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(Degree)
博士（医学）

(Date of Degree)
2008-03-25

(Date of Publication)
2012-03-13

(Resource Type)
doctoral thesis

(Report Number)
甲4323

TISSUE ENGINEERING
Volume 13, Number 5, 2007

DOI: 10.1089/ten.2006.0193

The Regenerative Effects of Platelet-Rich Plasma on Meniscal Cells *In Vitro* and Its *In Vivo* Application with Biodegradable Gelatin Hydrogel

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ABSTRACT

The objective of the study was to test the hypothesis that platelet-rich plasma (PRP) enhances meniscal tissue regeneration *in vitro* and *in vivo*. In the *in vitro* study, monolayer meniscal cell cultures were prepared, and 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt assay and 5-bromo-2′-deoxyuridine assay were performed to assess proliferative behavior in the presence of PRP. Alcian blue assay was performed to assess extracellular matrix (ECM) synthesis. To detect the fibrocartilage-related messenger ribonucleic acid (mRNA) expressions, real-time polymerase chain reaction was performed. In the *in vivo* study, 1.5-mm-diameter full-thickness defects were created in the avascular region of rabbit meniscus. Gelatin hydrogel (GH) was used as the drug delivery system for PRP growth factors. The defects were filled as follows: Group A, GH with PRP; Group B, GH with platelet-poor plasma; Group C, GH only. Each group was evaluated histologically at 4, 8, and 12 weeks after surgery. PRP stimulated deoxyribonucleic acid synthesis and ECM synthesis ($p < 0.05$). Meniscal cells cultured with PRP showed greater mRNA expression of biglycan and decorin ($p < 0.05$). Histological findings showed that remnants of gelatin hydrogels existed at 4 weeks, indicating that the hydrogels could control release for approximately 4 weeks. Histological scoring of the defect sites at 12 weeks revealed significantly better meniscal repair in animals that received PRP with GH than in the other two groups. These findings suggest that PRP enhances the healing of meniscal defects.

INTRODUCTION

THE MENISCI are wedge-shaped semi-lunar discs that play an important role in knee function.[1–8] Arthroscopic partial meniscectomy has been widely advocated for the treatment of meniscal injury, although biomechanical changes after meniscal injury were unavoidable.[9] Maintenance of the meniscus is important in the prevention of accelerated degeneration of the knee joint.

Because menisci tears located in the inner avascular part of the meniscus do not heal spontaneously, clinical repair techniques such as meniscal repair, rasping, and debridement[10,11] have been attempted to increase the healing potential of the meniscus.

Several growth factors, especially platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β), have been proven to be effective for meniscal tissue regeneration.[12–17] PDGF is considered to be one of most

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potent factors for mitogenic response of meniscal cells in monolayer and explant culture.[12–14] However, the cost of almost all genetically engineered growth factors is prohibitively high, and only some growth factors, such as basic fibroblast growth factor, hepatocyte growth factor, and insulin-like growth factor-1 (IGF-1) are available for clinical use.[18]

When the clinical application of growth factors was considered, platelet-rich plasma (PRP) provided one of the most attractive sources for growth factors. PRP could be easily prepared from a patient's own peripheral blood using a number of simple centrifugation steps and contained rich growth factors, such as PDGF and TGF-β. As an autologous source of growth factors, PRP has been investigated and proven to be useful for bone regeneration.[19–30] Thus, if PRP growth factors were also effective for meniscal tissue, PRP could be an attractive clinical source for meniscal tissue regeneration.

For the *in vivo* application of PRP, we used acidic gelatin hydrogels as a source of delivery for PRP growth factors. Gelatin is a biodegradable material that has been extensively used for pharmaceutical and medical purposes and has been proven to be biosafe throughout its long history of clinical applications. It was found that gelatin could control the release of PRP growth factors, such as TGF-β and PDGF, for an average of approximately 2 weeks in an *in vivo* environment.[31,32] Hokugo *et al.* previously reported that gelatin could effectively release growth factors containing PRP.[31]

Thus the purpose of this study was to investigate the effectiveness of PRP for the promotion of meniscal tissue regeneration. In our *in vitro* study, we first showed that PRP promoted meniscal cell proliferation and extracellular matrix (ECM) synthesis. We also examined the regenerative effects of PRP on meniscal tissues with rabbit meniscal defect *in vivo*.

