Enhancement of the osseointegration of a polyethylene terephthalate artificial ligament graft in a bone tunnel using 58S bioglass

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Received: 1 February 2011 / Accepted: 27 April 2011 / Published online: 17 May 2011
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Abstract

Purpose The aim of the study was to investigate whether a bioactive glass (BG) coating on the polyethylene terephthalate (PET) artificial ligament could enhance graft osseointegration by promoting bone regeneration at the interface between PET graft and bone tunnel.

Methods Thirty New Zealand white rabbits underwent artificial ligament graft transplantation in proximal tibial tunnels bilaterally. One limb was implanted with a 58S BG-coated PET graft, and the contralateral limb was implanted with a non-BG-coated PET graft as a control. The rabbits were randomly sacrificed at three, six and 12 weeks after surgery for biomechanical and histological examinations.

Results The maximum load to failures of the BG-coated experimental group were significantly higher than those of the control group at 12 weeks ($p=0.0051$). Histologically, at 12 weeks, the BG-coated PET graft induced great new bone formation between graft and host bone, and the average graft-bone interface width of the BG group became significantly lower than that of the control group. Furthermore, the BG coating on the ligament graft surface also stimulated greater expression of bone morphogenetic protein 2 (BMP-2) and vascular endothelial growth factor (VEGF) around the graft in vivo compared to the control group at three weeks ($p<0.05$).

Conclusions This study has shown that a BG coating on the PET artificial ligament surface has a positive effect in the induction of artificial ligament osseointegration within the bone tunnel.

Introduction

Although many types of artificial ligaments are no longer used following devastating effects in clinical applications, the Ligament Advanced Reinforcement System (LARS) ligament (Surgical Implants and Devices, Arc-sur-Tille, France), made of polyethylene terephthalate (PET), is one of the graft choices for ligament rupture management along with autograft and allograft in many countries [1]. Because many clinical investigations have shown the potential postoperative benefits of the LARS ligaments, they have become a good choice for use in reconstructing human ligaments, such as the anterior cruciate ligament (ACL) and posterior cruciate ligament (PCL) [2, 3]. Because it is a permanent artificial graft in the bone tunnel, the graft healing of the LARS artificial ligament is the major problem that must be considered, as the healing is directly related to the long-term stability of the graft in the bone tunnel. As reported previously [4, 5], the LARS ligament appears to have poor osseointegration with the surrounding bone because of its hydrophobicity and chemical inertia. Often, a band of fibrous scar tissue forms between the artificial ligament graft and the bone tunnel, and subsequently results in ligament loosening and graft failure. This phenomenon raises questions of how to promote and enhance the osseointegration of the artificial ligament graft in the bone tunnel after implantation.

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One strategy for graft osseointegration with host tissue is to stimulate new bone formation at the interface between the graft and host bone tunnel. The osseointegration of artificial grafts in the host bone could be greatly promoted by using coatings that are capable of stimulating both osteogenesis and angiogenesis [6]. Bioactive glass (BG), a biomedical material with extraordinary osteoconductivity and bioactivity, has been widely studied in bone tissue engineering to stimulate osteoblast proliferation, promote extracellular matrix production, enhance bone regeneration and facilitate the repair of bony defects [7–10]. Furthermore, several investigations have demonstrated that BG stimulates the production of vascular endothelial growth factor (VEGF) and contributes to angiogenesis *in vitro* and *in vivo* [11–13]. It has been widely accepted that BG not only stimulates new bone formation but also has some effect on angiogenesis [14].

Based on this background, we applied a BG coating to modify the surface of PET artificial ligament grafts. The aim of this investigation was to study the effect of the BG coating on the PET artificial ligament in a rabbit extra-articular tendon-to-bone healing model. We hypothesised that the BG surface coating would significantly enhance the osseointegration of the PET artificial ligament graft within the host bone.

