Ligament regeneration using an absorbable stent-shaped poly-L-lactic acid scaffold in a rabbit model

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Received: 12 July 2012 / Accepted: 29 August 2012 / Published online: 14 September 2012
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Abstract

Purpose Ligaments are frequently damaged in sports activities and trauma, and severe ligament injury can lead to joint instability and osteoarthritis. In this study, we aimed to regenerate the medial collateral ligament (MCL) using an absorbable stent-shaped poly-L-lactic acid (PLLA) scaffold in a rabbit model to examine the biocompatibility and mechanical properties.

Methods Twenty-three Japanese white rabbits were used in this study. MCL defects were surgically created in the knee joints and then reconstructed using stent-shaped PLLA scaffolds. As controls, flexor digitorum longus (FDL) tendons were implanted into the contralateral knees. Seven rabbits were sacrificed at three time points, conducted four, eight and 16 weeks after the operation. The regenerated tissues were histologically evaluated using fibre alignment scoring, morphology of fibroblast scoring and immunohistochemical analysis of types I and III collagen. The regenerated tissues were also biomechanically evaluated by measuring the ultimate failure load and stiffness.

Results At four weeks post-operation, spindle-shaped cells were observed on the inside of the scaffolds. At eight weeks, maturation of the regenerated tissues and collagen fibre alignment parallel to the ligaments was observed. At

16 weeks, the fibre alignment had become denser. The fibre alignment and morphology of fibroblast scores significantly increased in a time-dependent manner. Expression of type I collagen was more strongly observed in the scaffold group at eight and 16 weeks post-operation than at four weeks. Type III collagen was also observed at four, eight and 16 weeks post-operation. A thin layer of fibrocartilage was observed at the ligament-bone junction at eight and 16 weeks. The ultimate failure load of the scaffold group was 46.7 ± 20.7 N, 66.5 ± 11.0 N and 74.3 ± 11.5 N at four, eight and 16 weeks post-operation, respectively. There was no statistical difference between the normal MCL and the scaffold group at 16 weeks post-operation.

Conclusions The stent-shaped PLLA scaffold allowed for MCL regeneration with type I collagen expression and fibrocartilage formation and resulted in sufficient mechanical function.

Introduction

Ligaments are frequently damaged in sports activities and trauma. Severe injuries can lead to chronic instability of the joint and require surgical reconstruction of the ligament [1, 2]. While various procedures including reconstruction using autograft tissue [3], allograft tissue [4] and synthetic materials [5] have been reported for ligament reconstruction, these procedures have yet to be refined. For example, using autologous tissue for ligament reconstruction has been associated with donor site weakness and morbidity. Autologous tissue for ligament reconstruction can also result in an insufficient amount of tissue being available to allow for a second graft if re-injury occurs. Since the use of allogenic tissue increases the risk of disease transmission and an immunogenic response by the host, non-absorbable synthetic grafts have been used to reduce these disadvantages. However, non-absorbable synthetic grafts can cause bone

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erosion, which leads to joint instability and overall failure of the surgery [5, 6]. The outcome of anterior cruciate ligament (ACL) substitution using the Leeds–Keio polyester ligament at a mean of 13.3 years showed a high rate of rupture and joint laxity [7]. Non-absorbable synthetic grafts have shown excellent early results in stability; however, a large number of long-term ligament graft ruptures have been reported [8]. To overcome the disadvantages of using non-absorbable synthetic grafts, biologically absorbable grafts have been developed. However, absorbable scaffolds can cause adverse effects, including local inflammatory reactions [9, 10].

