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Effects of a cell-free method using collagen vitrigel incorporating TGF-β1 on articular cartilage repair in a rabbit osteochondral defect model

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Abstract: We studied the ability of collagen vitrigel material to repair cartilage *in vivo* when used alone or with transforming growth factor-β (TGF-β). We measured the time course and quantity of TGF-β1 released from the collagen vitrigel *in vitro* to quantify the controlled release of TGF-β1. Over 14 days, 0.91 ng of TGF-β was released from the collagen vitrigel. Osteochondral defects were made in the femoral trochlear groove in 36 Japanese white rabbits, which were divided into three groups: untreated group (group A), collagen vitrigel-implanted group (group B), and TGF-β1-incorporated collagen vitrigel-implanted group (group C). The weight distribution ratio between the affected and unaffected limbs served as an indicator of pain. Animals were sacrificed at 4 and 12 weeks after surgery, and their tissues were assessed histologically. The weight distribution ratio increased in all groups and did not differ significantly between groups at 12 weeks. Group A needed 6 weeks to attain maximum improvement, and groups B and C showed near-maximum improvement at 4 and 2 weeks, respectively. The International Cartilage Repair Society II score improved significantly in group C relative to the other groups. These findings suggest that sustained, slow release of TGF-β caused early pain mitigation and cartilage repair. © 2016 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater, 105B: 2592–2602, 2017.

Key Words: TGF-β, collagen vitrigel, controlled release, cartilage repair, articular cartilage

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INTRODUCTION

Since the report by Brittberg et al.[1] describing the implantation of cultured autologous chondrocytes, various cartilage regeneration treatments have been studied.[2–4] Treatments such as bone drilling and mosaicplasty are mainstays in the field of orthopedics. However, these methods have some problems, including narrowness of the treatment range, replacement of repair tissue with fibrous cartilage and not hyaline cartilage, and the need to harvest a graft.[5–7] We have developed a technique that uses cell sheets produced in temperature-responsive culture dishes developed by Okano et al.[8,9] to improve the repair of articular cartilage tissue and to treat a broad range of articular cartilage defects such as osteoarthritis of the knee. Chondrocyte sheets can help to repair articular cartilage defects by stimulating growth of hyaline-like cartilage.[10–17] A study of the mechanism of action of these cell sheets has confirmed that transforming growth factor-β (TGF-β), prostaglandin E_2, and other humoral factors also play important roles in the repair of articular cartilage tissue and that TGF-β plays a particularly important role.[17] TGF-β inhibits interleukin-1 (IL-1) and other catabolic cytokines, reportedly acts to promote cell proliferation and extracellular matrix production by articular chondrocytes, and promotes differentiation of mesenchymal stem cells into chondrocytes.[17,18]

There are five TGF-β isoforms, which have 70–80% homology in their amino acid sequences. Three isoforms

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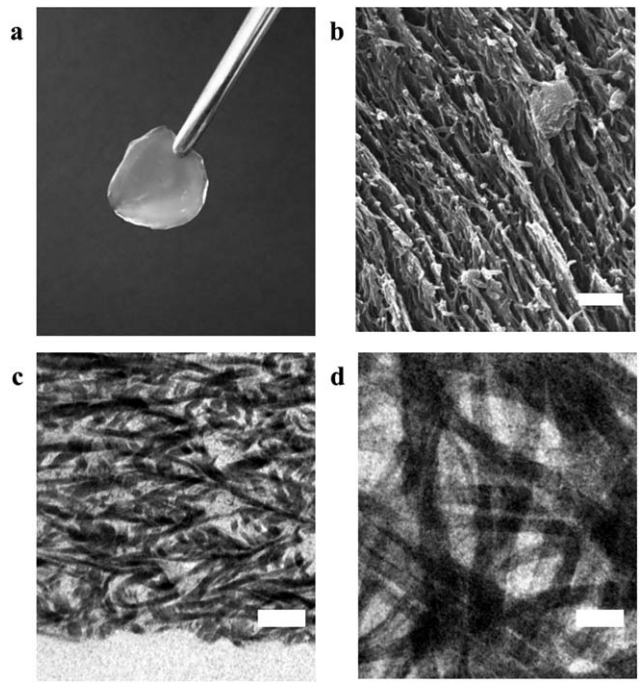

FIGURE 1. Collagen vitrigel. Photographs were obtained after the collagen vitrigel was rehydrated in PBS at room temperature 30°C for 30 min. (a) The collagen vitrigel used for this study was formed into a thin, round sheet 15 mm in diameter. The quantity of collagen in the vitrigel was 5.0 mg/cm^2. The collagen vitrigel was irradiated by an electron beam, stored in the dark at 25°C, and rehydrated in PBS just before use. (b) Scanning electron microscopy images of the collagen vitrigel membrane were obtained using a JSM 6510 instrument (JEOL, Tokyo, Japan) at an accelerating voltage of 20 kV. Original magnification is ×10,000. Bars = 1 μm. (c) and (d) Transmission electron microscopy images of the collagen vitrigel membrane obtained using a JEM-1210 instrument (JEOL). (c) Bars = 1 μm and (d) bars = 500 nm.

(TGF-β1, TGF-β2, and TGF-β3) exist in humans.[19] TGF-β1 is particularly useful for regenerating cartilage tissue.[20–22] TGF-β is most effective when a certain concentration is maintained in the lesion, although the brevity of its duration of activity has been reported as a disadvantage.[19,23,24] Although the short-term action of injected TGF-β alone has been studied extensively in animal models, such injection leads to the deposition of cartilage fibrocartilage, and a better method is needed to stimulate defect repair by hyaline cartilage.[20]