Materials and methods

Preparation of the PET sheet with BG coating

PET sheets (with a size of 2×0.9 cm) were taken from a LARS ligament (Surgical Implants and Devices, Arc-sur-Tille, France). They were cleaned in a 75% alcohol solution for four hours, washed with a large amount of deionised water and then dried in air for 24 hours. The BG solution was made with 0.2 g of BG powder (58S bioglass, Shanghai Research Centre of Biomedical Engineering, China) and 0.8 g of gelatin powder (porcine gelatin powder, Sigma-Aldrich, St. Louis, MO, USA). These powders were placed in a test tube with 50 ml phosphate-buffered saline (PBS) and stirred for two hours. The cleaned PET sheets were modified through plasma surface modification (HPD-100B Plasma Apparatus, Coronalab Co. Ltd., Nanjing, China) at 70 V for 40 seconds and then immersed in the BG solution for two hours. Then they were dried in air for 24 hours, after which a thin coating of 58S bioglass was observed on the PET sheet fibres by scanning electron microscopy (SEM, TESCAN VEGA 3, Brno, Czech Republic) (Fig. 1).

To guarantee a consistent BG coating on the sheets within the experimental group, we chose only BG-coated sheets with a weight of approximately 80 mg for our experimental group and uncoated PET sheets with a weight

Fig. 1 SEM micrograph of the 58S bioglass-coated PET sheets. Glass particles can be seen on and between the woven sheet fibres (magnification $\times 2000$)

of approximately 60 mg for the control group. These grafts were rolled into a cylinder that was 2 cm in length and 3 mm in diameter. Both the non-treated PET sheets and the BG-coated PET sheets were sterilised through a conventional gas sterilisation technique using ethylene oxide gas.

Animal study design and operative procedure

The animal experiment was approved by the Animal Care and Use Committee of our college. Thirty mature male New Zealand rabbits (mean weight 2.8 ± 0.4 kg) underwent an operative extra-articular graft-to-bone healing procedure. The rabbits were first anaesthetised with 3% pentobarbital (30 mg/kg). After disinfection and incision, a 3-mm-diameter bone tunnel was made in the proximal tibial metaphysis. The BG-coated graft was pulled into one limb, and the uncoated graft was implanted into the contralateral limb as the control. An approximately 0.5-cm-long graft end was left outside the lateral tunnel entrance for the subsequent biomechanical study. The graft ends were sutured with the adjacent periosteum and soft tissue at the bone entrance, and the wound was closed in layers. Postoperatively, the animals were returned to their cages and allowed free cage activity without immobilisation. These rabbits were sacrificed at three, six and 12 weeks after surgery for the following examinations.

Mechanical testing

The graft-tibia complex ($n=5$ limbs in each group at each time point) was harvested from each knee after sacrifice and prepared for mechanical testing immediately without being frozen. All scar tissue and sutures at the tibial tunnel exits were carefully removed. All mechanical testing was conducted using an Instron materials testing system machine (8874, Instron Co., Norwood, MA, USA). The graft portion outside of the lateral tunnel entrance was sutured by a No. 5 Ethibond suture for traction, and the tibial region was fixed firmly in a clamp. The bone tunnel was oriented parallel to the testing axis. Before the tensile test was conducted, the specimen was preloaded with a static preload of 1 N for five minutes. Immediately after preconditioning, the ultimate load to failure was performed with an elongation rate of 2 mm/min. The ultimate failure load (N) was recorded. For each specimen, testing was completed when the graft ruptured or was pulled out of the bone tunnel.

Histological examination

Graft-tibia complexes ($n=5$ limbs for each group at each time point) were prepared for the histological analysis of the graft-bone interface. Immediately after sacrifice, the graft-bone complex specimens were fixed in 10% formalin for 48 h and then embedded, undecalcified, in a methyl methacrylate compound. The samples were sectioned perpendicularly to the longitudinal axis of the tibial tunnel with a thickness of 5 μm using a microtome (SM2500, Leica, Nussloch, Germany). These sections were stained with haematoxylin and eosin (H&E) for routine histological evaluation. The slides were examined to visualise the graft-bone interface with inverted light microscopy (IX71SBF-2, Olympus Optical Co., Tokyo, Japan). Digital images were taken using a DP Manager (Olympus Optical Co., Tokyo, Japan). The two investigators performing the histological analysis were blinded to the animal treatment.

