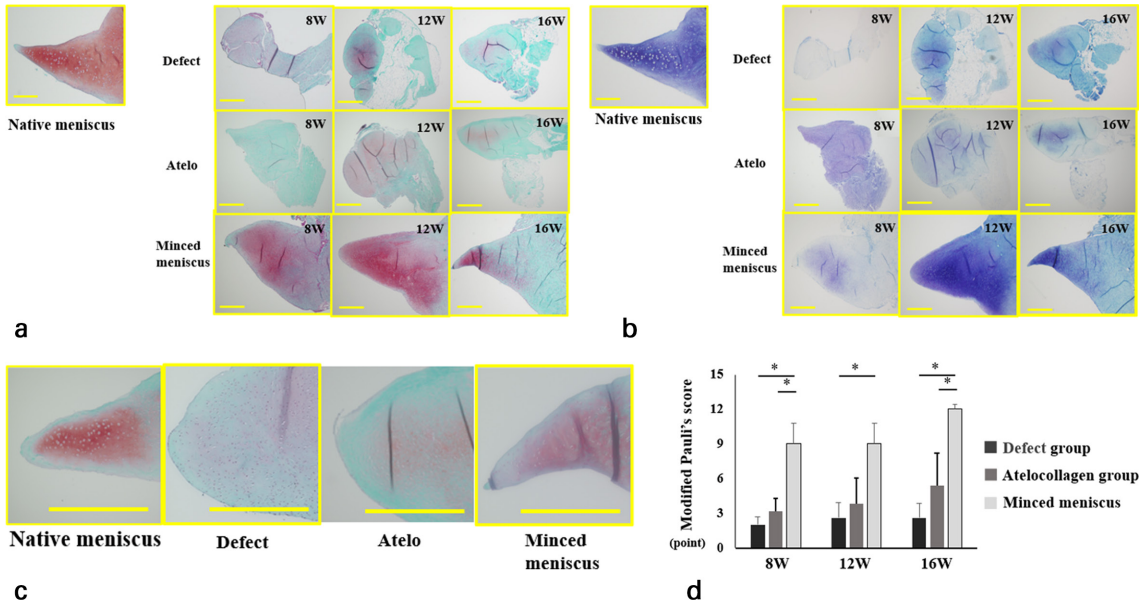

Fig. 4

a) Safranin-O/Fast green staining: representative pictures of each group at eight, 12, and 16 weeks. The left figure shows native meniscus. b) Toluidine blue staining: representative pictures of each group at eight, 12, and 16 weeks. The left figure shows native meniscus. c) Regenerated meniscus tissues at 16 weeks. Minced meniscus models presented more similar morphological and histological structures to the native meniscus than the other groups. d) Modified Pauli's scoring system for minced meniscus models is significantly better than that for the other groups. *p < 0.05.

eight and 16 weeks (eight weeks: 9.0 (SD 3.9) vs 3.2 (SD 1.5), p = 0.009; 12 weeks: 9.0 (SD 4.0) vs 3.8 (SD 4.3), p = 0.083; 16 weeks: 12.0 (SD 0.4) vs 5.4 (SD 2.8), p = 0.005) (Figure 4d).

To examine the effects of stem/progenitor cells in meniscus regeneration, immunohistochemistry using CD44 and CD271 staining was performed. No CD44 and CD271 positive cells were seen in any of the specimens at eight weeks (Supplementary Figure f).

