The use of autografts versus allografts for anterior cruciate ligament (ACL) reconstruction is controversial. The current popular options for ACL reconstruction are patellar tendon or hamstring autografts, yet advances in allograft technologies have made allogeneic grafts a favorable option for repair tissue. Despite this, the mismatched biomechanical properties and risk of osteoarthritis resulting from the current graft technologies have prompted the investigation of new tissue sources for ACL reconstruction. Previous work by our lab has demonstrated that tissue-engineered bone-ligament-bone (BLB) constructs generated from an allogeneic cell source develop structural and functional properties similar to those of native ACL and vascular and neural structures that exceed those of autologous patellar tendon grafts. In this study, we investigated the effectiveness of our tissue-engineered ligament constructs fabricated from autologous versus allogeneic BLB grafts. Our preliminary results demonstrate that 6 months postimplantation, our tissue-engineered auto- and allogeneic BLB grafts show similar histological and mechanical outcomes indicating that the autologous grafts are a viable option for ACL reconstruction. These data indicate that our tissue-engineered autologous ligament graft could be used in clinical situations where immune rejection and disease transmission may preclude allograft use.

Introduction

The anterior cruciate ligament (ACL) is the most commonly torn ligament in the knee with an estimated 350,000 ACL-related injuries occurring in the United States annually. Healing of the torn ACL is limited and often requires surgical intervention to restore knee stability and lessen degenerative features often found in the ACL-deficient knee. The current graft options used for ACL reconstructions are most commonly bone-patellar tendon-bone autografts (PTG) although the use of hamstring tendon autografts is increasing. Donor site morbidity, a major concern with tendon autografts, however, can lead to postoperative chronic pain and poor muscle function. Although allografts eliminate the issues related to donor site morbidity, they have other challenges including graft availability and the increased risks of disease transmission and immune rejection. Graft choice should therefore be dictated by patient-specific characteristics. There are also overarching concerns shared with the use of both tendon autografts and allografts. Mismatched biomechanical properties of the tendon graft tissue to replace the native ligament tissue can cause nonphysiological loading in the knee. These limitations may be associated with poorer outcomes from ACL reconstructions with reports of failure and recurrent instability in 10–15% of cases. Tissue engineering strategies are being studied to address the limitations associated with current tendon graft technologies. Most current tissue engineering techniques being investigated involve the use of natural or synthetic polymer scaffolds to provide an integrating and degradable structure for cellular attachment. Although they provide initial support, no scaffold technology has been developed that is capable of withstanding the multidirectional mechanical forces that native ACLs undergo in vivo. Prolonged mechanical support during graft healing can lead to issues with stress-shielding.
In our laboratory, we have developed a novel method of tissue engineering three-dimensional multiphase bone-ligament-bone (BLB) constructs from bone marrow stromal cells (BMSCs) capable of generating their own ECM without the use of scaffolds. In large animal models, we have shown that after 6 months in vivo, these engineered BLB constructs undergo ligamentization developing similar mechanical properties to those of native ACLs and induce vascular and neural development exceeding those of PTGs. These properties make our tissue-engineered BLB constructs an attractive alternative to traditional tendon grafts.

Previous work in our laboratory involved the use of allogeneic cell sources in our BLB grafts. Our previous method for BMSC harvest utilized euthanasia and extraction of bone marrow from the mid-substance of both femurs, rendering the host animal unavailable for tissue grafting. To allow for autologous cell sources, we developed a procedure to fabricate our BLB grafts from BMSCs isolated from bone marrow aspirations of the ACL compromised patient. Thus, the first part of this study was to validate the use of the BLB fabricated from autologous cells in a long-term ACL regeneration study. Second, we compared the efficacy of the BLB grafts from our well-described allogeneic cell source to an autologous derived cell source. We hypothesized that the structural and functional outcomes after 6 months of implantation would be similar in both autologous and allogeneic models. Autologous grafts would potentially allow various manufacturing approaches and patient-specific options with our technology. Our autologous derived BLB graft could be used if the patient wished to wait for the fabrication of the BLB graft from their own BMSCs. Conversely, patients who required expedited surgical intervention could benefit from possible “off-the-shelf” allogeneic BLB grafts.