Histomorphometric analysis

Each section, taken at the middle portion of the tunnel, was divided into four quadrants to determine the graft-bone interface width. In each of the four quadrants, the interface width was measured as the distance between the edge of the bone tunnel and the outer graft determined under $\times 200$ magnification. Four separate measurements were made in each of the quadrants for a total of 16 measurements for each specimen. The average interface width for each specimen was then determined by averaging these values obtained from each specimen. The two investigators who performed the histomorphometric analysis were blinded to the type of animal treatment.

Immunohistochemical analysis

The sections were deparaffinised and rehydrated. Endogenous peroxidase within the sections was blocked by incubating them in a 0.3% hydrogen peroxide solution for 30 min at 37°C. After they were washed three times with PBS, the sections were blocked with a blocking reagent containing goat serum in PBS for 20 minutes. After a brief wash, the sections were incubated with primary antibody (mouse anti-rabbit VEGF, diluted 1:50 (Calbiochem, San Diego, CA, USA) or mouse anti-bone morphogenetic protein 2 (BMP-2), diluted 1:50 (Boster Biotechnology, Wuhan, China) overnight at 4°C. After the sections were washed three times with PBS, they were incubated with a secondary antibody (MaxVision kit, Maixin Biotechnology, Fuzhou, China) for 15 min at room temperature. They were then washed three times with PBS and treated with dimethylaminoazobenzene (DAB) (simple stain DAB solution, Maixin Biotechnology, Fuzhou, China) for five minutes. The sections were washed again three times with PBS and then counterstained using haematoxylin.

Digital images were obtained using a DP Manager (Olympus Optical Co., Tokyo, Japan). Image-Pro Plus 6.0 software (Media Cybernetics Corp., Bethesda, MD, USA) was then used to measure the expression of VEGF and BMP-2 at the graft-bone interface of immunohistochemical sections. The expression of VEGF or BMP-2 was measured as the mean density value of VEGF- or BMP-2-positive staining at the graft-bone interface under $\times 200$ magnification. The two investigators who performed the histomorphometric analysis were blinded to the type of animal treatment.

Statistical analysis

Data analysis was performed using Stata 10.0 software (StataCorp, College Station, TX, USA) and the data are reported as the mean $\pm$ standard deviation. The experimental and control data were compared using the paired Student's *t* test. The statistical significance level was set at 0.05.

Results

Mechanical findings

All of the specimens failed when they were pulled out from the tunnel regardless of implantation duration (three, six or 12 weeks). No graft rupture occurred. After three weeks, there was no statistically significant difference in the load to failure between the experimental and control groups ($p>0.05$). After six weeks, the mean load to failure for the experimental group was higher than that of the control group (92 ± 12.0 N for the

Fig. 2 Mechanical examinations of the control group and BG-coated group at each time point after surgery: comparison of maximum failure load. The maximum failure load of the BG-coated group was significantly higher than that of the control group at 6 and 12 weeks (indicated by ##). There was no significant difference between the control group and the BG-coated group at 3 weeks (indicated by *N.S.*)

experimental limbs and 58.2 ± 9.4 N for the controls, $p = 0.0076$). After 12 weeks the mean load to failure for the experimental group was higher than that of the control group (114 ± 16.0 N for the experimental limbs and 68 ± 9.1 N for the controls, $p = 0.0051$) (Fig. 2).

Histological findings

At 3 weeks after surgery, inflammatory cells had infiltrated the graft-bone interface, both in the control group and in the BG-coated experimental group. At 6 weeks, newly formed bone growing from the host bone to the graft in the BG-coated group was found, and a thick fibrous tissue band was observed at the interface between the graft and the host bone in the control group at the same time point. At 12 weeks, a thin fibrous, scar tissue band was found between host bone and the graft in the control group, and no obvious new bone formation was observed at the