Conventional collagen gel comprises low-density collagen fibrils, which make it soft and difficult to handle. Takushi et al.[25] reported that drying a thermally denatured protein gel to remove gradually both free and bound water allows the gel to be converted into a hard transparent material similar to glass. Takezawa et al.[26] applied this vitrification process to conventional collagen gel and extended this work by rehydrating the vitrified collagen gel to produce a high-density gel comprising collagen fibrils—a novel biomaterial named "collagen vitrigel." Using this technology, the disk-shaped conventional gel of 0.25% (w/v) collagen fibrils can be concentrated up to 100–133 times and converted into a round collagen vitrigel membrane of 25–33% (w/v) collagen fibrils, which is equivalent to the composition of connective tissue found in the body.[27] Relative to conventional collagen gels, this collagen vitrigel membrane offers superior handling, strength, transparency, and permeability to proteins with a molecular weight of 100 kDa or higher.[26–28] The collagen vitrigel membrane comprises mainly collagen type I and has been reported to be an effective carrier for paracrine assays in three-dimensional *in vitro* culture and *in vivo* drug-delivery systems (DDSs).[29]

In the present study, we made use of the properties of collagen vitrigel as a DDS. By incorporating TGF-β into the collagen vitrigel, we prepared a carrier for the sustained, slow release of TGF-β without the need for cells. The purpose of this study was to examine the effects of the sustained, slow release of TGF-β on the repair of articular cartilage defects and the tissue-repairing effects of collagen vitrigel alone and as a sustained-release carrier. We implanted the sustained-release carrier into full-thickness articular cartilage defects in Japanese white rabbits, and we confirmed the therapeutic effects. We also conducted *in vitro* research to measure the quantity and duration of the sustained release of TGF-β from impregnated collagen vitrigel.

MATERIALS AND METHODS

We used 36 female Japanese white rabbits, aged ~18 weeks and weighing ~3 kg. All animal experimentation conformed to the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1996) and Tokai University guidelines on animal experimentation (animal experimentation approval number: 151075). In this study, one rabbit with implanted collagen vitrigel suffered a lower-leg fracture during rearing and was euthanized and excluded from the experimental results. No adverse events were observed in any of the other 35 rabbits.

Collagen vitrigel

Various studies of tissue regeneration have made use of the advantages of collagen vitrigel membrane.[30–36] Collagen xerogel membranes, defined as dried collagen vitrigel membranes without free water, can be prepared by simply revitrifying collagen vitrigel membranes on a separable sheet. Such collagen xerogel membranes are appropriate for long-term preservation because they can be easily converted into collagen vitrigel membranes by rehydration, as reported previously.[34,36,37] The thin, round membranes of collagen vitrigel with a collagen content of 5.0 mg/cm^2 and a diameter of 15 mm (Fig. 1) were prepared as reported previously.[36] Briefly, 1.8 mL of 0.5% porcine-derived atelocollagen solution was poured into a separable mold with an inner diameter of 15 mm, and the collagen vitrigel membrane was fabricated by gelation, vitrification, and rehydration with phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA). The membrane was then converted to a collagen xerogel membrane by revitrification on a separable sheet. This collagen xerogel membrane was irradiated with electron

beams and then stored in the dark at 25°C. Immediately before use, the membrane was rehydrated with PBS.

Incorporation of TGF-β1 into the collagen vitrigel

Human TGF-β1 (R&D Systems, Minneapolis, MN, USA) was dissolved to a final concentration of 10 ng/mL in PBS in a container precoated with 1% bovine serum albumin (BSA; Merck, Darmstadt, Germany) solution. Research on the mechanism of action of TGF-β in cultures of chondrocytes derived from mesenchymal stem cells shows that 10 ng/mL is the optimum concentration of TGF-β during culture.[19,22,38–42] Therefore, we used this concentration in our experiments. A thin, circular sheet of collagen vitrigel with 5.0 mg/cm^2 of collagen and a diameter of 15 mm was incubated in this solution for 24 h at 4°C before implantation.

Assessment of the quantity and duration of TGF-β1 released from TGF-β1-incorporated collagen vitrigel

After incubating the collagen vitrigel for 24 h at 4°C in a 10 ng/mL TGF-β1 solution, the collagen vitrigel was placed in 3 mL of 1% BSA solution and incubated at 37°C. The solution was collected and replaced every 2 days for 14 days ($n = 3$), and the collected samples were stored at −30°C. A human TGF-β1 quantikine ELISA Kit (R&D Systems) was used to measure the quantity of TGF-β1 in the samples.

Implantation

A drill was used to make an osteochondral defect with a diameter of 5 mm and depth of 3 mm in the articular cartilage of the right femoral trochlear groove in 36 Japanese white rabbits (Fig. 2). We irrigated the defect with physiological saline (Nipro, Osaka, Japan) during the drilling to prevent infection and thermal damage. The 36 rabbits were divided into three groups ($n = 12$ in each group): an untreated group (group A), a collagen vitrigel-implanted group (group B), and a TGF-β1-incorporated collagen vitrigel-implanted group (group C). After covering the defect with collagen vitrigel, we pressed the collagen vitrigel into the femur by reduction of the patella, which we had dislocated to create the defect. We then sutured the quadriceps femoris muscle and tendon to prevent dislocation. Six rabbits from each group were sacrificed at 4 weeks and another six rabbits at 12 weeks after surgery, and their tissues were examined histologically to assess the cartilage repair.

Pain assessment

We used the change in the weight distribution ratio between the affected limb and the unaffected limb as an indicator of pain. This ratio is used widely to assess pain in experimental animals.[15,43] The weight distribution ratio was measured with an incapacitance tester (Linton Instrumentation, Norfolk, UK). Each rabbit was placed on the tester and allowed to stand on its hind limbs, which were adjusted to rest on two separate sensors. The rabbit was then surrounded by a box to restrict its movement and was allowed to rest in this position for ∼5 min, after which the weight distribution was measured. The mean weight distribution ratios were calculated from five measurements taken for each rabbit in all groups, and group mean values were calculated from those mean ratios. Measurements were taken at 1, 2, 3, 4, 6, 8, 10, and 12 weeks after surgery. The weight distribution ratio (expressed here as the affected limb weight as a percentage of the total weight) was calculated using the following formula: weight distribution ratio (%) = (affected limb weight/[unaffected limb weight + affected limb weight]) × 100.