Materials and Methods

Experimental design and animal care

We used our previously established model of ACL replacement in adult Black Suffolk sheep for their anatomical and functional similarities to the human knee. In this study, BMSCs were harvested from marrow aspirations from five adult sheep to fabricate our tissue-engineered constructs for use as ACL replacement graft tissue. Two constructs were produced from each aspiration with one implanted into the aspirated sheep (autologous) and the other implanted into another sheep (allogeneic) as shown in Figure 1 for a total of 10 sheep. Animals were acclimated to the Unit for Laboratory Animal Medicine husbandry facilities at the University of Michigan for at least 1 week before any procedure. Sheep were given access to food and water ad libitum. All animal care and animal surgeries were performed in accordance with the Guide for Care and Use of Laboratory Animals (Public Health Service, 1996, NIH Publication No. 85–23); the experimental protocol was approved by the University Committee for the Use and Care of Animals.

Preparation of cell culture supplies

Unless otherwise indicated, all solutions and media were prepared and stored at 4°C before the isolation and culture of primary cells and were warmed to 37°C in a heated bead bath before use. The media used in this experiment have been previously described. Briefly, growth medium (GM) consisted of 78% Dulbecco’s modified Eagle medium (DMEM; Gibco, Grand Island, NY), with 20% fetal bovine serum (Gibco), 2% antibiotic antimycotic (ABAM; Gibco), 6 ng/mL basic fibroblast growth factor (Peprotech),...
0.13 mg/mL ascorbic acid-2-phosphotase (Sigma-Aldrich, St. Louis, MO), and 0.05 mg/mL L-proline (Sigma-Aldrich); differentiation medium (DM) consisted of 91% DMEM, 7% horse serum albumin (Gibco), 2% ABAM, 0.13 mg/mL asc-2-phos, 0.05 mg/mL L-proline, and 2 mg/mL transforming growth factor beta (Peprotech). About $10^{-8}$ M dexamethasone (Sigma-Aldrich) was added to GM and DM for the culture of bone cells. 7

Construct dishes were prepared as described previously13 to form and maintain three-dimensional constructs. Briefly, 100 mm diameter cell culture plates were coated with 12 mL Sylgard (type 184 silicon elastomer; Dow Chemical Corp., Midland, MI) and allowed to cure for 3 weeks at room temperature. Before use, plates were decontaminated with UV light (wavelength 253.7 nm) for 60 min and rinsed with 70% EtOH and DBPS.

Isolation and expansion of BMSCs

Marrow aspirations were used to obtain BMSCs for construct fabrication. Bone marrow was aspirated from the iliac crest of a sheep using a Monoject Illinois needle (Sherwood Medical Company, St. Louis, MO) with the animal under general anesthesia induced by intravenous propofol and sustained with inhalation of isoflurane in oxygen. Marrow was collected using heparinized needles and dispensed into EDTA blood collection tubes (BD, San Jose, CA) at room temperature at 600 g for 30 min. The upper layer of plasma was discarded and the mesenchymal cells contained in the mononuclear cell layer were transferred into a new conical. The isolate was then centrifuged at 500 g for 10 min and the supernatant removed. An equivalent volume to the pellet of DPBS added. A layer of 15 mL Ficoll-Paque Premium (MNC; GE Healthcare, Munich, Germany) was added on top of the aspirate solution and was centrifuged (AccuSpin FR; Beckman Coulter, Inc., Fullerton, CA) at room temperature of 30 min. The upper layer of plasma was discarded and the mononuclear cell layer was transferred into a new conical. The supernatant was removed, the pellet was resuspended in 20 mL GM and a cell count was taken. Cells were then plated at 40,000–60,000 cells/cm$^2$ in cell culture dishes.

