Potency of double-layered Poly L-lactic Acid scaffold in tissue engineering of tendon tissue

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Abstract A successful scaffold for use in tendon tissue engineering requires a high affinity for living organisms and the ability to maintain its mechanical strength until maturation of the regenerated tissue. We compared two types of poly(L-lactic acid) (PLLA) scaffolds for use in tendon regeneration, a plain-woven PLLA fabric (fabric P) with a smooth surface only and a double layered PLLA fabric (fabric D) with a smooth surface on one side and a rough (pile-finished) surface on the other side. These two types of fabric were implanted into the back muscles of rabbits and evaluated at three and six weeks after implantation. Histological examination showed collagen tissues were highly regenerated on the rough surface of fabric D. On the other hand, liner cell attachment was seen in the smooth surface of fabric P and fabric D. The total DNA amount was significantly higher in fabric D. Additionally, mechanical examination showed fabric P had lost its mechanical strength by six weeks after implantation, while the strength of fabric D was maintained. Fabric D had more cell migration on one

side and less cell adhesion on the other side and maintained its initial strength. Thus, a novel form of double-layered PLLA fabric has the potential to be used as a scaffold in tendon regeneration.

Introduction

Tendons and ligaments are connective tissues, which join muscle to bone or bone to bone. Tendon or ligament injuries including flexor tendon injuries in the hand or rotator cuff tears in the shoulder are quite common and negatively impact the quality of life for those injured [10, 18]. The majority of acute tendon injuries can be treated by suturing the tendon to tendon or tendon to bone. However, primary repair of chronic tendon injuries such as massive rotator cuff repairs in the shoulder are usually impossible. To improve the clinical results in these conditions, tendon transfer or autografting using fascias or another tendon have been performed [14, 22]. However, there are some disadvantages to these techniques, for example, tendon transfer is not an anatomical reconstruction and autograft requires the sacrifice of normal tissue. To overcome these disadvantages, some nonabsorbable synthetic materials have been used in reconstruction after massive rotator cuff tear or anterior cruciate ligament injury [20, 26]. Although, using nonabsorbable synthetic materials such as polytetrafluoroethylene (PTFE) has been reported to induce foreign body reaction or bone erosion several years after operation [15, 20]. Extracellular matrix derived from animals such as porcine small intestinal submucosa is another alternative; however, these may cause immune reactions and have the risk of zoonoses resulting from xenotransplantation [6, 12].

Currently, some alternative therapies for tendon repair are being developed. Tissue engineering, which requires

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scaffolds, cultured cells and growth factors, has made innovative progress in research into musculoskeletal regeneration or orthopaedic surgery [1, 5, 21]. Funakoshi et al. reported rotator cuff regeneration using synthetic scaffolds and cultured fibroblasts and concluded that transplantation of fibroblasts had better results than using scaffold only [8]. However, these methods require two-step operations of which the first step is harvesting the stem cells and the second step is transplanting the cells. If a scaffold could induce stem cells from the surrounding tissues and cultivate the cells by itself, pre-expansion of the cells for transplantation would not be required. We postulated that certain forms of bioabsorbable scaffold can induce stem cells from the surrounding tissues and regenerate tendon defects without requiring harvesting, culture, and transplantation of stem cells. We prepared two types of bioabsorbable fabrics from synthetic poly (L-lactic acid) (PLLA) fibres and evaluated their use in a rabbit model.

Materials and methods

Preparation of PLLA fabric

PLLA fibers, with molecular weight of 86,000 and diameter of 23 μm , were textured into two types of fabric. The first type was textured into plain-woven fabric (fabric P), which had a smooth surface only (Fig. 1a). The second type was textured into a double layered structure (fabric D), which has a smooth surface on one side (Fig. 1b) and a rough (pile-finished) surface on the other side (Fig. 1c).

Implantation of PLLA fabrics into back muscle in rabbits

All the animal experiments were approved by the Animal Research Committee of Kobe University Graduate School of Medicine. Sixteen female Japanese white rabbits (2.7–3.5 kg) were used in this experiment. General anaesthesia

using intravenous phenobarbital (30 mg/kg) was administered to the rabbits. The surgical area was disinfected and 3 ml of 1% lidocaine was injected subcutaneously. Longitudinal axis skin incisions were made on both sides of the back. A piece of fabric P (10×60 mm) was implanted into the back muscle on the right side, and a piece of fabric D was similarly implanted on the left side. Four rabbits were sacrificed at three and six weeks after surgery and half (10×30 mm) of each PLLA fabric was evaluated histologically while the DNA was extracted from the remaining fabric. Then the total DNA amount was measured using QIAquick DNA (QIAGEN, Hilden, Germany). Four rabbits were examined mechanically at three and six weeks after surgery.