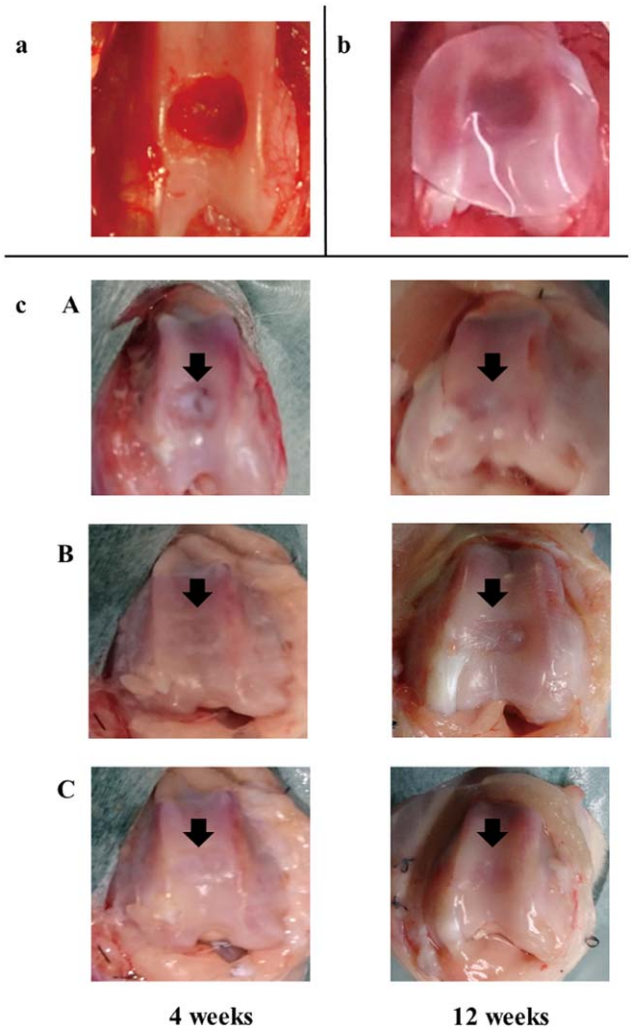

FIGURE 2. Osteochondral defect model and gross appearance of the defects 4 and 12 weeks after surgery. (a) Osteochondral defect model. We created an osteochondral defect (diameter, 5 mm; depth, 3 mm) by drilling into the articular cartilage of the right femoral trochlear groove. (b) Model with transplanted collagen vitrigel. Collagen vitrigel was transplanted to cover the defect in groups B and C. (c) Gross appearance of the defects 4 and 12 weeks after surgery. Filling in of the defect in groups A and B was insufficient at 4 and 12 weeks after surgery. In group C, the defect was filled with cartilaginous tissue at 4 and 12 weeks after surgery.

Tissue assessment

Animals were sacrificed at 4 and 12 weeks after surgery, and their tissues were examined histologically to confirm the repair of the osteochondral defect at the right femoral trochlear groove of the right knee joint. After sacrifice, the

TABLE I. ICRS II Histological Grading System

Ti: **Tissue morphology (viewed under polarized light)**	**Basl**: **Basal integration**	**Vasc**: **Vascularization (within the repaired tissue)**
0%: Full-thickness collagen fibers	0%: No integration	0%: Present
100%: Normal cartilage birefringence	100%: Complete integration	100%: Absent
Matx: **Matrix staining (metachromasia)**	**Ftide**: **Formation of a tidemark**	**SurfSp**: **Surface/superficial assessment**
0%: No staining	0%: No calcification front	0%: Total loss or complete disruption
100%: Full metachromasia	100%: Tidemark	100%: Resembles intact articular cartilage
Cmorph: **Cell morphology**	**SubBA**: **Subchondral bone abnormalities/marrow fibrosis**	**MDzone**: **Mid/deep zone assessment**
0%: No round/oval cells	0%: Abnormal	0%: Fibrous tissue
100%: Mostly round/oval cells	100%: Normal marrow	100%: Normal hyaline cartilage
Cclus: **Chondrocyte clustering (4 or more grouped cells)**	**Infl**: **Inflammation**	**Hgtot** 1: Tissue morphology (Ti). 2: Matrix staining (Matx) 3: Cell morphology (Cmorph). 4: Chondrocyte clustering (Cclus). 5: Surface architecture (SurfA). 6: Basal integration (Basl). 7: Formation of a tidemark (Ftide). 8: Subchondral bone abnormalities/marrow fibrosis (SubBA). 9: Inflammation (Infl). 10: Abnormal calcification/ossification (AbnCO). 11: Vascularization (Vasc). 12: Surface/superficial assessment (SurfSp). 13: Mid/deep zone assessment (MDzone).
0%: Present	0%: Present	
100%: Absent	100%: Absent	
SurfA: **Surface architecture**	**AbnCO**: **Abnormal calcification/ ossification**	
0%: Delamination, or major irregularity	0%: Present	
100%: Smooth surface	100%: Absent	

This system evaluates repair tissue based on 14 items: 1: Tissue morphology (Ti). 2: Matrix staining (Matx) 3: Cell morphology (Cmorph). 4: Chondrocyte clustering Cclus). 5: Surface architecture (SurfA). 6: Basal integration (Basl). 7: Formation of a tidemark (Ftide). 8: Subchondral bone abnormalities/marrow fibrosis (SubBA). 9: Inflammation (Infl). 10: Abnormal calcification/ossification (AbnCO). 11: Vascularization (Vasc). 12: Surface/superficial assessment (SurfSp). 13: Mid/deep zone assessment (MDzone). The maximum score is 100 points and the minimum score is 0 points for each item. The total score range is from 0 (no repair) to 1300 (normal articular cartilage).

right femur was collected and immobilized in 10% formalin solution, delipidated for 2–3 days with acetone solution, washed with water, and decalcified with 10% ethylenediaminetetraacetic acid for 5–7 days. This process was repeated four times. After trimming, samples were embedded in paraffin. With the defect placed at the center, sections were prepared at 3 μm thickness at right angles and were stained with safranin O and hematoxylin and eosin. And we performed immunohistochemical staining using mouse primary monoclonal antibodies which react with human collagen type II (Daiichi Fine chemical, Toyama, Japan). To immunostain for collagens type II, deparaffinized sections were treated with 0.4% pepsin (DAKO) for 30 min. at 37°C. After washing in distilled water, sections were treated with 0.3% hydrogen peroxide/methanol solution at RT for 15 min. After washing in PBS, sections were blocked with 2.5% normal goat serum (MP-7402, Vector Laboratories, Burlingame, CA, USA) for 10 min. at RT. They were then treated for 3 h at RT with mouse monoclonal antibodies for human type II collagen diluted at 1:100 with 1% BSA in PBS. After washing in PBS, they were treated for 1 h at RT with ImmPRESS polymer anti-mouse IgG reagent (MP-7402, Vector Laboratories, Burlingame, CA, USA). Finally, they were immersed for 2–8 min. in Tris–HCl buffer (pH 7.6) containing 0.02% diaminobenzidine and 0.005% hydrogen peroxide, and then counterstained with Mayer's hematoxylin.