Fabrication of BLBs

Using previously described methods, the BMSCs were expanded into ligament and bone lineages.13–15 Briefly, passage-3 cells in the ligament pathway and passage-4 cells in the bone pathway were seeded at a density of 21,000 cells/cm$^2$ and switched to DM after 8 days plating. After 2 days in DM, the bone monolayers were rolled with sterile tweezers into a tube shape and transferred to Sylgard plates. After 1 to 2 additional days, the bone constructs were then ready to be incorporated into a ligament monolayer to create our BLB. Each confluent ligament monolayer was removed intact from the cell culture plate surface and transferred to Sylgard plates where they were pinned back into a single layer. Bone constructs were then pinned on each end of the ligament monolayer and subsequently surrounded by ligament tissue. The length of the BLB was adjusted with minucien pins to the desired length of ~60 mm comprised of at least a 30 mm ligament portion and two 15 mm bone ends. Four of these constructs were placed side-by-side and allowed to fuse. The DM was changed every 2–3 days. After 2 weeks of formation, two fused sets of four constructs were combined for an implantation width of ~4–5 mm at the ligament region. After an additional 1 week in culture, the fully formed BLB was ready for implantation. Before implanting, the bone ends of the BLB were threaded with nonabsorbable 5-0 silk suture to allow for passage into the bone tunnel and fixation onto the periosteum.

Surgical procedure

ACL replacements were performed arthroscopically based on a previously described procedure.13 Briefly, after induction of general anesthesia and preparation of the surgical site, two 6 mm incisions were made at the joint line on either side of the patellar tendon for arthroscopic access. After excising the native ACL, remnants of the stump on both the femur and tibia were left to aid in anatomical positioning of the BLB. Drill guides were used to position Steinmann pins in the center of the tibial and femoral footprints. Cannulated reamers were used over the pins to drill 5–6 mm bone tunnels. The BLB was then passed through the bone tunnels with suture and the proximal and distal ends were secured with suture to the periosteum with the knee in 30 degrees of flexion. Incisions were closed with staples and the surgical site was sprayed with Alushield (Neogen Corp., Lansing, MI).

Explantation

After a 6-month recovery, both knees were explanted for morphological and mechanical analyses. Following euthanasia, the intra-articular space was exposed by cutting the patellar tendon and reflecting the patella backward to collect synovial fluid for analysis. The knee was subsequently harvested by removing the tibia and femur and taken for mechanical testing. Anterior drawer testing of the knee at 45 degrees was performed with the knee capsule intact. The knee was then dissected to the BLB or contralateral/native ACL (C-ACL) and the medial condyle was removed for visualization of the entire graft for mechanical testing. Following testing, the BLB and ACL were dissected from the bone insertions and harvested for histology.

Histological analysis

For histological preparation, samples were fixed in 10% neutral buffered formalin for 8 days and stored in 70% ethanol at 4°C until processing. Each tissue was sectioned in a microtome (Leica RM 2155) at 6 μm, placed onto Superfrost Plus microscopy slides, and placed in 60°C oven for 1 h to dry. For morphological characteristics, sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry (IHC) staining with specific antibodies was performed to detect the presence of elastin and collagen type 1. Picrosirius red staining was used for analysis of collagen content and fiber organization. Semi-quantitative analysis was performed of the collagen fiber birefringence using previously established methods.16 Under uniform conditions,
sections stained for picrosirius red were imaged at 10× magnification in a single session under monochromatic polarized light rotated in the plane for maximum brightness. Images were imported using ImageJ software into 8 bit gray scale showing collagenous material representing a gray scale value 1–255 and noncollagenous components as dark with a gray scale value of zero. Mean gray scale values of 10 randomly selected rectangular areas (50×50 μm) from representative sections of each animal were obtained using ImageJ to assign a brightness value for comparison.