Poly-L-lactic acid (PLLA), an organic polymer of L-lactate that dissolves into H₂O and CO₂, is widely used as an orthopaedic synthetic material in ligament and tendon tissue engineering because of its biocompatibility and initial strength. Clinical studies have shown that a local inflammatory reaction to PLLA might arise because its degradation products are acidic [9] and that the slow degradation rate of PLLA fibres might inhibit host tissue maturation [10]. Therefore, we hypothesised that fewer synthetic fibres would reduce the inflammatory reaction and accelerate host tissue maturation, resulting in better ligament reconstruction. In this study, the PLLA scaffolds were designed in a stent shape and used as a synthetic graft in a rabbit ligament reconstruction model. The purpose of this study was to regenerate the medial collateral ligament (MCL) using an absorbable stent-shaped PLLA scaffold, which was examined for its biocompatibility and mechanical properties using a rabbit model.

Materials and methods

Scaffolds

A stent-shaped PLLA scaffold was fabricated from 0.2-mm diameter PLLA fibres, the ultimate failure load of which was 4.9 ± 0.7 N. Since the average ultimate failure load of a normal MCL in rabbit knees is 82.5 ± 2.0 N, 16 PLLA fibres were used for 1 scaffold. PLLA fibres were radially arranged in a round template (Hamanaka, Kyoto, Japan) at equal intervals. Using this template, the PLLA fibres were manually knitted into a stent-shaped scaffold. One end of the scaffold was looped, and the other end was bound and bonded by thermocompression (Fig. 1a). The knitted scaffold was 3.0 mm in diameter and 34.0 mm in overall length, with an ultimate failure load of 94.1 ± 10.7 N and stiffness of 13.9 ± 8.5 N/mm. Both ends of the scaffold were clamped directly. The interval of the clamped end was 25 mm. The scaffold was attached vertically to a tensile sensor (AG-I Shimadzu Co., Kyoto, Japan). Before the tensile test was conducted, the scaffold was preconditioned with a static preload of 0.5 N for five seconds, followed by ten cycles of loading and unloading at a strain amplitude of approximately

0.5 % at a rate of 20 mm/min [11]. Immediately after preconditioning, the ultimate load to failure was recorded in uniaxial tension at 20 mm/min. The load–deformation curve was recorded, from which the ultimate load at failure and the stiffness were measured. Each scaffold was sterilised using ethylene oxide gas before use in the animal experiments.

Surgical treatment of the medial collateral ligament defect in a rabbit model

All animal experiments were approved by the Institutional Animal Care and Use Committee of Kobe University. Twenty-three skeletally mature female Japanese white rabbits (Kitayama Labs, Nagano, Japan) were used in this study. Their mean age was 16.1 weeks (range 14–18 weeks), and their mean weight was 3.0 kg (range 2.6–3.5 kg). For general anaesthesia, the rabbits were administered intravenous pentobarbitone (30 mg/kg). Once under anaesthesia, the surgical area was disinfected and 3 ml of 1 % lidocaine was injected subcutaneously. A 3-cm long incision was then made in the medial portion of the right knee. The MCL was exposed and resected from its femoral and tibial insertion. On the femoral side, a bone tunnel was created from the MCL insertion to the contralateral cortical bone using a 2.0-mm diameter drill. On the tibial side, a 2.0-mm hole was drilled perpendicular to the cortical bone. The scaffold was then threaded into the bone tunnels, and the looped end of the scaffold was fixed with a suture button (diameter, 5.0 mm) (Meira, Nagoya, Japan) on the femoral side. The thermocompression-bonded end of the scaffold was fixed with a T-J screw (diameter 2.5 mm, length 5 mm) (Meira, Nagoya, Japan) on the tibial side (Fig. 1b) [12]. A flexor digitorum longus (FDL) tendon was harvested from a donor rabbit. The tendons were stored at -80 °C and thawed before their day of use. The FDL tendon was implanted into the left side of the limb using same technique as the scaffold group and the knee reconstructed with the FDL allograft was used as a control. After MCL reconstruction, the subcutaneous tissue and skin were sutured in separate layers. All rabbits were allowed free cage movement. For macroscopic and histological evaluations, three rabbits were randomly selected at four, eight and 16 weeks after the operation. For mechanical analysis, four rabbits were randomly selected at four, eight and 16 weeks after the operation. Four knees from two rabbits that did not undergo surgery were examined mechanically as normal controls.