Fig. 3 Histological characterisation of the control group (a, c, e) and the BG-coated group (b, d, f) at 3 weeks (a, b), 6 weeks (c, d) and 12 weeks (e, f) after surgery. The interface between the artificial ligament graft and the host bone are depicted (H&E staining, $\times 200$). There is new bone formation at the graft-bone interface of the BG-coated group at 6 weeks, and newly formed bone at the interface and connected to the artificial graft at 12 weeks after surgery. HB host bone, IF interface, G graft, NB new bone. Bar = 200 μ m

interface. In the BG-coated group at 12 weeks, bone formation around the graft was significantly enhanced; additionally the new bone that was formed at the graft-bone interface created a dense trabecular network, the interface width appeared to be much narrower, and there was less scar tissue formation (Fig. 3).

Histomorphometric analysis

Treatment of the BG-coated grafts resulted in less apparent scar tissue formation at the graft-bone interface than did the non-coated grafts, as evidenced by interface widths at 12 weeks (Fig. 4). There were no differences in the interface width between the control group and the BG-coated group at three weeks ($p>0.05$). However, at six weeks, there was a significant difference between the control group and the BG-coated group (204 ± 17.8 vs 110 ± 15.8 μm , $p=0.022$). Likewise, the mean interface width of the BG-coated group was significantly lower than that of the control group at 12 weeks (50 ± 11.2 vs 118 ± 18.9 μm , $p<0.001$).

Immunohistochemical analysis

The BG coating on the PET artificial ligament surface resulted in more BMP-2 and VEGF expression at three weeks, as evidenced by the density value of the related protein (Fig. 5). The density value of BMP-2 in the BG-coated group was 0.151 ± 0.007 compared to 0.128 ± 0.006 for the control group at three weeks ($p=0.016$), and the density value of VEGF in the BG group was 0.345 ± 0.038 compared to 0.251 ± 0.029 for the control group at three weeks ($p=0.0029$). The amounts of BMP-2 and VEGF decreased with time in both the BG-coated group and the control group. There

Fig. 4 Interface width between the graft and the host bone of the control group and the BG-coated group. The BG-coated group had narrower interface widths than did the control group at 12 weeks, respectively, indicating less fibrous tissue formation at the interface (## $p<0.05$). BG bioglass, N.S. no significant difference

Fig. 5 Immunohistochemical analysis shows the relative expression levels of BMP-2 (a) and VEGF (b) at 3, 6 and 12 weeks after surgery. Both BMP-2 and VEGF expression decreased with time. Specimens in the BG-coated group had significantly elevated BMP-2 and VEGF expression at the graft-bone interface compared to the controls at 3 weeks. (## $p<0.05$ compared to the control group, N.S. no significant difference)

were no significant differences in BMP-2 or VEGF expression between the control group and the BG-coated group at 12 weeks ($p>0.05$, respectively).

Discussion

Previous investigations of biomaterial implants in orthopaedic and dental applications have demonstrated that the success of the graft in vivo depends on the osseointegration of the graft with the host bone tissue, which requires new bone formation at the interface between the graft and the host bone [15]. Furthermore, several important proteins and

growth factors, such as BMP, located around the graft, have been shown to favour osteogenesis around the graft [16–18]. The lack of these functional proteins or growth factors near the graft is thought to hamper bone formation, because these proteins play an important functional role in graft osseointegration within host bone [19]. For these reasons, there is much interest in developing strategies that can effectively accelerate and improve the expression of these proteins and growth factors to enhance graft osseointegration [15, 20].

In a dog ACL reconstruction model using the ipsilateral flexor digitorum superficialis tendon, Sasaki et al. [15] evaluated the ability of a granulocyte colony-stimulating factor (G-CSF) to enhance autograft tendon-to-bone healing. They found that significantly more new bone and microvessels were formed in the grafts coated with G-CSF compared to the control group, which contained a graft without a G-CSF coating. In addition, real-time polymerase chain reaction (PCR) results revealed significantly elevated mRNA expression levels of VEGF and osteocalcin in the tibial bone tunnel and graft compared to the controls. In our study, we used BG as a coating material to stimulate angiogenesis and osteogenesis around the PET artificial ligament. More new bone formation was indeed observed at the interface between the graft and the host bone in the BG-coated group compared with the control group at six and 12 weeks after surgery. At three weeks after surgery, the expression of VEGF and BMP-2 in the BG-coated group was significantly higher than it was in the control group. We propose that the bioglass coating on the artificial ligament graft may play an important role in osteogenesis and angiogenesis at the interface between the graft and the host bone.