Discussion

In this study, we found that minced meniscus embedded in atelocollagen gel exhibits high cell migration and proliferation in vitro, and implantation of the same produces tissue resembling the native meniscus tissue in vivo. Tissue engineering has attracted significant attention for meniscus regenerative treatment.[26] A variety of cells, including meniscal cells, chondrocytes, and MSCs derived from bone marrow, fat tissue, or synovium, have been used.[27-30] However, there is no consensus regarding the best cell resource for meniscal regeneration. Desirable results were reported in experiments using MSCs as a cell source, but it is not clear whether MSCs differentiate into meniscus cells due to a lack of markers for meniscal genes.[31,32] There are some reports on the use of minced meniscus for meniscus regeneration. Kobayashi et al[21] proposed the use of meniscus fragments as a cell source not requiring isolation and culture. Mincing the meniscus increases the tissue surface area, enabling cells that are essential in meniscal repair to migrate to defects and providing more space for proliferation.[20] We considered the meniscus adequate for meniscal regeneration because it contains cells, such as fibrochondrocytes, that are beneficial for tissue regeneration. In vitro, migrated cells from minced meniscus in atelocollagen gel showed comparably higher staining for Ki67 in all groups, indicating that the cells could proliferate. In addition, 11.3 × 10^4 cells per 10 mg meniscus fragment could migrate and proliferate in the gel. This demonstrates that minced meniscus could be a potential cell source for meniscus regeneration.

Scaffolds for meniscus tissue engineering can be categorized into four broad classes: synthetic polymers, hydrogels, natural matrices, or tissue-derived materials. Similar to cell sources, there is no consensus regarding the ideal materials for scaffolds for meniscal regeneration. However, it is important to know the material properties of each potential scaffold to select the most appropriate scaffold for the target environment.[33,34] Collagen is often used as a scaffold for cartilage regeneration. Collagen gels have been used for in vivo cartilage regeneration experiments with some clinical applications.[35–41] In our in vivo experiment, the minced meniscus and atelocollagen scaffold complex remained in the transplanted area for 12 weeks without deviation to the cavity of the knee joint, as observed in MRI. There was no significant difference between the defect and atelocollagen groups observed via macroscopic and histological analysis. Thus, the regenerated tissues in the minced meniscus group were not derived from the surrounding tissues but rather from the implanted minced meniscus. Atelocollagen may be an appropriate scaffold for implantation of minced meniscus owing to its ease of use and ability to restore tissue at the defect site. MSCs are widely accepted to have

the potential to regenerate the meniscus; previously, MSCs from synovial tissues were found to contribute to meniscus regeneration.[42,43] These cells have multidifferentiation capacity and recruit cellular and growth factors. Synovial cells also contribute to meniscus repair.[44] In this study, the expression of MSC markers was not observed in the regeneration process until eight weeks. It was also confirmed that meniscus cells were capable of chondrogenesis, suggesting that the meniscus cells from the fragment itself may contribute to the regenerated meniscus. Moreover, the in vitro study revealed that the migrated cells from the fragment have a high cell proliferation capacity. These cells may produce extracellular matrix in the gel during the process. It is also possible that the production of the extracellular matrix from the meniscus fibrochondrocytes from the fragments might be promoted by the surrounding synovium.

This study has some limitations. First, the mechanical properties of the regenerated tissue were not evaluated. However, the morphology of the regenerated tissue was similar to that of native meniscal tissue. Second, this study was based on a rabbit model; meniscal cell proliferation, matrix production, and the strength of meniscal tissue may differ between humans and rabbits. Third, the appropriate amount of minced meniscus for embedding in atelocollagen was not established. However, the amount of minced meniscus was unrelated to cell proliferation, as all groups exhibited similar cell growth. Fourth, the anterior half of the medial meniscus was excised to produce the massive meniscal defect, however, the defect area was not loaded with body weight. Further, the meniscal area loaded with body weight is larger in humans than in rabbits. Whether tissues can be regenerated in humans, as achieved in this study, is uncertain. Regardless, the proper scaffold material for implantation of the minced meniscus at the defect site is required.

In conclusion, this study showed that cells in the minced meniscus can proliferate and implantation of the minced meniscus within atelocollagen induced meniscus regeneration. Thus, this novel meniscus regeneration method may be a potential therapeutic alternative to a one-step surgery for treating meniscus tears.

Supplementary material

Details of the methodology used in our experiment, including evaluation of cell migration and proliferation, three lineage differentiation, MRI evaluation, and evaluation of multipotent cells. ARRIVE checklist shows that the ARRIVE guidelines were adhered to in this study.

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