The International Cartilage Repair Society (ICRS) II histological grading system was used to assign scores. Two blinded board-certified orthopedic surgeons scored the samples, and a mean score was calculated. The ICRS II histological grading system is an assessment method developed by Mainil-Varlet et al.[44] This assessment method assigns scores for 13 items: 1: Tissue morphology (Ti). 2: Matrix staining (Matx), 3: Cell morphology (Cmorph), 4: Chondrocyte clustering (Cclus), 5: Surface architecture (SurfA), 6: Basal integration (Basl), 7: Formation of a tidemark (Ftide), 8: Subchondral bone abnormalities/marrow fibrosis (SubBA), 9: Inflammation (Infl), 10: Abnormal calcification/ossification (AbnCO), 11: Vascularization (Vasc), 12: Surface/superficial assessment (SurfSp), 13: Mid/deep zone assessment (MDzone). The maximum score is 100 points and the minimum score is 0 points for each item

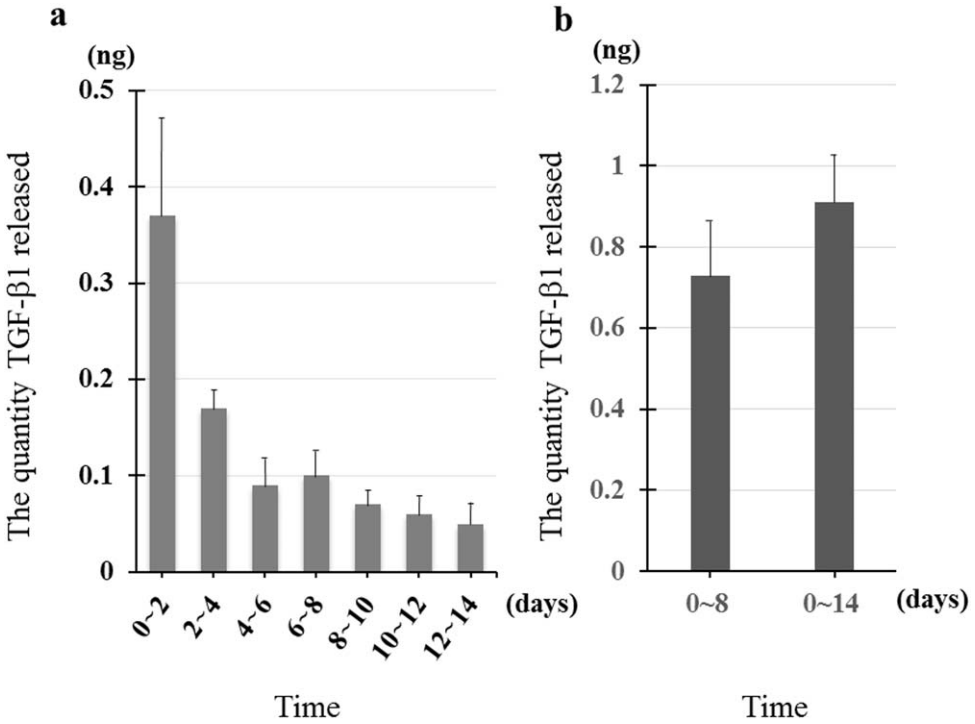

FIGURE 3. Quantity of released TGF-β1 measured every 2 days and total quantity released. (a) Quantity of TGF-β1 released measured every 2 days up to 14 days after surgery. (b) Total quantity of released TGF-β1 measured up to 8 and 14 days after surgery.

(Table I). We did not do a single score overall assessment in this study.

Statistical analysis

All data except the weight distribution ratio are expressed as mean ± standard deviation. The weight distribution ratio is expressed as mean ± standard error (SE). Analysis of variance was used to analyze all data. The Holm–Bonferroni method was used for *post hoc* testing. Statistical significance was set at $p < 0.05$.

RESULTS

Quantity of TGF-β1 released *in vitro* from TGF-β1-incorporated collagen vitrigel over 2 weeks

The duration and quantity of TGF-β1 released from the TGF-β1-incorporated collagen vitrigel were measured. The quantity of TGF-β1 released at different times after incorporation was 0.37 ± 0.10 ng at 0–2 days, 0.17 ± 0.02 ng at 2–4 days, 0.09 ± 0.02 ng at 4–6 days, 0.10 ± 0.01 ng at 6–8 days, 0.07 ± 0.01 ng at 8–10 days, 0.06 ± 0.01 ng at 10–12 days, and 0.05 ± 0.01 ng at 12–14 days. We next measured the sustained release of TGF-β1 for 2 weeks (Fig. 3). The total quantity of TGF-β released was 0.73 ± 0.13 ng over the first 8 days and 0.91 ± 0.12 ng over the entire 14 days after incorporation (Fig. 3).