Histological analysis

After macroscopic examination, the regenerated tissues were fixed in 4 % paraformaldehyde for 24 hours, decalcified with 0.25 mol/l ethylenediaminetetraacetic acid in phosphate-buffered saline at pH7.5, dehydrated in graded alcohol solutions and embedded in paraffin wax. Long axis sections (6 µm

Fig. 1 Macroscopic view of scaffold (a). A schematic drawing of the reconstruction of the medial collateral ligament using a stent-shaped PLLA scaffold in a rabbit model. The femoral end of the scaffold and graft were fixed with a suture button. The tibial end was fixed with an interference screw (T-J screw) (b). Macroscopic view of the scaffold at sacrifice at four weeks post-operation. *F* femoral side, *T* tibial side, the arrow shows the scaffold covered with smooth connective tissue (c)

thick) were assessed by haematoxylin and eosin and Masson's trichrome staining. The tissues were evaluated using fibre alignment scoring [13], and the appearance of the cells' shapes were assessed using morphology of fibroblast scoring [13]. Histological findings were assessed by two (H.N. and T.M.) independent investigators. Toluidine blue staining was performed to evaluate the femoral bone-ligament junction.

Immunohistochemical analysis

For immunohistochemical analysis of types I and III collagen, deparaffinised sections were digested with proteinase K (DakoCytomation Inc., Carpinteria, CA, USA) for 10 min, then treated with 3 % hydrogen peroxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to block any endogenous peroxidase activity. The sections were treated in a 1:50 dilution of mouse anti-type I and III collagen antibodies (Southern Biotechnology Associates Inc., Birmingham, AL, USA) at 4 °C overnight, then subsequently treated with peroxidase-labelled anti-mouse immunoglobulin [Histofine Simple Stain MAX PO (M), Nichirei Bioscience, Tokyo, Japan] at room temperature for 30 min. A positive signal appeared as a brown reaction product using peroxidase substrate 3,3-diaminobenzide (Histofine Simple Stain DAB Solution, Nichirei Bioscience, Tokyo, Japan), which was then counterstained using haematoxylin.

Mechanical analysis

The femur-ligament-tibia complex was harvested from each limb immediately after sacrifice, and all soft tissues, except

the scaffold and allograft, were resected by sharp dissection. The femur and tibia were potted in specially designed devices using polymethyl methacrylate (PMMA) resin, and then the complexes were placed vertically in a tensile sensor (AG-I Shimadzu Co., Kyoto, Japan). The ultimate failure load and stiffness were measured as described previously in the "Scaffolds" section [11]. Four normal ligaments were also measured using the same method.

Statistical analysis

To compare the mechanical properties of the scaffold reconstruction with the control group at the same time points, a two-sided Mann–Whitney U test was used. To compare the mechanical properties at each time point within the same group, analysis of variance (ANOVA) was performed. If the ANOVA was significant, post hoc pairwise comparisons were conducted using Tukey's test. In all cases, a significance level of $P < 0.05$ was used. All data are presented as mean $\pm$ SD.

Results

Histological examination

Ligament

No infection, contracture, mobility disability or inflammatory reaction was observed in any rabbit throughout the post-operative period. Macroscopically, the PLLA scaffolds were

covered with smooth connective tissue at four weeks post-operation (Fig. 1c), showing that the regenerated tissues were thicker than normal ligaments. Microscopically, randomly aligned spindle-shaped cells associated with vascular formation were seen in the scaffold group at four weeks post-operation (Fig. 2a). At eight weeks post-operation, vascularity

had decreased and the regenerated fibres had aligned in parallel (Fig. 2b). At 16 weeks, fibre alignment had become denser (Fig. 2c). In the control group, the fibres showed hyalinisation, and ruptures of the fibre were observed on the inside of the allograft at four weeks post-operation (Fig. 2d). By eight weeks, ruptures of the fibres were not observed on