In another study, Leach et al. [14] investigated the potential of VEGF-releasing polymeric scaffolds with a bioactive glass coating in vitro and in vivo using a rat critical-sized defect model. They suggested that the bioactive glass coating on the polymeric substrate participates in bone healing through indirect processes which promote angiogenesis and bone maturation and not directly on osteoprogenitor differentiation and bone formation. As previously reported [21], bioglass releases ions, such as Ca^{2+} and Si^{4+} , after implantation in vivo, which stimulate osteoblastic proliferation and differentiation. Thus, we hypothesise that the ions released from the bioglass in vivo might play an important role in new bone formation at the graft-bone interface.

Previously, Day et al. [11] implanted polyglycolic acid (PGA)/45S5 bioglass composite scaffolds subcutaneously into rats and demonstrated that the experimental group with the bioglass coating stimulated neovascularisation from the surrounding tissue into the mesh to a greater extent than did the control group. However, the amount of bioglass on the PGA mesh was not quantified, and it was suggested that only approximately 20% of the outer surfaces of the mesh were coated with bioglass particles. In our study, we used the BG-coated sheets with a weight of approximately

80 mg as our experimental group and uncoated PET sheets with a weight of approximately 60 mg as the blank control group, which means that there was 20 mg of the bioglass and gelatin composite coating on each PET graft. According to the weight ratio of the bioglass and gelatin (1:4), we assumed that each graft was coated with approximately 4 mg of BG, and 3 mg of BG in the bone tunnel, as three fourths of the graft was placed in the bone tunnel.

Leu and Leach [22] cultured human microvascular endothelial cells (HMVECs) in indirect contact with bioglass-loaded collagen sponges and found that the greatest proliferative response, achieved with collagen sponges loaded with 1.2 mg of bioglass particles and HMVECs exposed to 0.12 and 1.2 mg of bioglass, resulted in greater VEGF mRNA production after 72 hours of bioglass exposure than either the negative control group (0 mg) or the highest bioglass-content group (12 mg). In our in vivo study, we coated the PET artificial ligament with approximately 4 mg bioglass. The VEGF expression by the BG group was higher than that of the control group. It is presumed that 4 mg of BG is a safe and effective dose to stimulate and promote angiogenesis around the artificial ligament graft in our study.

Acknowledgments This project was subsidised by the 863 Project (No. 2007AA021902, 2007AA021903) and 973 Project (No. 2009CB930000) from the Ministry of Science and Technology of China.

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Conflict of interest The authors declare that they have no conflict of interest.

References

- Legnani C, Ventura A, Terzaghi C, Borgo E, Albisetti W (2010) Anterior cruciate ligament reconstruction with synthetic grafts. A review of literature. *Int Orthop* 34:465–471
- Li B, Wen Y, Wu H, Qian Q, Wu Y, Lin X (2009) Arthroscopic single-bundle posterior cruciate ligament reconstruction: retrospective review of hamstring tendon graft versus LARS artificial ligament. *Int Orthop* 33:991–996
- Nau T, Lavoie P, Duval N (2002) A new generation of artificial ligaments in reconstruction of the anterior cruciate ligament. Two-year follow-up of a randomised trial. *J Bone Joint Surg Br* 84:356–360
- Gao K, Chen S, Wang L, Zhang W, Kang Y, Dong Q et al (2010) Anterior cruciate ligament reconstruction with LARS artificial ligament: a multicenter study with 3- to 5-year follow-up. *Arthroscopy* 26:515–523
- Guidoin MF, Marois Y, Bejui J, Poddevin N, King MW, Guidoin R (2000) Analysis of retrieved polymer fiber based replacements for the ACL. *Biomaterials* 21:2461–2474
- De Giglio E, Cometa S, Ricci MA, Zizzi A, Cafagna D, Manzotti S et al (2010) Development and characterization of rhVEGF-loaded poly(HEMA-MOEP) coatings electrosynthesized on tita-