Pain assessment as measured by the weight distribution ratio between the affected and unaffected limbs

The weight distribution ratio was compared from 1 to 12 weeks after surgery (Fig. 4). From 1 to 12 weeks, the ratio improved from 35.6% ± 2.1% to 43.6% ± 1.2% in Group A, from 37.0% ± 1.4% to 43.6 ± 1.4% in group B, and from 38.8% ± 2.1% to 44.5% ± 1.5% in group C. At 1 week after surgery, the difference between groups A and C was significant. At 2 weeks after surgery, the ratio was 36.7% ± 0.7% in group A, 39.0% ± 0.9% in group B, and 45.5% ± 0.3% in group C; the differences between groups A, B, and C were significant. At 3 weeks after surgery, the ratio was 41.4% ± 1.5% in group A, 43.7% ± 0.4% in group B, and 45.4% ± 0.4% in group C; the differences between groups A, B, and

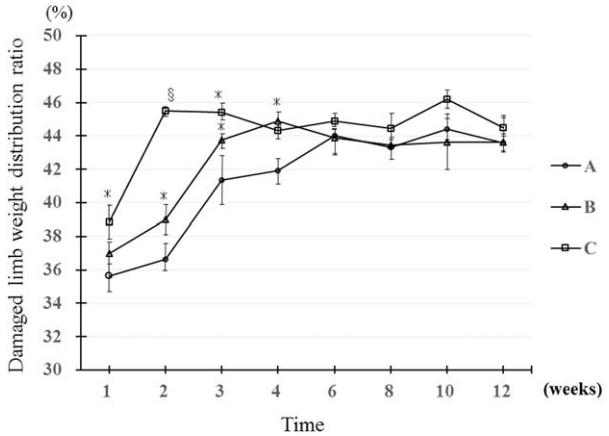

FIGURE 4. Damaged limb weight distribution ratio (%) after surgery. $p < 0.05$ was considered significant. (*) versus group A, (§) versus group B. Damaged limb weight distribution ratio (%) = damaged limb load (g)/[undamaged limb load (g) + damaged limb load (g)] × 100. The damaged limb weight distribution ratios (mean ± SE) in weeks 1, 2, 3, 4, 6, 8, 10, and 12 after surgery are shown.

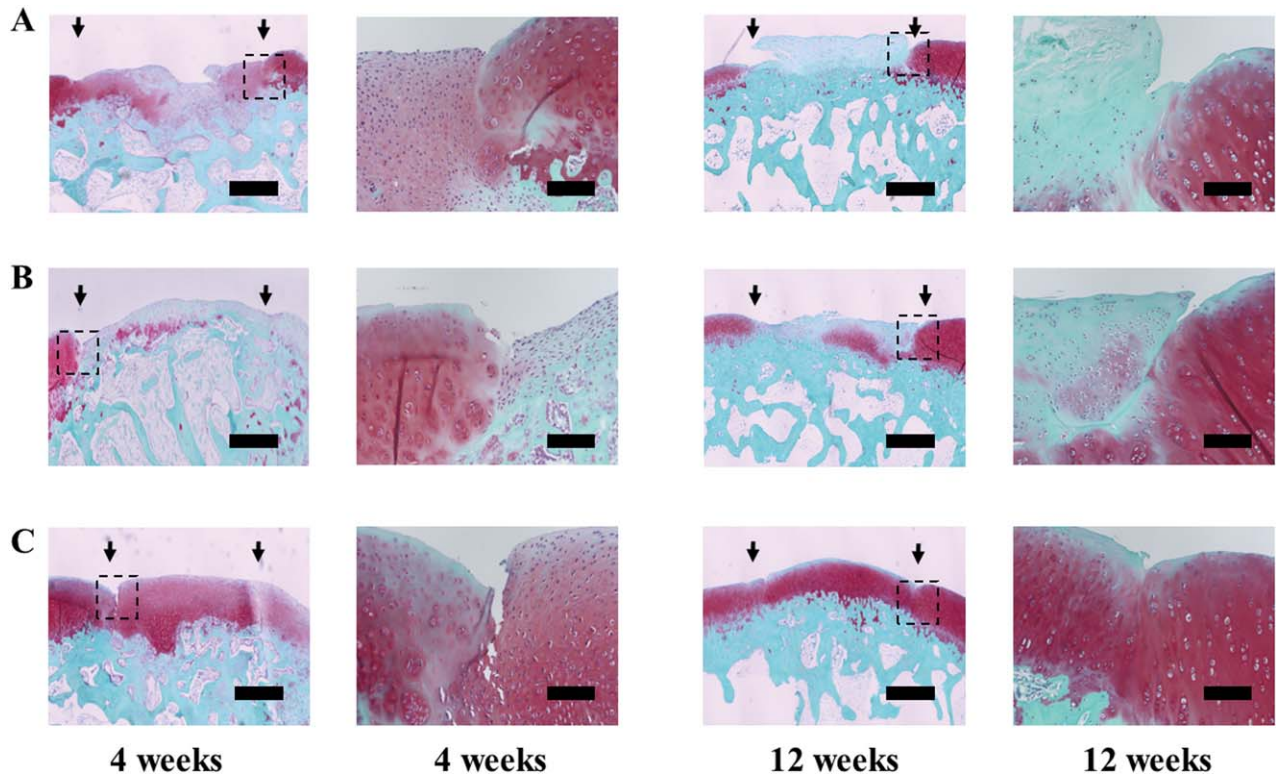

FIGURE 5. Histological observations of repair tissue. Samples were stained with Safranin O at 4 and 12 weeks after surgery (bar = 1000 μm). Higher magnification images for each sample are shown to the right of each image (bar = 100 μm). The higher-magnification images represent the tissue enclosed by black squares in the lower magnification images. Group A: untreated group. Group B: collagen vitrigel-implanted group. Group C: TGF-β1-incorporated collagen vitrigel-implanted group.

C were significant. At 4 weeks after surgery, the ratio was 41.9% ± 0.8% in group A, 44.9% ± 0.1% in group B, and 44.3% ± 0.5% in group C; the differences were significant between groups A and B, and between groups A and C. However, no significant differences were observed between the groups at 6, 8, 10, and 12 weeks after surgery.

The time needed for significant recovery differed between groups: group A took 6 weeks, group B took 4 weeks, and group C took 2 weeks. Group A showed significant improvement of the weight distribution ratio between 1 and 6 weeks after surgery, and group B showed significant improvement between 1 and 4 weeks after surgery but no further significant improvement both groups. Group C showed significant improvement between 1 and 2 weeks after surgery but no further significant improvement. At 2 and 3 weeks after surgery, the differences of the weight distribution ratio between groups B and C were significant. At 2, 3, and 4 weeks after surgery, the differences between group A and the other groups were significant.