Fig. 2 Histological findings of regenerated tissue stained with haematoxylin and eosin (H&E) and Masson's trichrome (MT). The regenerated tissue stained with H&E: the defect was reconstructed with the stent-shaped PLLA scaffold (a–c) and FDL tendon (d–f). At four (a,

d), eight (b, e) and 16 weeks post-operation (c, f) (bar 10 μ m). The regenerated tissue stained with MT: the defect was reconstructed with the stent-shaped PLLA scaffold (g–i) and FDL tendon (j–l). At four (g, j), eight (h, k) and 16 weeks post-operation (i, l) (bar 10 μ m)

the inside of the allograft (Fig. 2e). The cellularity increased with dense fibre regeneration at 16 weeks (Fig. 2f). Masson's trichrome staining showed minor collagen fibre formation at four weeks in the scaffold group (Fig. 2g). At eight weeks, the collagen fibres had realigned to the long axis of the ligament (Fig. 2h). Dense fibre formation was observed at 16 weeks post-operation (Fig. 2i). In the control group, collagen fibres were sparse at four weeks (Fig. 2j); however, they become denser in a time-dependent manner (Fig. 2k, l). Fibre alignment (Table 1) and morphology of fibroblast scoring (Table 2) both significantly increased in a time-dependent manner. There was no significant difference between the scaffold group and the control group at each time point for either score.

Immunostaining showed that stronger expression of type I collagen was observed in the scaffold group at eight and 16 weeks post-operation than at four weeks (Fig. 3a–c). The expression of type I collagen in the scaffold group at 16 weeks post-operation resembled a normal ligament (Fig. 3d). In the control group, expression of type I collagen was not observed at four and eight weeks post-operation and only observed at 16 weeks post-operation (Fig. 3e–g). Type III collagen was also present at four, eight and 16 weeks post-operation in the scaffold group (Fig. 3h–j). The expression of type III collagen in the scaffold group at 16 weeks was stronger than a normal ligament because of the regenerative process (Fig. 3k). The expression of type III collagen was not detected at any of the time points in the control group (Fig. 3l–n).

The femoral bone-ligament junction

In the scaffold group, the fibrocartilage layer at the scaffold-bone interface was not seen at four weeks post-operation (Fig. 4a), although a thin fibrocartilage layer was observed at eight weeks and had increased in width at 16 weeks (Fig. 4b, c). In the control group, fibrocartilage regeneration was not observed at any of the time points (Fig. 4d–f).

Mechanical evaluation

The results of mechanical examination are described in Tables 3 and 4. All of the surgically treated samples were ruptured at the scaffold-femoral bone interface, while

Table 2 Morphology of fibroblast score (points), mean $\pm$ SD

	4 weeks	8 weeks	16 weeks
Scaffold	0.6 $\pm$ 0.2	1.7 $\pm$ 0.6 ^a	2.3 $\pm$ 0.3 ^b
Control	0.6 $\pm$ 0.1	1.3 $\pm$ 0.2 ^c	2.3 $\pm$ 0.4 ^d

^a $P < 0.01$ versus scaffold at 4 weeks

^b $P < 0.05$ versus scaffold at 8 weeks

^c $P < 0.05$ versus control at 4 weeks

^d $P < 0.01$ versus control at 8 weeks

normal MCLs were ruptured on the ligament tibial side. The ultimate failure load of the scaffold group had increased in a time-dependent manner, and a significant difference was not seen between the scaffold and control groups at any of the time points. In addition, a statistically significant difference was not seen between the scaffold group and normal MCLs at 16 weeks (Table 3). The stiffness of the scaffold group increased significantly in a time-dependent manner. There was no significant difference between the scaffold and control groups at eight and 16 weeks post-operation. In addition, no significant difference was seen between the scaffold group and normal MCLs at 16 weeks (Table 4).