MATERIALS AND METHODS

Collection and preparation of PRP (rabbit)

Blood samples ($n = 4$ in each group) were obtained from Japanese white rabbits (Kitayama Labs, Nagano, Japan), weighing 2.8 ± 3.2 kg. The Animal Research Committee of Kobe University Graduate school of Medicine approved all procedures. PRP was prepared using 2 centrifugation techniques, as previously reported.[29,30] Briefly, rabbits were anesthetized using intravenous injection of 2.0 to 2.4 mL Pentobarbital sodium solution at a dose of 30 mg/kg body weight. Sixteen mL of whole blood was drawn from each subject into tubes containing 4 mL of acid citrate dextrose-A solution as an anticoagulant and equally separated into 2 sterile tubes. An aliquot was removed from each tube to determine the platelet count. The tubes were then spun in a laboratory centrifugation apparatus (6800; Kubota, Tokyo, Japan) at 4°C for 15 min at 800 rpm, and all plasma was transferred to 2 new sterile tubes to be further centrifuged at 4°C for 10 min at 2000 rpm. The supernatant plasma was discarded and the remaining approximately 0.8 mL of plasma and precipitated platelet was designated PRP. Platelet-poor plasma (PPP) was collected using a further centrifugation step (at 4°C for 10 min at 3000 rpm) in the remaining red cell fraction after the first centrifugation. Platelet counts were also performed for samples of PRP and PPP. Samples were thawed and stored at −80°C until used.

Enzyme-linked immunosorbent assay analysis of growth factors in PRP and PPP

To measure the concentration of growth factors secreted from PRP and PPP, enzyme-linked immunosorbent assay (ELISA) was performed ($n = 7$ in each group). Briefly, the PRP and PPP samples were thawed and centrifuged at 4°C for 10 min at 3000 rpm to remove platelet membranes, and then the supernatants were used. Commercial human PDGF, TGF-β1, and vascular endothelial growth factor (VEGF) ELISA kits (R&D Systems, Minneapolis, MN) were used according to the manufacturer's instructions. All samples for TGF-β1 analysis were acid activated with 1 N hydrochloric acid.

In vitro *study*

Tissue and culture preparation. In this study, meniscal tissue was prepared from skeletally mature female Japanese white rabbits (Kitayama Labs), weighing 2.8 ± 3.2 kg. The rabbits were euthanized with an overdose of pentobarbital sodium solution injected intravenously. Soon after sacrifice, the knee joint was opened, and the bilateral meniscus were harvested and carefully dissected free from the adherent synovium and the capsule. Only the inner two-thirds, avascular zone of the meniscus was used. The pieces were diced manually into smaller pieces and digested using 0.2% collagenase (Sigma, St. Louis, MO) for 1 h at 37°C, followed by a subsequent digestion in 0.05% trypsin for 30 min at 37°C to release the cells from the ECM. The digests were filtered to remove any undigested material using cell strainers with a pore size of 45 μm and then centrifuged and washed 2 times with phosphate-buffered saline (PBS). The pellets of the cells were resuspended and cultured in 75-cm^2 culture flasks containing Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10% FBS, penicillin G (100 U/mL), and streptomycin (100 μg/mL). The cultures were maintained at 37°C in a humidified 5% carbon dioxide (CO_2) atmosphere. The cells reached confluence at an average of 3 weeks.

Cell viability: 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt assay. Meniscal cell viability was assayed using the 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay technique (CellTilter 96 Aqueous; Promega, Madison, WI) ($n = 5$

in each group).[33,34] Cells were trypsinized and seeded at a density of approximately 5000 cells/well in 96-well cell culture plates in 100 μL of culture medium with 10% FBS. After 24 h, the medium was refreshed with the culture medium using 1% FBS containing growth factors, PRP, and PPP at the indicated concentrations (3%, 10%, 30%). After 48 h, the medium was removed and washed with PBS, then refreshed with fresh medium containing MTS reagent (100 μL medium with 1% FBS plus 20 μL MTS reagent/well). The optical density was measured at 490 nm using an automatic microplate reader after 1.5 h of further incubation at 37°C in a humidified atmosphere of 5% CO_2. The percentage viability of each well was calculated.

Cell proliferation ELISA and 5-bromo-2′-deoxyuridine assay. Meniscal cell proliferation was determined using a colorimetric immunoassay, based on the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporated in newly synthesized cellular deoxyribonucleic acid (DNA) ($n = 5$ in each group).[35,36] The cells were seeded, and the medium was refreshed using the same method as MTS assay for 48 h, after which BrdU was added to a final concentration of 10 μM. After incubation for 4 h, DNA synthesis was assayed using the Cell Proliferation ELISA, BrdU kit (Roche Molecular Biochemicals, Penzberg, Germany) according to the manufacturer's instructions.