Tissue assessment (characteristic of the tissue of each group and ICRS II scores)

We divided the 36 Japanese white rabbits into three groups ($n = 12$) and sacrificed six animals from each group at 4 and 12 weeks after surgery. One rabbit in group B, which was to be sacrificed at 12 weeks after surgery, experienced a lower-leg fracture and was euthanized. No other adverse events, such as infections, were observed during the experiment. After sacrifice, the right femur was collected and the osteochondral defects were assessed using the ICRS II histological grading system (Table I, Fig. 5). We used collagen type II staining as an indicator of the restoration of cartilage tissue, and hematoxylin and eosin staining as an indicator of inflammation. We visually confirmed that collagen vitrigel was absorbed by 4 weeks after surgery.

The histology of regenerated cartilage representing the average score for group A 4 weeks after surgery indicates defect filling with fibrous tissue and poor defect filling rates. At best, the histology indicates defect filling with hyaline cartilage-like repair tissue. At worst, the defect was not repaired at all. The histology of regenerated cartilage representing the average score for group A 12 weeks after surgery indicates defect filling with fibrous tissue and cartilage-like repair tissue. At best, the histology indicates defect filling with hyaline cartilage-like repair tissue. At worst, the defect was not repaired at all.

The histology of regenerated cartilage representing the average score for group B 4 weeks after surgery indicates defect filling with fibrous tissue and fibrocartilage-like repair tissue. At best, the histology indicates defect filling with hyaline cartilage-like repair tissue. At worst, the defect was not repaired at all. The histology the average and best score for group A 12 weeks after surgery indicates histology

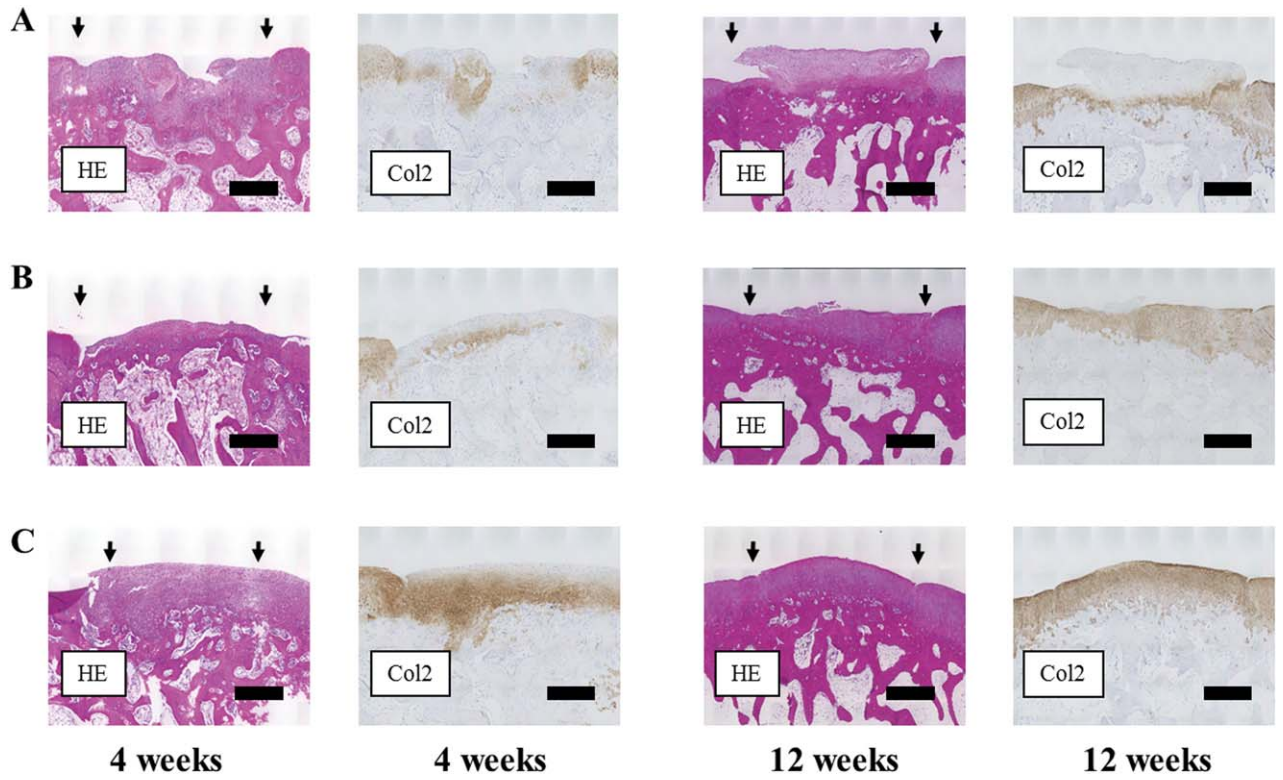

FIGURE 6. Histological observations of repair tissue. Samples were stained with hematoxylin and eosin and antibody to collagen type II at 4 and 12 weeks after surgery (bar = 1000 μm). Group A: untreated group. Group B: collagen vitrigel-implanted group. Group C: TGF-β1-incorporated collagen vitrigel-implanted group.

almost similar to group B 4 weeks after surgery. At worst, the histology indicates defect filling with fibrous tissue and fibrocartilage-like repair tissue but defect-filling rate was about 60%.

The histology of regenerated cartilage representing the average score for group C 4 weeks after surgery indicates defect filling with tissue staining for safranin O and collagen II. At best, the histology indicates repair with cartilage like properties. At worst, the histology indicates defect filling with cartilage tissue but with poor lateral integration and a lack of formation of a tidemark. The average, best, and worst scores of group A 12 weeks after surgery indicates histology almost similar to group C 4 weeks after surgery.

We evaluated collagen type II staining (Fig. 6). We observed pale weak staining in groups A and B at 4 and 12 weeks but did not observe staining in most of the repair tissue. We observed staining in most of the repair tissue in group C at 4 and 12 weeks. The defect in group C appeared to contain more collagen type II compared with that in with groups A and B. We used hematoxylin and eosin staining to identify inflammation (Fig. 6). At 4 weeks, only a few sections of tissue from groups A and B showed mild invasion and accumulation of lymphocytes, which confirmed that there was no inflammatory reaction in almost all of the repair tissue.