Histological analysis

After macroscopic examination, PLLA fabric or scaffolds were fixed in 4% paraformaldehyde for 24 hours, dehydrated in graded alcohol solutions, and embedded in paraffin wax. Sagittal sections (7 μm thick) were cut through the fabrics or scaffold. Haematoxylin and eosin (H-E) and Azan staining were performed and examined by light microscopy.

Mechanical analysis

Four rabbits were sacrificed at three and six weeks after implantation and PLLA fabric was harvested immediately after sacrifice, and the end of the fabric was clamped by a specially designed device. The fabric was placed vertically to a tensile sensor (AG-I SHIMAZU Co, Kyoto, Japan). Before the tensile test was conducted, the fabric 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 per minute. Immediately after preconditioning, the ultimate load to failure was recorded in uniaxial tension at

Fig. 1 Microscopic appearance of PLLA fabric (scale bar represents 1 mm). **a** Fabric P. **b** Smooth side of fabric D. **c** Pile-finished side of fabric D

20 mm per minute. The load–deformation curve was recorded, from which the ultimate load to failure and the energy were measured.

Statistical analysis

To compare the total DNA amount of fabric D with that of fabric P, the unpaired *t* test was used. To compare the mechanical properties of PLLA fabric with each time point, Dunnett's test was performed. In all cases a significance level of $p < 0.05$ was used. All data are presented as mean $\pm$ standard error (SE).

Results

Implantation of PLLA fabrics into back muscle in rabbits

All the wounds healed uneventfully. On gross appearance both fabrics were covered with thin smooth membranes.

Microscopically, some spindle shaped cells adhered to the outer surface of both PLLA fabrics, and no inflammatory reaction was seen. Three weeks after surgery, in fabric P, few cells were observed in the interstices of the PLLA fibres (Fig. 2a). In contrast, fabric D showed some cell adhesions on the smooth side and a large amount of cell migration internally on the pile-finished side (Fig. 2b). Six weeks after surgery, few cell migrations were seen in the interstices of the fibres in fabric P (Fig. 2c). In fabric D, linear alignment of regenerated tissues was observed in the pile-finished side; however, a crimp pattern of collagenous fibres was not seen (Fig. 2d). Absorption of PLLA fibres was not seen in this study. Azan staining six weeks postoperatively showed that collagenous tissue stained blue was not observed in the interstices of PLLA fibres of fabric P, although collagenous tissue regenerated around the fabric P (Fig. 3a). On the other hand, in fabric D, some collagenous tissue was regenerated in the interstices of the pile-finished PLLA fibres and extended to the outside of the fabric (Fig. 3b).

Fig. 2 Microscopic appearance of two types of PLLA fabric in the back muscle of the rabbits (H-E staining). Linear cell attachment was seen in both fabrics. Cell migration was not seen interstices of the fibres in fabric P. On the other hand, a large amount of cell migration was seen internally on the pile-finished side of fabric D (scale bar represent 100 μ m). **a** Fabric P after three weeks implantation. **b** Fabric D after three weeks implantation. **c** Fabric P after six weeks implantation. **d** Fabric D after six weeks implantation

Fig. 3 Azan staining of fabrics at six weeks implantation. Blue stained area shows collagenous tissue. Both fabrics were covered with collagenous tissue. More collagenous tissue was seen on the pile-finished side of fabric D (scale bar represent 100 μ m). **a** Fabric P. **b** Fabric D (the left side of the figure is the smooth side of the fabric and the right side is the pile-finished side)

Measuring total DNA amount of each PLLA fabric

Total DNA amount of fabric D per unit area was $63.4 \pm 12.2 \mu\text{g}/\text{cm}^2$ and that of fabric P was $14.6 \pm 2.2 \mu\text{g}/\text{cm}^2$. Fabric D showed a significantly higher amount of total DNA (Fig. 4).