Assessment of ECM formation. Quantitative dye-binding assays and Alcian blue-binding assays were performed to measure the amounts of ECM and sulphated glycosaminoglycans (sGAGs) in the culture medium ($n = 4$ in each group).[37] There was no interference from proteins or nucleic acids using this method, and this assay could detect sGAGs in blood samples.[37] Briefly, after seeding the meniscal cells at a density of approximately 10,000 cells/well in 96-well cell culture plates, the medium was refreshed the next day with a culture medium with 1% FBS containing 10% PRP. On days 2, 4, 6, and 8, the supernatants were collected and replaced with a new culture medium containing 10% PRP. The gathered supernatants were assayed using Alcian blue-binding assays (Wieslab AB, Ideon, Sweden) according to the manufacturer's instructions. Culture medium containing 10% PPP was also collected and assayed.

Gene expression analysis. To investigate the transcriptional expression of meniscal cell-related genes, we used real-time quantitative reverse transcriptase polymerase chain reaction (PCR) ($n = 5$ in each group) according to the manufacturer's instructions (Takara Bio, Shiga, Japan). Briefly, after culturing meniscal cells for 48 h, total ribonucleic acid (RNA) was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription of RNA into complementary DNA (cDNA) was performed by incubating approximately 1 μg of RNA using oligo (dT) primer, deoxyribonucleotide triphosphate, 10×PCR buffer, magnesium chloride, RNase inhibitor, and Mulv Reverse Transcriptase (all from Applied Biosystems, Foster City, CA). The converted cDNA (2 μL) samples were amplified in triplicate using real-time PCR (ABI PRISM 7700) in a final volume of 50 μL using SYBR Green Master Mix reagent at a final concentration of 1×(Applied Biosystems). The primer pairs of rabbit-specific genes were obtained using a previously reported method[38] and were designed by the manufacturer (Takara Bio) according to the published sequences available in GenBank (Table 1). Levels of transcripts for 18S ribosomal RNA proved to be stable and were therefore elected as a reference gene. Melting curve analysis was performed to ensure that only a single product was amplified using Dissociation Curves software (Applied Biosystems). Specificity of the reactions was confirmed using 2.5% agarose gel electrophoresis. Results were obtained using sequence detection software (ABI Prism 7700) and evaluated using Microsoft Excel (Microsoft Corp., Redmond, WA).

In vivo *study*

Preparation of gelatin hydrogels incorporating PRP. Gelatin hydrogels were prepared using chemical cross-linking

TABLE 1.

Gene	*Sequences*	*GeneBank accession no.*
Col 1	Sense: 5′-ATGGATGAGGAAACTGGCAACT-3′ Antisense:5′-GCCATCGACAAGAACAGTGTAAGT-3′	D49399
Aggrecan	Sense: 5′-TCTACCGCTGTGAGGTGATGC-3′ Antisense: 5′-TTCACCACGACCTCCAAGG-3′	L38480
Biglycan	Sense: 5′-AGGATCTGCTCCGATACTCCAA-3′ Antisense: 5′-CAGGCTCCCGTTCTCAATCA-3′	AF020290
Decorin	Sense: 5′-CTGGACAAAGTGCCCAAGGA-3′ Antisense: 5′-TGACGAGGATCAATGCGTGAA-3′	U03394
18S	Sense: 5′-CGGACACGGACAGGATTGAC-3′ Antisense: 5′-CCAGACAAATCGCTCCACCA-3′	X06778

of aqueous gelatin solution with glutaraldehyde (GA; Wako Pure Chemical Industries, Osaka, Japan) according to a previously reported method.[31,32,39–41] The hydrogels were designed to biodegrade some of the individual growth factors for an average of approximately 2 weeks in *in vivo* conditions.[32,42] Briefly, gelatin with an isoelectric point of 5.0 (Nitta Gelatin, Osaka, Japan) was prepared as an acidic gelatin. After mixing 400 μL of aqueous GA (Wako Pure Chemical Industries) solution (25 wt%) with 80 mL of aqueous gelatin solution (5 wt%) preheated at 40°C, the mixed aqueous solution was cast into balance dishes and left for 12 h at 4°C to allow for chemical cross-linking of gelatin. The resulting hydrogel sheets were placed in 100 mM glycine aqueous solution and then agitated at 37°C for 1 h to block the residual aldehyde groups of unreacted GA. The cross-linking hydrogel sheets were thoroughly washed with double-distilled water, freeze-dried, and sterilized with ethylene oxide gas. The water content of gelatin hydrogel was 95 wt% when calculated from the hydrogel weight in the wet and dry state. The gelatin hydrogel sheets were cut into small squares (2×2 mm).

PRP (30 μL) was added by drops onto the freeze-dried gelatin hydrogel squares and left for 1 h at 37°C to allow it to impregnate the hydrogel. Similarly, empty gelatin hydrogel and gelatin hydrogel incorporating PPP were prepared as controls, in addition to using PPP and PBS.