In the groups sacrificed at 4 weeks after surgery, the ICRS II scores were 701.2 ± 116.5 in group A, 744.2 ± 97.6

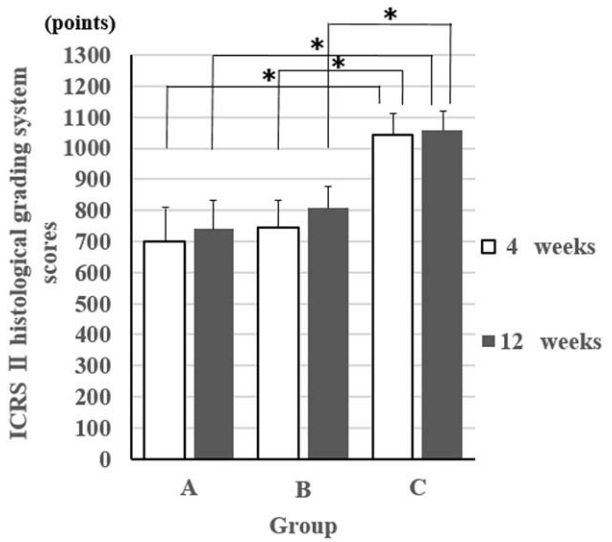

FIGURE 7. ICRS II histological grading system scores. $p < 0.05$ was considered significant. The total score ranges from 0 (no repair) to 1300 (normal articular cartilage). Group A received an osteochondral defect only, group B received the defect and collagen vitrigel transplantation, and group C received the defect and TGF-β1-incorporated collagen vitrigel transplantation. The animals were sacrificed and the tissues were examined 4 and 12 weeks after surgery. (note: one rabbit in group B was injured and euthanized during the experiment, and was excluded from the data analysis.)

TABLE II. ICRS II Histological Grading System Results at 4 Weeks (Values are the Mean ± SE)

	A	B	C
Ti	53.7 ± 11.0	49.8 ± 14.5	82.0 ± 5.8
Matx	42.2 ± 13.7	45.9 ± 18.2	75.2 ± 12.7
Cmorph	44.2 ± 13.6	48.1 ± 16.3	76.3 ± 9.1
Cclus	28.4 ± 13.5	25.3 ± 9.6	64.6 ± 12.6
SurfA	68.7 ± 10.4	53.0 ± 13.5	88.3 ± 3.9
Basl	40.8 ± 7.9	53.6 ± 12.5	73.1 ± 8.6
Ftide	25.6 ± 8.8	37.0 ± 9.7	69.4 ± 9.8
SubBA	43.9 ± 10.7	52.1 ± 15.4	80.1 ± 4.7
Infl	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
AbnCO	65.9 ± 12.0	79.8 ± 6.8	85.7 ± 6.31
Vasc	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
SurfSp	38.7 ± 11.9	42.3 ± 13.0	75.2 ± 6.9
MDzone	49.2 ± 14.3	57.3 ± 14.6	73.9 ± 12.4
Total	701.2 ± 116.5	744.2 ± 97.6	1043.8 ± 60.4

The maximum score is 100 points and the minimum score is 0 points for each item. The total score range is from 0 (no repair) to 1300 (normal articular cartilage). The results were as follows group (A): 701.2 ± 116.5; group (B): 744.2 ± 97.6; group (C): 1043.8 ± 60.4. Group (C) exhibited higher Ti, Matx, Cclus, SurfA, Basl, Ftide, SubBA, SurfSp, and MDzone than groups (A) and (B). Groups (A) and (B) did not have major differences.

in group B, and 1043 ± 60.4 in group C (Fig. 7). The differences were significant between groups A and C, and between groups B and C. For the individual ICRS II items assessed, Matx, Cmorph, and Ftide differed significantly between groups C and A, and between groups B and C. The values for all ICRS II items excluding Infl and Vasc were higher in group C than in groups A and B. For the individual ICRS II items assessed, groups A and B did not have major differences (Table II).

In the groups sacrificed at 12 weeks after surgery, the ICRS II score was 740.9 ± 79.8 in group A, 809.9 ± 68.9 in group B, and 1059.5 ± 58.2 in group C (Fig. 7). The difference was significant between groups A and C, and between groups B and C. For the individual ICRS II items assessed, Ti, Matx, Cmorph, and Ftide differed significantly between groups C and A, and between groups C and B. The values for all ICRS II items excluding Infl and Vasc were higher in group C than in groups A and B. For the individual ICRS II items assessed, groups A and B did not have major differences (Table III). In group A, no significant differences were observed for total ICRS II scores between 4 and 12 weeks after surgery. Similarly, no significant differences were observed between 4 and 12 weeks for either groups B or C.

DISCUSSION

Several *in vitro* studies of carriers for the sustained release of TGF-β have been reported.[45–47] This study is the first to perform *in vitro* experiments to assess collagen vitrigel as a sustained-release carrier for TGF-β1. Measurement of the duration and quantity of TGF-β1 released showed a sustained release of 0.37 ng over the first 2 days after impregnation, which accounted for 40% of the total quantity released over the 14 days of measurement. Thereafter, the quantity released decreased gradually. During the second week following incorporation, sustained release of ~0.2 ng was observed, which confirmed the sustained, slow release of TGF-β1. Nicoll et al.[45] reported a sustained release of ~0.5% TGF-β1 injected into a gel during the first week. We measured the sustained release of ~3% of the injected TGF-β1 during the first week after incorporation. This confirmed the sustained release of TGF-β from the collagen vitrigel.

Research on the mechanism of action of TGF-β in cultures of chondrocytes derived from mesenchymal stem cells has shown that 10 ng/mL is the optimum concentration of TGF-β during culture and that 1 week is the optimum duration of action after administration.[19,22,38–42] Continued sustained release of a large quantity of TGF-β inside a joint should be prevented because this can increase the formation of cartilaginous tissue.[48,49] Thus, it is believed that both the environment and carrier must allow for early sustained release of an appropriate quantity of TGF-β to allow it to act effectively on the cartilage defect. In this regard, because of the confirmed sustained-release duration of 2 weeks, we feel that the collagen vitrigel satisfies this requirement. Analysis of the affected parts of rabbits sacrificed at 4 weeks after surgery also showed that no collagen vitrigel remained; therefore, the collagen vitrigel is believed to have been absorbed within 4 weeks.