Mechanical property of PLLA fabric

The ultimate failure load of fabric D was 24.3 ± 0.9 N preoperatively, 32.0 ± 4.2 N at three weeks and 23.6 ± 5.4 N at six weeks, and that of fabric P was 76.6 ± 3.5 N preoperatively, 67 ± 16.4 N at three weeks, and 44.0 ± 3.4 N at six weeks. The energy of fabric D was 0.28 ± 0.02 J preoperatively, 0.3 ± 0.11 J at three weeks and 0.21 ± 0.11 J at six weeks, and that of fabric P was 0.83 ± 0.12 J preoperatively, 0.89 ± 0.09 J at three weeks and 0.59 ± 0.1 J at six weeks. The change ratio of the ultimate failure load or energy was calculated by dividing the measured value of each time point by the value of

preoperative data (Fig. 5a, b). Fabric P lost 43% of the ultimate failure load and 62% of the energy by six weeks while fabric D lost 4% of the ultimate failure load and 24% of the energy. Both fabrics lost their mechanical strength; however, no significant statistical difference was observed in fabric D.

Discussion

A combination of scaffolds, cultured cells, and growth factors has been used in successful tissue regeneration [5]. The ideal scaffold should be biocompatible, highly porous, and biodegradable. It should permit cell migration and easy attachment of cells to scaffold and provide an environment that is suitable for cell proliferation and differentiation, thus allowing the cells to secrete their own extracellular matrices to form tissue-like organization, while the scaffold degrades [11].

In tendon surgery, maintaining the initial strength of the repaired site and preventing adhesion after operation are required. To avoid these problems we consider the ideal structure of the scaffold in tendon regeneration to be less adhesive outside, have more cell migration on the reverse side, and to maintain its mechanical strength until the maturation of the regenerated matrix. We prepared a novel form of PLLA fabric as a scaffold for tendon surgery that had a smooth surface because of dense PLLA fibre weaving and a highly porous surface on the other side because of its pile-finished structure. Previous researchers have shown in in vitro studies that a highly porous scaffold has a better cell migration because of enhanced diffusion of the cultured medium into the scaffold [9, 24]. However, in an in vivo study the best porosity of scaffold may be one that changes relative to the condition. In a cartilage repair study, Ikeda et al. reported that a lower porosity of poly (DL-lactide-co-

Fig. 4 Total DNA amount. Fabric D showed a significantly higher amount of total DNA. **a** Fabric D. **b** Fabric P

Fig. 5 Mechanical analysis. The change ratio of ultimate failure load or energy was calculated by dividing the measured value of each time point by the value of preoperative data. Both fabrics lost their mechanical strength by six weeks after implantation; however, no significant loss was seen in fabric D. **a** Ultimate failure load of fabric. **b** Energy of fabric

glycolide) (PLG) scaffolds inhibits cell migration and a higher porosity leads to weakness in the regenerated tissue [13]. Ozaki et al. compared three forms of PTFE scaffold (PTFE felt, fabric and mesh) and concluded that the felt type scaffold had higher cell migration than the other structures [20].

We compared two types of PLLA fabric and implanted them into the back muscle of rabbits to select the optimal structure of PLLA fabric for our scaffold. Histologically, both fabrics showed linear cell attachment on their smooth surfaces. More spindle cells were observed in the interstices on the PLLA fibres of the pile-finished side of fabric D, compared to the smooth side of fabric D. Azan staining showed collagenous fibres extended to the outside of the fabric. The total DNA amount of fabric D was significantly higher than fabric P. This indicates that fabric D contains more cells inside. Therefore, we consider that fabric D had a tendency for preventing adhesion on its smooth surface and incorporating the surrounding cells internally because of its pile-finished structure.