Animal experiments. Eighteen skeletally mature female Japanese white rabbits (Kitayama Labs) with a mean weight of 3.1 kg (2.8 ± 3.3 kg) were used in this study. Surgical procedures were performed according to a previously reported method.[43–45] Briefly, after general anesthesia was administered, the rabbits were placed in the supine position and surgeries were performed on the bilateral knees. In each rabbit, the limbs were disinfected, and 5 mL of 1% lidocaine was injected subcutaneously, where the incision was to be made. A medial parapatellar approach was used to expose the knee joint, and the patella was everted. The knee was flexed to the maximum, and a 1.5-mm-diameter full-thickness circular defect was produced in the anterior portion of the inner two-thirds avascular zone of the medial meniscus using a biopsy punch (Kai Medical, Gifu, Japan). The defects were divided into 3 groups according to treatment as follows: Group A, defects were filled with gelatin hydrogel with PRP; Group B, defects were filled with gelatin hydrogel with PPP; Group C, defects were filled with gelatin hydrogel with PBS. Joint capsule and skin were sutured as separate layers in all groups. After surgery, all rabbits were returned to their cages and allowed to move freely without joint immobilization. Four, 8, and 12 weeks postoperatively, rabbits were euthanized with an intravenous injection of a fatal dose of pentobarbital sodium, and the operated meniscus was taken from the knee joint and prepared for histological evaluation ($n = 4$ in each group and at each time point).

Histological examination. The specimens were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer solution for 4 h and embedded in paraffin wax. Each specimen was cut into slices, 7 μm thick, along the radial plane. For histological analysis, the section was stained with hematoxylin and eosin staining and Safranin-O fast green staining. The reparative tissue was evaluated using original semi-quantitative scoring (Table 2) using three different observers blind to treatment. Assessment points were divided into 3 as follows: 1) reparative tissue with bonding, assessing whether the reparative tissues had bonded with the surrounded normal meniscus tissues; 2) existence of fibrochondrocytes, and 3) stainability with Safranin-O. The total attainable score was 6 points, 2 for each category. The points were calculated and evaluated statistically and histologically for each specimen.

Statistical analysis

Data obtained were statistically analyzed using analysis of variance. A value of $p < 0.05$ was regarded as statistically significant.

RESULTS

In vitro *study*

Assessment of the prepared PRP. The mean number of counted thrombocytes in the peripheral blood was 21.33 $\times 10^4$/μL. The mean PRP platelet count was 104.45$\times 10^4$/μL. The mean PPP platelet count was 1.38$\times 10^4$/μL. The concentration of thrombocytes in PRP increased 4.89-fold according to the mechanical count ($p < 0.005$).

Analysis of growth factor concentrations in PRP samples and PPP samples using ELISA revealed that levels of growth factors were significantly higher in PRP samples (Table 3A).

TABLE 2.

1. Reparative tissues with bonding
   - 2 points: Bilaterally bonds with surrounding meniscus.
   - 1 point: Partially bonds with surrounding meniscus.
   - 0 points: No bond with surrounding meniscus.
2. Existence of fibrochondrocytes
   - 2 points: Fibrochondrocytes exist diffusely in the reparative tissues.
   - 1 point: Fibrochondrocytes are localized in the reparative tissues.
   - 0 points: No fibrochondrocytes in the reparative tissues.
3. Staining with Safranin-O
   - 2 points: Densely stained with safranin-O.
   - 1 point: Faintly stained with safranin-O.
   - 0 points: Not stained with safranin-O.

Effects of PRP on meniscal cell proliferation and sGAG synthesis. MTS assay was used as a measurement of cell viability. MTS assay revealed that PRP upregulated the viability of meniscal cells in a dose-dependent manner. PPP slightly activated meniscal cell viability, although the effect of PPP was limited, and downregulation of meniscal cell viability was observed when 30% PPP was added. The effect of PRP was significantly greater than that of PPP (Fig. 1). Comparable results for differences in proliferation behavior, expressed as the amount of newly synthesized DNA, are shown in Table 3B. BrdU assay revealed that there was more DNA synthesis in meniscal cells in the presence of PRP than in the presence of PPP or in the control (supplement-free medium). These results indicate that PRP promotes meniscal cell proliferation.

Quantification of the sGAG synthesized by meniscal cells in medium after 8 days of culture was determined to evaluate the cellular function using Alcian blue-binding assay. As shown in Table 3C, the density of sGAG in the mixed gathered mediums was significantly higher in the presence of PRP than in supplement-free medium (control) or PPP ($p < 0.01$). These results indicate that PRP promotes sGAG synthesis in meniscal cells.