- nium to enhance bone mineralization and angiogenesis. *Acta Biomater* 6:282–290
7. Au AY, Au RY, Al-Talib TK, Eves B, Frondoza CG (2008) Consil bioactive glass particles enhance osteoblast proliferation and maintain extracellular matrix production in vitro. *J Biomed Mater Res A* 86:678–684
 8. Välimäki VV, Yrjans JJ, Vuorio E, Aro HT (2005) Combined effect of BMP-2 gene transfer and bioactive glass microspheres on enhancement of new bone formation. *J Biomed Mater Res A* 75:501–509
 9. Vogel M, Voigt C, Gross UM, Müller-Mai CM (2001) In vivo comparison of bioactive glass particles in rabbits. *Biomaterials* 22:357–362
 10. Xu C, Su P, Chen X, Meng Y, Yu W, Xiang AP et al (2011) Biocompatibility and osteogenesis of biomimetic Bioglass-Collagen-Phosphatidylserine composite scaffolds for bone tissue engineering. *Biomaterials* 32:1051–1058
 11. Day RM, Boccaccini AR, Shurey S, Roether JA, Forbes A, Hench LL et al (2004) Assessment of polyglycolic acid mesh and bioactive glass for soft-tissue engineering scaffolds. *Biomaterials* 25:5857–5866
 12. Day RM (2005) Bioactive glass stimulates the secretion of angiogenic growth factors and angiogenesis in vitro. *Tissue Eng* 11:768–777
 13. Gorustovich AA, Roether JA, Boccaccini AR (2010) Effect of bioactive glasses on angiogenesis: a review of in vitro and in vivo evidences. *Tissue Eng Part B Rev* 16:199–207
 14. Leach JK, Kaigler D, Wang Z, Krebsbach PH, Mooney DJ (2006) Coating of VEGF-releasing scaffolds with bioactive glass for angiogenesis and bone regeneration. *Biomaterials* 27:3249–3255
 15. Sasaki K, Kuroda R, Ishida K, Kubo S, Matsumoto T, Mifune Y et al (2008) Enhancement of tendon-bone osteointegration of anterior cruciate ligament graft using granulocyte colony-stimulating factor. *Am J Sports Med* 36:1519–1527
 16. Ma CB, Kawamura S, Deng XH, Ying L, Schneidkraut J, Hays P et al (2007) Bone morphogenetic proteins-signaling plays a role in tendon-to-bone healing: a study of rhBMP-2 and noggin. *Am J Sports Med* 35:597–604
 17. Rodeo SA, Suzuki K, Deng XH, Wozney J, Warren RF (1999) Use of recombinant human bone morphogenetic protein-2 to enhance tendon healing in a bone tunnel. *Am J Sports Med* 27:476–488
 18. Mihelic R, Pecina M, Jelic M, Zoricic S, Kusec V, Simic P et al (2004) Bone morphogenetic protein-7 (osteogenic protein-1) promotes tendon graft integration in anterior cruciate ligament reconstruction in sheep. *Am J Sports Med* 32:1619–1625
 19. Anderson K, Seneviratne A, Izawa K, Atkinson B, Potter H, Rodeo S (2001) Augmentation of tendon healing in an intraarticular bone tunnel with use of a bone growth factor. *Am J Sports Med* 29:689–698
 20. Raines AL, Olivares-Navarrete R, Wieland M, Cochran DL, Schwartz Z, Boyan BD (2010) Regulation of angiogenesis during osseointegration by titanium surface microstructure and energy. *Biomaterials* 31:4909–4917
 21. Välimäki VV, Aro HT (2006) Molecular basis for action of bioactive glasses as bone graft substitute. *Scand J Surg* 95:95–102
 22. Leu A, Leach JK (2008) Proangiogenic potential of a collagen/bioactive glass substrate. *Pharm Res* 25:1222–1229