The weight distribution ratio between the affected and unaffected limbs was measured as an indication of postoperative pain. This ratio improved at 12 weeks after surgery and did not differ significantly between groups. However, group A required 6 weeks to reach maximum improvement, whereas groups B and C showed near-maximum improvement at 4 and 2 weeks after surgery, respectively. Ito et al.[15] used a similar method to assess the weight

TABLE III. ICRS Histological Grading System Results at 12 Weeks (Values are the Mean ± SE)

	A	B	C
Ti	52.6 ± 10.6	63.6 ± 9.6	91.4 ± 2.4
Matx	35.2 ± 11.9	39.9 ± 10.3	85.0 ± 7.5
Cmorph	38.9 ± 13.6	37.1 ± 9.6	79.6 ± 7.9
Cclus	24.3 ± 10.5	24.5 ± 6.0	70.7 ± 12.9
SurfA	67.1 ± 6.1	63.8 ± 13.8	82.3 ± 4.3
Basl	58.8 ± 12.3	72.8 ± 7.0	73.2 ± 5.9
Ftide	30.3 ± 8.2	47.6 ± 8.0	71.0 ± 7.4
SubBA	65.1 ± 12.3	70.1 ± 7.9	70.0 ± 6.8
Infl	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
AbnCO	69.8 ± 11.0	78.3 ± 6.14	80.8 ± 6.3
Vasc	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
SurfSp	54.4 ± 8.8	55.6 ± 11.8	75.1 ± 6.5
MDzone	44.3 ± 11.7	56.7 ± 9.9	80.6 ± 7.9
Total	740.9 ± 79.8	809.9 ± 68.9	1059.5 ± 58.2

The maximum score is 100 points and the minimum score is 0 points for each item. The total score range is from 0 (no repair) to 1300 (normal articular cartilage). The results were as follows group (A): 740.9 ± 79.8; group (B): 809.9 ± 68.9; group (C): 1059.5 ± 58.2. Group (C) exhibited higher Ti, Matx, Cclus, Ftide, SurfSp, and MDzone than groups (A) and (B). Groups (A) and (B) did not have major differences.

distribution ratio in their research on the repair of articular cartilage defects using cell sheets and reported changes up to 4 weeks. Ito et al.[15] reported that the weight distribution ratio improved from 35.6% ± 1.1% to 49.6% ± 0.1% in the first 4 weeks in articular cartilage treated with cartilage cell sheets. Group C in our study exhibited improvement by 3 weeks after surgery similar to that in the animals in the study by Ito et al.[15] However, we found no improvement in the weight distribution ratio after this point. We believe that the cell sheets provide sustained release of various cytokines in addition to TGF-β1 and that these also contribute to articular cartilage repair.[17] Regardless of whether other cytokines are involved, the sustained release of TGF-β1 alone seems to be related to the similar improvements in early pain assessment in group C in this study and the cell sheet-treated group in the study by Ito et al.

The histological assessment showed that group C exhibited significant improvement relative to groups A and B at 4 and 12 weeks after surgery. No significant differences were observed between 4 and 12 weeks within each group. These results suggest that the early, sustained, slow release of TGF-β1 into the cartilage defect contributed to improved tissue repair starting immediately after surgery. For the items of the ICRS II score, improvements were observed in many items, and these improvements are considered to be a characteristic effect of TGF-β1. We confirmed that subchondral bone intruded into the repair cartilage area with some samples of the group for 12 weeks. We believe that subchondral bone intruded into the space that was not filled because of slow or poor repair. In a study in which liposomal TGF-β1 was injected to treat articular cartilage defects, Abe et al.[20] found that the extent of improvement did not differ between the group injected with TGF-β1 alone and the control group but that the tissue was repaired with fibrous cartilage tissue. In our study, we observed that the repair tissue contained hyaline cartilage, which suggests that the sustained, slow release of TGF-β1 stimulated regeneration by a better quality of cartilage. By contrast, in a study using a hydrogel scaffold and TGF-β used to treat articular cartilage defects, Holland et al.[46] reported that the sustained, slow release of TGF-β alone failed to produce a more favorable improvement in tissue compared with an untreated group. The possible reasons for the different findings include differences in the environment between *in vivo* and *in vitro* experimentation or differences in the defect position and size. Collagen vitrigel is a carrier with good tissue-engraftment capacity and whose implantation has been reported to induce tissue repair.[32,33,35,36] This implies that, compared with other carriers, the sustained, slow release of TGF-β into articular cartilage produces more effective results.

The ICRS II score did not show significant differences between groups A and B for the items. And there were no significant differences in the total score between these groups, which suggests that there was no effective cartilage repair in the vitrigel-only-treated group (group B).

The present study suggests that sustained, slow release of TGF-β1 over 2 weeks contributes to early improvement in pain and tissue repair of articular cartilage. The results also confirm that collagen vitrigel is useful as a sustained-release carrier. However, although we identified areas where collagen vitrigel improved the quality of the repaired cartilage tissue, treatment with collagen vitrigel alone did not produce the same effects as those of the TGF-β-incorporated collagen vitrigel. Our study confirmed that sustained, slow release of TGF-β1 occurred for 2 weeks and that 40% of the total quantity was released in the first 2 days; however, the mean sustained release could not be confirmed. The quantity of TGF-β1 released (0.91 ng) was also less than the intended 10 ng. Further improvement to the carrier to achieve the sustained release of a greater quantity of TGF-β1 into articular cartilage defects is a future challenge.

Further evaluation of the repair tissue is needed before this technology can be applied to humans. Collagen vitrigel offers the advantages of easy manufacturing and handling of various sizes. In this study, we confirmed that collagen vitrigel was effective as a controlled-release carrier in a rabbit osteochondral defect model. We plan to evaluate further the duration of the controlled-release effect and optimum quantity of TGF-β incorporated into the gel using both *in vitro* and larger animal studies.

CONCLUSION

Collagen vitrigel is an effective carrier for the sustained release of TGF-β1. Implantation of TGF-β1-incorporated collagen vitrigel increased the early mitigation of pain and articular cartilage repair.

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