Effects of PRP on gene expression of meniscal cell-related proteins. The results of meniscal cell-related messenger ribonucleic acid (mRNA) expressions after culture with 10% PRP, 10% PPP, and control medium are shown in Table 3D. All results are reported as relative mRNA expression in the control. First we examined the effect of PRP on the gene expression level of collagen 1. No significant change was found in the expression of collagen 1 mRNA in each sample. Next we examined the effect of PRP on the gene expression level of proteoglycans (PGs). Aggrecan mRNA expression fell approximately twice as much in the PRP samples as in the control samples ($p < 0.05$). There

Table 3. *In Vitro* Data

(a) ELISA

	PRP	*PPP*
PDGF-BB (ng/mL)	*3.23 ± 1.50	0.12 ± 0.06
TGF-β1 (ng/mL)	**78.41 ± 52.71	4.66 ± 3.67
VEGF (pg/mL)	**138.79 ± 53.79	19.44 ± 2.35

Values are expressed as the mean ± standard deviation.
*$p < 0.001$ compared with PPP group.
**$p < 0.005$ compared with PPP group.

(b) BrdU assay

	Control	*PRP*	*PPP*
absorbance (450–690 nm)	0.14 ± 0.01	*1.00 ± 0.02	0.30 ± 0.07

Values are expressed as the mean ± standard deviation.
*$p < 0.05$ compared with control and PPP groups.

(c) Alcian blue-binding assay

	Control	*PRP*	*PPP*
sGAG synthesis (μg/mL)	3.63 ± 0.50	*10.32 ± 0.96	8.11 ± 1.71

Values are expressed as the mean ± standard deviation.
*$p < 0.01$ compared with control and PPP groups.

(d) Real-time PCR (Relative mRNA expression in the control)

	PRP	*PPP*
Col 1	1.26 ± 0.90	1.38 ± 1.13
Aggrecan	+0.52 ± 0.22	1.28 ± 0.77
Biglycan	*2.10 ± 1.00	1.45 ± 0.88
Decorin	*4.71 ± 2.31	1.45 ± 0.49

Values are expressed as the mean ± standard deviation.
+Down regulation ($p < 0.05$) compared with control and PPP groups.
**Up regulation ($p < 0.05$) compared with control and PPP groups.

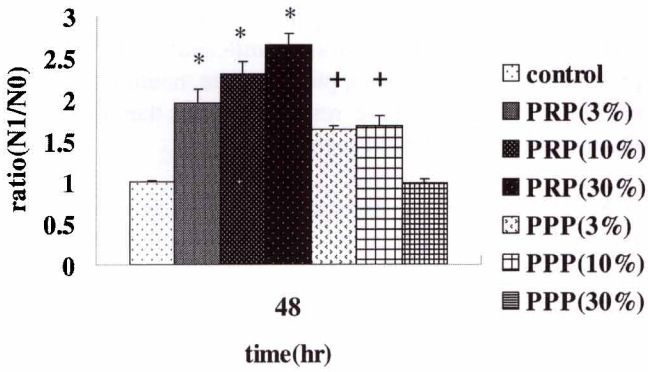

FIG. 1. 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium inner salt assay measurement to evaluate cell viability. Platelet-rich plasma (PRP) upregulates the viability of meniscal cells in a dose-dependent manner ($*p < 0.05$). The effect of PRP is significant compared with the same dose of platelet-poor plasma ($^{+}p < 0.05$).

was no significant change in aggrecan mRNA expression between PPP and control. Measurements of mRNA expression of small PGs were also performed. Biglycan mRNA expression was more than twice as high in the PRP samples as in control samples ($p < 0.05$) and a fold increase in decorin mRNA expression was more than four times as high ($p < 0.05$). Furthermore, mRNA expression of both small PGs were upregulated significantly more in PRP samples than in samples ($p < 0.05$).

In vivo *study*

All the rabbits were moving freely in their cages by the second postoperative day. No evidence of postoperative infection at the wound site was observed, and all the wounds healed uneventfully. In the synovial tissue, slight hypertrophy was observed in the knees of some animals. No clear degeneration of articular cartilage of the femur or tibia was found.

Histological findings at 4 and 12 weeks are shown in Figures 2A–F and 3A–F. The results of semi-quantitative scoring are shown in Figure 4. Reparative tissue bonding scores over time in group A (treated with PRP) and group B (treated with PPP) are shown. In group C (treated with hydrogel only), the scores were almost the same each week. At every time point, the group A scores were higher than those in the other two groups, but these results were not statistically significant, except for the difference in results between groups A and B at 4 weeks.

The reparative tissues of groups B and C were mostly occupied with fibrous connective tissue and spindle-shaped fibroblast-like cells at every time point. At 8 weeks, some samples in group B contained a few oval-shaped cells, although the cells were localized in their sections, and the number had not increased much by 12 weeks. In group A, some oval-shaped cells could be found as early as 4 weeks.