To deparaffinize the slides with paraffin sections for IHC, slides were washed three times in xylene for 3 min, twice in 100% ethanol for 2 min, twice in 95% ethanol for 2 min, and once in 70% ethanol for 2 min. Following deparaffinization, slides were washed in phosphate buffered saline (PBS)-0.1% Triton X-100 (PBST; Sigma-Aldrich) for 15 min at room temperature. Using a hydrophobic pen, sections were circled for blocking and antibody staining. Sections were covered in blocking solution containing PBST with 3% bovine serum albumin (Sigma-Aldrich) for 30 min at room temperature. Primary antibodies were diluted in blocking solution and added to the sections and incubated overnight at 4°C in a hydration chamber. The dilutions of the primary antibodies used were as follows: 1:35 of rabbit anti-elastin (#AB2389; Millipore, Billerica, MA); and 1:100 of rabbit anti-collagen type I (#AB292; Abcam, Cambridge, MA). Slides were then washed for 5 min in PBST at room temperature three times for a total of 15 min. Secondary antibodies were diluted at 1:500 in blocking solution in the dark and added to the sections. The slides were kept in a hydration chamber and incubated at room temperature for 2.5 h. The slides were then washed for 15 min in PBS at room temperature three times for a total of 45 min. Nuclei were stained using Prolong Gold with DAPI (Sigma-Aldrich) to each section. Slides were then coverslipped and incubated overnight at room temperature before imaging with an Olympus BX-51 microscope.

Knee laxity testing

Knee laxity was measured using a custom-designed anterior drawer tester using an MTS 810 servohydraulic test system with a 25 kN load cell. The bones were potted in grips with a polymer and secured with two ¼”-20 screws. Ink markings were placed at a known distance onto the femur and tibia grips for displacement tracking. The test comprised of a 0.5 mm/s extension until a 50 N force was achieved. Images were collected with a Grasshopper IEEE-1394b digital camera (Point Grey, British Columbia, CA) and analysis for displacement was determined using MetaMorph software.

Mechanical testing

After the drawer test, knee tissue was further dissected away leaving only the BLB or C-ACL attached at both tibial and femoral insertions. The length of the ligament and the width and thickness of the proximal, middle, and distal regions were recorded. The cross-sectional areas from these three locations were averaged and used as the representative area for stress calculations. The knee was repositioned for a flexion angle of 30 degrees by fixing the tibia and femur grips at 90 and 60 degrees respectively in the sagittal plane. Graphite powder was blown onto the specimen to create a surface pattern for optical displacement measurement using digital image correlation (DIC) to compute full-field strain contours. Uniaxial tension tests at a strain rate of 0.05/s for a loading time of 7.5 s were then conducted on the BLB and ACL specimens to obtain the stiffness using previously developed testing protocols. A Photon high-speed camera was used for synchronized force and image acquisition with a custom-developed LabView program. The load-unload cycle for each specimen was run in triplicate. VIC-2D Software (Correlated Solutions, Columbia, SC) was used for DIC analysis.

Statistical analysis

Comparisons among the three groups were done using one-way analysis of variance (ANOVA) with Tukey’s post-hoc test. A p-value < 0.05 for all statistical tests was considered significant. All data were reported as mean ± standard deviation.

Results

The BLB constructs fabricated from bone marrow aspirates using our prior methodology were all produced and implanted successfully demonstrating similar morphology and robustness to the constructs fabricated from whole femoral bone marrow extractions used in our previous study. Two sheep from the allogeneic BLB group were excluded from the study. One of the sheep developed an illness unrelated to the experiment and was unable to recover. The explanted ligament from a different sheep was compromised by an equipment error during uniaxial tensile testing precluding determination of tangent modulus but was able to be saved for histology.

Size of explanted BLB compared to C-ACL

After 6 months in vivo, explanted autologous BLBs achieved an average length of 18.7 ± 2.7 mm with allogeneic BLBs reaching 18.5 ± 1.2 mm (Fig. 2A). The average CSAs of autologous BLBs were 27.7 ± 5.0 cm² and allogeneic BLB CSAs were 29.7 ± 10.5 mm² (Fig. 2B). The average length and CSA of the C-ACLs were 21.4 ± 3.1 mm and 39.1 ± 9.2 mm² respectively. One-way ANOVA showed no statistically significant difference (p > 0.05) between the average lengths F(5,51) = 1.7, and CSAs F(5,54) = 2.3 of the groups.