Discussion

We demonstrated that the MCL could be regenerated by implantation of a stent-shaped PLLA scaffold in a rabbit model. Many previous studies have used the combination of scaffolds with either growth factors or cultured cells [14–16] to encourage cell retention and migration within the scaffolds. The high-density scaffolds, however, were largely unable to maintain any migrated cells within their interstices [17]. Therefore, we designed the scaffold in a stent shape, so that it contains fewer synthetic fibres. The scaffold was implanted into the MCL defect, and its mechanical property and ability to regenerate the ligament was evaluated. The collagen fibre alignment of the regenerated tissue was parallel to the long axis of the ligament, and the histological evaluation showed maturation of the tissue in a time-dependent manner. In addition, expression of type I collagen was observed in the regenerated ligamentous tissue. Furthermore, the scaffold-bone junction on the femoral side showed regeneration of the fibrocartilage layer at eight and 16 weeks post-operation. Conversely, the control group, which was reconstructed by allograft, did not show fibrocartilage regeneration inside of the bone tunnel.

For ligament tissue engineering, usage of various materials including silk, polytetrafluoroethylene, poly(lactic-co-glycolic acid) and PLLA has been reported [18–22]. Ideally, scaffolds should have good mechanical strength during the

Table 1 Fibre alignment score (points), mean $\pm$ SD

	4 weeks	8 weeks	16 weeks
Scaffold	0.9 $\pm$ 0.1	1.8 $\pm$ 0.2 ^a	2.4 $\pm$ 0.2 ^b
Control	0.9 $\pm$ 0.1	1.3 $\pm$ 0.2	2.6 $\pm$ 0.4 ^{c, d}

^a $P < 0.01$ versus scaffold at 4 weeks

^b $P < 0.01$ versus scaffold at 8 weeks

^c $P < 0.01$ versus control at 4 weeks

^d $P < 0.05$ versus control at 8 weeks

Fig. 3 Immunostaining of the regenerated tissue in the reconstructed MCLs. The regenerated tissue was stained using anti-type I collagen antibody, where the defect was reconstructed with the stent-shaped PLLA scaffold (a–c). The normal MCL stained with anti-type I collagen antibody (d). The regenerated tissue was stained using anti-type I collagen antibody, reconstructed with FDL tendon (e–g). At four (a, e), eight (b, f) and 16 weeks post-operation (c, g) (bar 10 μ m). The

regenerated tissue was stained with anti-type III collagen antibody, where the defect was reconstructed with the stent-like PLLA scaffold (h–j). The normal MCL stained with anti-type III collagen antibody (k). The regenerated tissue was stained with anti-type III collagen antibody, reconstructed with FDL tendon (l–n). At four (h, l), eight (i, m) and 16 weeks post-operation (j, n) (bar 10 μ m)

early stages, which it can adequately maintain during tissue regeneration. Several studies have performed mechanical testing after rabbit knee ligament reconstruction using synthetic scaffolds. One of these studies demonstrated that compared to normal rabbit ACL at eight weeks post-operation, the reconstruction of a rabbit's ACL using chitin-coated polyester fabrics yields 30.8 % of peak resistance force [23]. Another study used a cell-seeded silk scaffold for ACL reconstruction, which led to a 34.5 % ultimate failure load with 7.4 % stiffness compared to native ACLs after eight weeks of reconstruction [15]. Yet another report found that compared to normal rabbit MCLs at 12 weeks post-operation, rabbit MCLs reconstructed using chitosan and hyaluronan hybrid scaffolds resulted in an ultimate failure load of 78 % and stiffness of 34 % [14]. In this

study, we selected PLLA fibres as our scaffold material because PLLA has better initial strength than other materials [24]. When compared to normal MCLs, the ultimate failure load of the stent-shaped scaffold was 56.6, 80.6 and 90.1 % at four, eight and 16 weeks, respectively. Stiffness was 27.1, 68.9 and 83.0 % at four, eight and 16 weeks, respectively. Although the fixation method of the scaffolds and the mechanical testing methods are not identical to other previous reports, the stent-shaped scaffolds allowed for sufficient mechanical function. However, the ultimate failure load of the post-operative scaffold complex was less than the scaffold itself. It is possible that the mechanical properties of the scaffold might be influenced by the fixation method, including the use of screws and buttons.