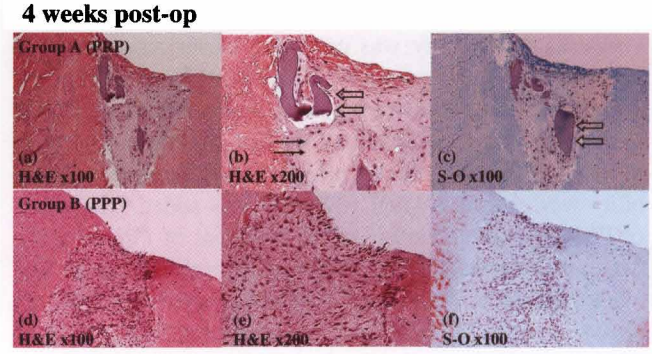

FIG. 2. Photomicrographs of the reparative tissues at 4 weeks. Sections of (**A–C**) are treated with platelet-rich plasma, Group A. Sections of (**D–F**) are treated with platelet-poor plasma, group B. **B** and **D** are the sections stained with hematoxylin and eosin (H–E) (magnification ×100), and **B** and **E** are the same staining with high magnification (×200). **C** and **F** are the sections stained with Safranin-O (S-O) (×100). In group A (**B**), the group A defects contained oval-shaped cells and relatively rich extracellular matrix components (black arrow), which were faintly stained with safranin-O. (**C**) Gelatin hydrogels remained in the defects at 4 weeks (open arrow). Group B defects are filled with Safranin-O negative fibrous tissue (**D–F**).

The scores of group A increased in a time-dependent manner, and at 12 weeks, many oval-shaped chondrocyte-like cells with unevenly spaced lacunae were diffusely present in the reparative tissues in almost all group A samples. The morphology resembled the inner zone of the meniscus. The group A score was statistically higher than the other 2 groups at 12 weeks.

The Safranin-O–positive ECM in the reparative tissues were examined and scored. In groups B and C, the reparative

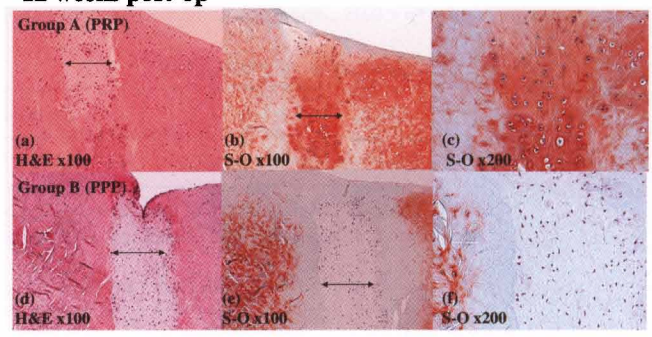

FIG. 3. Photomicrographs of the reparative tissues (arrow) at 12 weeks. Sections of **A–C** are group A and sections of **D–F** are group B. **A** and **D** are the sections stained with hematoxylin and eosin (H-E). (×100). **B** and **E** are the sections stained with Safranin-O (S-O) (×100), and **C** and **F** are the same staining with high magnification (×200). In group A, (**B, C**) the reparative tissues consist of Safranin-O–positive matrix with unevenly spaced lacunae. In contrast, group B defects are filled with Safranin-O negative fibrous tissue (**E, F**).

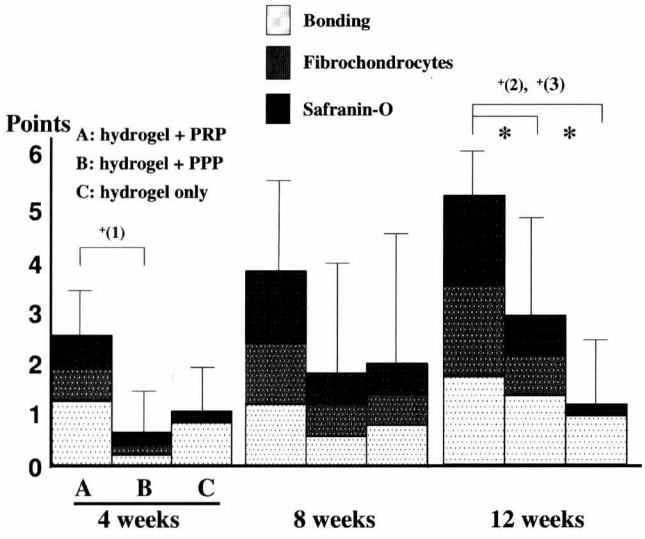

FIG. 4. The results of semi-quantitative scoring *in vivo*. Each average score is presented. Group A total score was significantly higher than that of the other 2 groups at 12 weeks ($*p < 0.05$). $^{+}$(1) Reparative tissue bonding score in group A was significantly higher than in group B at 4 weeks. $^{+}$(2) Scores showing the existence of fibrochondrocytes in group A were statistically higher than in the other 2 groups at 12 weeks. $^{+}$(3) The scores of Safranin-O (S-O) staining in group A were significantly higher than in the other 2 groups at 12 weeks.

tissues looked fibrous with hematoxylin and eosin staining, and the tissues showed almost no staining with Safranin-O at any time point. In group A, although a relatively abundant ECM with oval-shaped cells existed at 4 weeks, the matrix was not stained with safranin-O. However, the group A score increased in a time-dependent manner, and at 12 weeks, the group A filled tissues were well stained with Safranin-O in almost all samples. The group A score was also statistically higher than the other 2 groups at 12 weeks.