Analysis of structure, vascularization, innervation, and elastin of the BLB

Histological observations showed similarities between both autologous and allogeneic derived BLBs. H&E sections demonstrated advanced remodeling and tissue regeneration exhibited by a native collagen crimp pattern with fibers aligned along vertical axis of the tissue (Fig. 3). Compared to the C-ACL, both autologous and allogenic graft types similarly showed an advance in phenotype after implantation and demonstrated signs of regeneration suggested by the prominent vascularization, innervation, and increased cellular density seen on H&E, collagen type I, and elastin staining (Figs. 3 and 5). Semi-quantitative analysis of collagen birefringence was performed to compare collagen alignment and maturation (Fig. 4). Explanted allograft and
autologous BLBs had very similar mean gray scale values (allo: 90±11, auto: 90±9) and were lower than C-ACL knees (115±16), with the difference being statistically significant \((p<0.05)\), \(F(2,13)=7\).

**Knee laxity**

The knee laxity for the autologous implanted knees averaged 1.8±1.2 mm \((n=5)\) while that of the allograft knees was 1.9±0.4 mm \((n=4)\). The C-ACL knee laxity averaged 0.5±0.3 mm \((n=15)\) (Fig. 6A). One-way ANOVA showed no significant difference between autologous and allograft knee laxity \((p>0.05)\), but both groups had significantly increased laxity \((p<0.001)\) compared to the C-ACL knees, \(F(3,26)=7.9\). When comparing allogeneic and autologous knees within BLB cohorts there did not appear to be an appreciable trend (Fig. 6B). Set #5 was excluded from this analysis due to the unfit allogeneic animal in this group described earlier.

**Modulus analysis of BLB**

The tangent modulus (the slope of the stress–strain curve at a specified strain range) for the autologous group averaged 46±15 MPa \((n=5)\), whereas the tangent modulus of the allograft BLBs averaged 35±13 MPa \((n=3)\) at a strain range of 0.035–0.080 (Fig. 7A). The tangent modulus data indicated no significant differences between the two graft types \((p>0.05)\). Both grafts achieved ~23–30% of the average C-ACL modulus, which was 158±32 MPa \((n=12)\) at a strain range of 0.040–0.10. Comparison of the allogeneic and autologous explanted BLBs when matched within BLB cohorts did not show a consistent trend (Fig. 7B). Sets #3 and #5 were excluded from the analysis due to the loss of the two allogeneic test subjects mentioned previously.

**FIG. 2.** Comparisons of length (A) and cross-sectional area (B) of C-ACL, explanted Allo BLB, and Auto BLB after 6 months *in vivo*. Values are mean±standard deviation. There were no significant differences found in the length and cross-sectional area of the C-ACL, Allo BLB, and Auto BLB. C-ACL, contralateral/native ACL.

**FIG. 3.** Longitudinal sections of C-ACL and explanted BLB (Allo/Auto) after 6 months *in vivo*. H&E staining (A–C) was used to ascertain general structure and morphology. Immunohistochemical staining for Elastin (D–F) and Type I Collagen (G–I) indicated positive staining in both Allo (E, H) and Auto (F, I) with organization and staining showing similarities to C-ACL (D, G). All images taken at 40× magnification with scale bars indicating 100 μm. H&E, hematoxylin and eosin. Color images available online at www.liebertpub.com/tea
Discussion

The decision to use patient-derived tendon autografts versus cadaveric tendon allografts for current ACL reconstruction procedures is multifactorial. Autograft use has demonstrated earlier graft incorporation but warrants concern of donor site morbidity at the site of harvest, whereas allogeneic tissues can present with immune rejection and disease transmission but requires less time in surgery. Both of these tendon grafts, however, are poor biomechanical matches for native ACL tissue.