As the scaffold material we chose PLLA fibre, which has a slow absorption rate. PLLA has been used in screws or rods in orthopaedic surgery [4, 23, 25] and in several studies involving tissue engineering in ligaments or tendons [7, 16, 19]. The advantages of PLLA over poly glycolic acid (PGA) or PLGA as a material for scaffold have been previously reported. Li et al. compared PLG, poly-L-lactate-epsilon-caprolactone (PLC) and PLLA scaffold in vitro and concluded PLLA and PLC scaffold are the best materials for tendon regeneration because the slow absorption rate showed better mechanical properties [17]. Lu et al. compared three materials (PLLA, PGA and PLGA) and reported PGA scaffolds had the highest tensile strength but rapid degradation resulted in matrix disruption and cell death over time. In contrast PLLA-based scaffolds

maintained their structural integrity and exhibited superior mechanical properties over time [19]. In an in vivo study, Yokoya et al. implanted three materials (PTFE, PLC, and PGA) into the joint capsules of knees and concluded that PGA was the best material for scaffold because of its faster absorption rate. However, they were concerned that the fast absorption rate may lead to rapid deterioration of its mechanical strength [27]. In this study, both fabrics lost their mechanical strength by six weeks postoperatively. Fabric P significantly lost its mechanical strength, on the other hand, no significant statistical difference was observed in fabric D. This result indicates that fabric D has the potential as scaffold for tendon regeneration where initial strength is important for obtaining satisfactory results.

However, there are some limitations to this study. First, the PLLA fabrics were not absorbed by six weeks. This study showed that fabric D has sufficient initial strength and migration of surrounding cells. However, some clinical experiments have shown that significant systematic or local reaction may arise because degradation products of PLLA are natural metabolites but also acidic [2, 3, 18]. In our experiments histological examination did not show any inflammatory reaction, but further examination until the PLLA fibres are absorbed should be made. Second, the fabrics did not implant into the tendons. Tensile force was not provided to the fabrics; therefore, the results of mechanical examination could not be applied to tendon regeneration research.

In conclusion, the double-layered PLLA fabric, which has a pile-finished structure on one side and smooth structure on the other side, has good cell migration on the pile-finished side and less cell adhesion on the smooth side. It also has sufficient initial strength. This novel form of double layered PLLA fabric has the potential as scaffold for tendon regeneration.