There were some samples showing remnants of the hydrogel in every group at 4 weeks, although the remnants had disappeared by 8 weeks.

DISCUSSION

The menisci play important roles in knee function, including loading, shock absorption, proprioception, maintenance of joint stability, and lubrication.[1–8] Injury to the inner avascular part of the meniscus does not heal spontaneously; therefore, arthroscopic resection of the meniscus has been widely performed. However, repair of the inner avascular part of the meniscus is challenging for orthopedic surgeons.

In tissue-engineering approaches from cytokine therapy, several growth factors have been investigated and proven to be effective in meniscal tissue regeneration.[12–14,16] In the current study, we used PRP as an autologous growth factos, and the results showed the remarkable healing properties of PRP for the repair of the inner avascular part of meniscal injury *in vitro* and *in vivo*.

PRP was known as a concentrated suspension of growth factors, such as PDGF, TGF-β, VEGF, IGF-I, and endothelial cell growth factor.[20] These individual growth factors are well known to be effective for meniscal cells. For example, it has been demonstrated that the effect of TGF-β1 was dose-dependent, focusing on the PG synthesis of meniscal tissues,[15,16] and PDGF was considered to be one of most potent factors for mitogenic response in meniscal cells.[12,13,17] Because PRP was an autologous source, there was no risk of antibody formation or infection. Thus, platelets are a promising source of autologous growth factors, and if PRP growth factors could be effective for meniscal tissue, growth factor–promoted meniscal healing was anticipated.

In our study, PRP preparations concentrated over 1 million/μL platelets and therefore could be recognized as "therapeutic PRP."[20,30,31] Furthermore, the contained growth factors in our PRP preparations were also comparable with the PRP, prepared from commercial tube technique, further described as PRP1 (Curasan AG, Kleinostheim, Germany).[25–29] Thus, we consider our PRP preparations sufficient for investigating its effects on meniscal cells.

In the current *in vitro* study, we showed the effectiveness of PRP not only for its proliferating effect on meniscal cells, but also for synthesizing GAG. GAG was important for the meniscus, as well as hyaline cartilage, and the phenotype of the inner avascular region of the meniscus was fibrocartilage-like, and it was known that the cells isolated from this region produce more GAGs in culture than cells from the peripheral region.[15,46] In Table 3A, PPP also stimulated GAG synthesis more than control on meniscal cells; we supposed that it depended on a few platelets contained in PPP.

The results of real-time PCR study to assess the PRP were interesting. Aggrecan was a main, large aggregating PG in hyaline cartilage. Aggrecan in meniscus showed an organized, spatial network, in contrast to its diffuse distribution in articular cartilage, and contributed to the pool in the synovial fluid.[47] When hyaline cartilage regeneration was considered, the downregulation of aggrecan mRNA expression would not be desired. In fact, some reports have revealed that platelet supernatant activated chondrocyte proliferation but induced a dedifferentiation of chondrocytes toward fibroblast-like phenotypes in monolayer and 3-dimensional alginate cultures.[48,49] However, when considering meniscal tissue regeneration, it remained to be seen whether these changes in mRNA expression would lead to the fibrocartilage desired, for the following reasons. The native meniscus itself actually had a low PG content,[14] and the ratio of PG was different from hyaline cartilage, which was largely composed of aggrecan. Small PG (decorin and biglycan) and aggrecan have previously been

extracted in a weight ratio of approximately 1:1 in the adult human meniscus; thus, the amount of small PG was high.[50] Roughley *et al.* stated that the presence of aggrecan and small PG might be a common feature of the need to resist compressive and tensile forces in the meniscus.[50] As we saw, real-time PCR study in monolayer meniscal cells cultured with PRP revealed upregulations of small PG mRNA expressions. This result indicates that PRP induces the meniscal cells toward fibrocartilage-like phenotypes, suggesting that PRP is a more attractive and favorable source for fibrocartilage than for hyaline cartilage.

In general, the lives of many growth factors are too short to sustain biological activity *in vivo*. PRP growth factors were initiated and secreted from platelets through blood clotting, and more than 95% of the presynthesized growth factors were secreted within 1 h.[20] It was therefore likely that free PRP diffused into the knee joint rapidly and would result in no exertion of growth factor activity. Therefore, a single local application of PRP would be unlikely to achieve successful tissue regeneration.