Fig. 4 Toluidine blue staining of the regenerated tissue at the ligament-bone junction in the reconstructed MCLs. *L* ligament, *B* bone, *FC* fibrocartilage. The regenerated tissue, where the defect was

reconstructed with the stent-shaped PLLA scaffold (a–c) and FDL tendon (d–f). At 4 (a, d), 8 (b, e) and 16 weeks post-operation (c, f) (bar 10 μ m)

Interestingly, the failure mode of the regenerated tissue in this study was not the same as the native MCLs. While the normal MCL ruptured on the ligament tibial side, the regenerated MCL containing the stent-shaped scaffold ruptured on the femoral side. One possible reason for this discrepancy might be because of the different methods of fixation for the femoral and the tibial sides, where the femoral side of the scaffold was fixed by a suture button, while the tibial side was fixed by an interference screw [12]. Okuno et al. reported that suture button fixation is biomechanically weaker than interference screw fixation [25]. Fibrocartilage regeneration was observed at the scaffold-bone interface; however, it was not identical to a normal enthesis. Furthermore, the ligament-bone junction of the femoral side might be histologically weaker than that of the tibial side because the regenerated fibrocartilage was still immature at 16 weeks post-operatively. Normal direct ligament-bone insertion consists of four zones (bone, mineralised fibrocartilage, fibrocartilage and ligament). Only three zones (bone, fibrocartilage and ligament) could be found on the femoral side,

and regenerated fibrocartilage was not mineralised by 16 weeks post-operation.

There were some limitations in this study. A detailed analysis of the regenerated tissues' origin was not performed. Since the MCL is an extra-articular ligament, there is an abundant blood and tissue supply, which might help encourage the surrounding cells to migrate inside of the scaffolds. Assessment of cell migration patterns and origins may be useful for improving biological ligament regeneration. Another limitation is that the PLLA fibres had not been absorbed by 16 weeks. Since by-products of PLLA fibres can be acidic and cause a local inflammatory reaction, a longer follow-up period for observing PLLA fibre degradation may be necessary.

In conclusion, the stent-shaped PLLA scaffold allowed for MCL regeneration with type I collagen expression and fibrocartilage formation and resulted in sufficient mechanical function. This provides novel insight for new techniques to aid in regeneration of ligament tissues.

Table 3 Ultimate failure load of regenerated tissue (N), mean $\pm$ SD

	4 weeks	8 weeks	16 weeks
Normal	82.5 $\pm$ 2.0		
Scaffold	46.7 $\pm$ 20.7 ^a	66.5 $\pm$ 11.0 ^{a, b}	74.3 $\pm$ 11.5 ^b
Control	61.7 $\pm$ 14.4 ^a	50.9 $\pm$ 9.2 ^a	96.0 $\pm$ 25.4

^a $P < 0.05$ versus normal ligament

^b $P < 0.05$ versus scaffold at 4 weeks

Table 4 Stiffness of regenerated tissue (N/mm), mean $\pm$ SD

	4 weeks	8 weeks	16 weeks
Normal	44.7 $\pm$ 4.6		
Scaffold	12.1 $\pm$ 0.7 ^{a, b}	30.8 $\pm$ 3.7 ^c	37.1 $\pm$ 2.6 ^c
Control	26.5 $\pm$ 3.1 ^a	20.8 $\pm$ 1.2 ^a	35.4 $\pm$ 3.8

^a $P < 0.05$ versus normal ligament

^b $P < 0.05$ versus control at 4 weeks

^c $P < 0.05$ versus scaffold at 4 weeks

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