References

- Aspenberg P (2007) Stimulation of tendon repair: mechanical loading. GDFs and platelets. A mini-review. *Int Orthop* 31:783–789. doi:10.1007/s00264-007-0398-6
- Bostman O, Pihlajamäki H (2000) Clinical biocompatibility of biodegradable orthopaedic implants for internal fixation: a review. *Biomaterials* 21:2615–2621. doi:S0142961200001290
- Bostman OM, Pihlajamäki HK (2000) Adverse tissue reactions to bioabsorbable fixation devices. *Clin Orthop Relat Res* 371:216–227
- Brand JC Jr, Nyland J, Caborn DN, Johnson DL (2005) Soft-tissue interference fixation: bioabsorbable screw versus metal screw. *Arthroscopy* 21:911–916. doi:S0749-8063(05)00672-9
- Butler DL, Juncosa-Melvin N, Boivin GP, Galloway MT, Shearn JT, Gooch C, Awad H (2008) Functional tissue engineering for tendon repair: a multidisciplinary strategy using mesenchymal stem cells, bioscaffolds, and mechanical stimulation. *J Orthop Res* 26:1–9. doi:10.1002/jor.20456
- Dejardin LM, Arnoczky SP, Ewers BJ, Haut RC, Clarke RB (2001) Tissue-engineered rotator cuff tendon using porcine small intestine submucosa. Histologic and mechanical evaluation in dogs. *Am J Sports Med* 29:175–184
- Freeman JW, Woods MD, Laurencin CT (2007) Tissue engineering of the anterior cruciate ligament using a braid-twist scaffold design. *J Biomech* 40:2029–2036. doi:S0021-9290(06)00350-2
- Funakoshi T, Majima T, Iwasaki N, Suenaga N, Sawaguchi N, Shimode K, Minami A et al (2005) Application of tissue engineering techniques for rotator cuff regeneration using a chitosan-based hyaluronan hybrid fiber scaffold. *Am J Sports Med* 33:1193–1201. doi:0363546504272689
- Gugala Z, Gogolewski S (2005) The in vitro growth and activity of sheep osteoblasts on three-dimensional scaffolds from poly(L/DL-lactide) 80/20%. *J Biomed Mater Res A* 75:702–709. doi:10.1002/jbm.a.30479
- Hoffmann A, Gross G (2007) Tendon and ligament engineering in the adult organism: mesenchymal stem cells and gene-therapeutic approaches. *Int Orthop* 31:791–797. doi:10.1007/s00264-007-0395-9
- Hokugo A, Takamoto T, Tabata Y (2006) Preparation of hybrid scaffold from fibrin and biodegradable polymer fiber. *Biomaterials* 27:61–67. doi:S0142-9612(05)00427-8
- Iannotti JP, Codsi MJ, Kwon YW, Derwin K, Ciccone J, Brems JJ (2006) Porcine small intestine submucosa augmentation of surgical repair of chronic two-tendon rotator cuff tears. A randomized, controlled trial. *J Bone Jt Surg Am* 88:1238–1244. doi:88/6/1238
- Ikeda R, Fujioka H, Nagura I, Kokubu T, Toyokawa N, Inui A, Makino T et al (2008) The effect of porosity and mechanical property of a synthetic polymer scaffold on repair of osteochondral defects. *Int Orthop* 33(3):821–828. doi:10.1007/s00264-008-0532-0
- Karas SE, Giachello TL (1996) Subscapularis transfer for reconstruction of massive tears of the rotator cuff. *J Bone Jt Surg Am* 78:239–245
- Kimura A, Aoki M, Fukushima S, Ishii S, Yamakoshi K (2003) Reconstruction of a defect of the rotator cuff with polytetrafluoroethylene felt graft. Recovery of tensile strength and histocompatibility in an animal model. *J Bone Jt Surg Br* 85:282–287
- Kimura Y, Hokugo A, Takamoto T, Tabata Y, Kurosawa H (2008) Regeneration of anterior cruciate ligament by biodegradable scaffold combined with local controlled release of basic fibroblast growth factor and collagen wrapping. *Tissue Eng Part C Methods* 14:47–57. doi:10.1089/tec.2007.0286
- Li WJ, Cooper JA Jr, Mauck RL, Tuan RS (2006) Fabrication and characterization of six electrospun poly(alpha-hydroxy ester)-based fibrous scaffolds for tissue engineering applications. *Acta Biomater* 2:377–385. doi:S1742-7061(06)00021-3
- Liu Y, Ramanath HS, Wang DA (2008) Tendon tissue engineering using scaffold enhancing strategies. *Trends Biotechnol* 26:201–209. doi:S0167-7799(08)00055-3
- Lu HH, Cooper JA Jr, Manuel S, Freeman JW, Attawia MA, Ko FK, Laurencin CT (2005) Anterior cruciate ligament regeneration using braided biodegradable scaffolds: in vitro optimization studies. *Biomaterials* 26:4805–4816. doi:S0142-9612(04)01054-3
- Ozaki J, Fujimoto S, Masuhara K, Tamai S, Yoshimoto S (1986) Reconstruction of chronic massive rotator cuff tears with synthetic materials. *Clin Orthop Relat Res* 202:173–183
- Pecina M, Vukicevic S (2007) Biological aspects of bone, cartilage and tendon regeneration. *Int Orthop* 31:719–720. doi:10.1007/s00264-007-0425-7
- Sano H, Kumagai J, Sawai T (2002) Experimental fascial autografting for the supraspinatus tendon defect: remodeling process of the grafted fascia and the insertion into bone. *J Shoulder Elbow Surg* 11:166–173. doi:S1058274602937821
- Tuompo P, Arvela V, Partio EK, Rokkanen P (1997) Osteochondritis dissecans of the knee fixed with biodegradable self-reinforced polyglycolide and polylactide rods in 24 patients. *Int Orthop* 21:355–360
- Wake MC, Patrick CW Jr, Mikos AG (1994) Pore morphology effects on the fibrovascular tissue growth in porous polymer substrates. *Cell Transplant* 3:339–343
- Waris E, Ashammakhi N, Kaarela O, Raatikainen T, Vasenius J (2004) Use of bioabsorbable osteofixation devices in the hand. *J Hand Surg [Br]* 29:590–598. doi:S0266768104000415
- Woods GA, Indelicato PA, Prevot TJ (1991) The Gore-Tex anterior cruciate ligament prosthesis. Two versus three year results. *Am J Sports Med* 19:48–55
- Yokoya S, Mochizuki Y, Nagata Y, Deie M, Ochi M (2008) Tendon-bone insertion repair and regeneration using polyglycolic acid sheet in the rabbit rotator cuff injury model. *Am J Sports Med* 36:1298–1309. doi:0363546508314416