One method that was needed for *in vivo* application was controlled release. Tabata *et al.* reported a controlled-release system composed of acidic gelatin with an isoelectric point of 5.0, which forms a poly-ionic complex with growth factors, and they demonstrated that the controlled release of growth factors from the hydrogel of acidic gelatin promoted bone regeneration in rabbit and monkey skull defects.[39,40,42] The growth factors were immobilized in the hydrogel through a physicochemical interaction with gelatin molecules, and the immobilized growth factors were released from the hydrogel as a result of hydrogel degradation.[32] As a source of sustained delivery of PRP growth factors, we also used acidic gelatin hydrogels.

In the current *in vivo* study, almost all the defects in group A (hydrogels with PRP) were healed with ECM-rich fibrocartilages at 12 weeks. We considered the healing process to occur as in Figure 5. First, it was the healing process called "intrinsic repair" (meniscal fibrochondrocytes activation).[51] In our *in vivo* study, round cells and rich ECM formations were seen as early as 4 weeks, and progress toward fibrocartilage occurred gradually. Furthermore, we have shown the effectiveness of PRP with rabbit meniscal cells *in vitro*. It was therefore possible that the surrounding meniscal cells played some part in the process of meniscal repair.

In addition to intrinsic repair, extrinsic repair (neovascularization and formation of granulation tissue) has also been reported to be important in meniscal regeneration.[51,52] In the early stages of our study, some samples in the histological analysis showed pannus-like tissues invading the surface of the meniscus through the synovium to the meniscal defect. It was possible that synovial cells (containing synovium-derived MSCs[53]) also played a part in the process of meniscal repair.

In some samples at 4 weeks, like Figure 2A–C, there were still some remnants of hydrogel in the meniscal defect. The

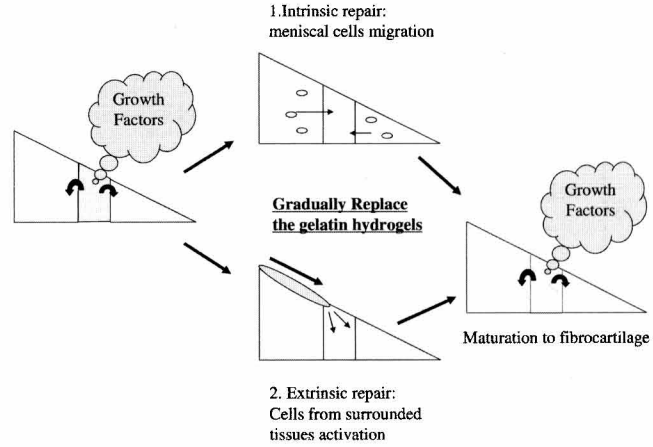

FIG. 5. The process of meniscal repair with hydrogels incorporating platelet-rich protein (PRP). When hydrogels incorporating PRP fill the defect, the growth factors are released as the hydrogels biodegrade. We believe that the growth factors activated 2 processes as follows. 1. Fibrochondrocytes derived from the surrounding meniscus were activated and migrated into the defect (intrinsic repair) (middle upper schema). 2. Synovium-derived MSCs or other multi-potent cells were activated and migrated into the defect through the surface of the surrounding meniscus (extrinsic repair) (middle lower schema) together. These cells migrated into the defects and gradually replaced the gelatin hydrogels. PRP growth factors further stimulated these migrated cells, which eventually directly matured to fibrocartilage (right side schema).

acidic hydrogels were designed to biodegrade some individual growth factors in an average of approximately 2 weeks under the *in vivo* conditions,[32,42] although the detailed degradation characteristics were unclear when various growth factors, like PRP, were added to the meniscal defect. We hypothesized that *in vivo* conditions might be different for different locations and different degradation characteristics could occur when rich growth factors, like PRP, were applied. In our histological findings, there were no remnants of the hydrogel after 8 weeks, so we were convinced that the acidic hydrogels were biodegraded and could control release of PRP growth factors for about 4 weeks in our *in vivo* study.

In conclusion, it appeared that PRP enhanced the healing properties of the inner avascular part of meniscal cells. It is likely that growth factors contained in PRP enhanced the biological activities of the meniscal cells for meniscal tissue regeneration. In the current *in vivo* application for rabbit meniscal defects, we found that the reparative fibrocartilaginous tissues closely resembled the inner portions of the normal meniscus, whereas borders between normal and reparative tissues existed histologically. Further investigation was needed to evaluate the biomechanical properties of this reparative tissue, although we believed that PRP was an effective biomaterial for meniscal tissue regeneration.

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