Our scaffoldless tissue engineering approach to develop allogeneic ACL grafts has been shown to be effective in restoring function, even surpassing outcomes seen with current tendon graft options in our allogeneic models. For this study, we developed a BMSC isolation protocol using bone marrow aspirates for autologous construct implantation adapted from our previously established non-recovery allogeneic isolation method of utilizing the whole marrow contents of a femur. From this new bone marrow aspiration technique, we were able to produce BLBs in vitro that were structurally and functionally similar to those made via our allogeneic model. Furthermore, by fabricating BLBs for each set of autologous and allogeneic animals from the same initial aspirate, we were able to eliminate batch to batch variation between donation methods. Postimplantation, as was seen with our previous studies, these constructs also did not appear to elicit an immune rejection response in our animals. After 6 months in vivo, both autologous and allogeneic BLBs showed similar mechanical outcomes. Histological evaluation of both BLB graft types indicated an analogous regeneration containing vasculature, innervation, and increased cellular density. These results demonstrate that using autologous cells for fabrication of BLBs is a viable option with our methods for ACL reconstruction.
The use of allogeneic cells and tissues has been comprehensively investigated both in research and clinical settings. The safety and efficacy of these treatments continue to advance by taking additional precautions of donor selection and sterile processing and preservation of the tissues. However, patient and physician perceptions of allogeneic sources can be negative due to risks of disease transmission and immune rejection. In some patients, there are concerns associated with the use of cells and tissues other than their own. Even for ubiquitous and routine procedures such as blood transfusion, which has extensive donor screening and laboratory testing to minimize risk of disease transmission, many patients are opting for autologous blood donations. In the field of tissue engineering, although BMSCs have been shown to be immunomodulatory and utilized in tissue transplantation for their immunosuppressive effects to combat graft-versus-host disease, the notion of implanting constructs derived from allogeneic sources may still carry negative connotations for the patient. In this study, we have shown that autologous derived BLBs have very similar outcomes to allogeneic BLBs. In theory, patients seeking peace of mind by using autologous derived constructs would be able to do so after only needing to undergo a simple bone marrow aspiration preoperative procedure.

By incorporating our technology within the autologous versus allogeneic cell source paradigm, we have determined that our constructs have increased versatility in that they have the potential to be used in either application. Further work is needed to fully characterize our autologous and allogeneic BLBs in vivo to determine the fate of the implanted cellular and extracellular components. Additionally, while the findings of this study suggest that outcomes from autologous derived grafts are not superior to those of allogeneic grafts, continued investigation is required to develop our technology by finding interventions in vitro during construct fabrication or in vivo during construct remodeling to enhance biomechanical outcomes to further restore native knee function.

A limitation of this work that is shared with fresh tissue-engineered grafts is the length of time required for graft fabrication and the subsequent viable time of the graft after formation. With our methods, total in vitro time required is ~5–6 weeks from aspiration to implantable graft. Additionally, due to the cost and resource demands of this project, it was necessary to have mechanical and histological assessments performed on the same tissue. Mechanical tests were performed first at a conservative level to prevent damage to the tissue. As a result, there is a higher variation in the selection of the strain region from the linear portion of the stress–strain curve to calculate tangent modulus. It should also be noted that due to these constraints and the loss of two animals in the allogeneic group, there were a limited number of test subjects and that these data therefore represent preliminary findings.

Our BLB technology has been shown to exceed morphological and biomechanical outcomes of patellar tendon grafts by actually regenerating ligament tissue and recovering...
native biomechanics to the knee.\textsuperscript{13} In this study, we were able to expand on our fabrication technology to the harvesting of BMSCs from bone marrow aspirations for performing autologous donations. We have shown that both autologous and allogeneic grafts result in similar biomechanical and histological outcomes after 6 months implantation. Both graft types grew to the size of the C-ACL in vivo and when compared to each other, had similar biomechanical and histological outcomes. Each graft exhibited a ligamentous crimp pattern with collagen alignment, demonstration of remodeling through vascularization, and comparable knee laxities and mechanical moduli. These results demonstrate that our tissue-engineered BLB graft could be manufactured from autologous or allogeneic cell sources for use in clinical situations where immune rejection and disease transmission are concerns for the patient, thereby expanding the versatility of our technology.

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Disclosure Statement

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