Appendix

Scaffold Preparation

Human freeze-dried Achilles tendon allografts were provided by the Musculoskeletal Transplant Foundation (Edison, New Jersey). Each allograft was transferred under aseptic conditions to an individual clean, autoclaved, 1000-mL glass flask, and 1000 mL of DNase-free and RNase-free distilled water (Gibco, Grand Island, New York) was added. The bottle was placed onto a rotating shaker (MaxQ 400; Barnstead, Dubuque, Iowa) at 200 rpm at 37°C for twenty-four hours. After twenty-four hours, the water was discarded and replaced, and the cycle was repeated. At the conclusion of the second cycle, the water was discarded and 500 mL of 0.05% trypsin-EDTA (ethylenediaminetetraacetic acid) (Gibco) was added. The sample was returned to the rotating shaker at 200 rpm at 37°C for one hour. At the end of the cycle, the trypsin solution was discarded and 500 mL of high-glucose DMEM (Dulbecco's modified Eagle medium) (Gibco) containing 10% FBS (fetal bovine serum) (Valley Biomedical, Winchester, Virginia) with 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Gibco) was added to halt trypsin digestion of the sample and to maintain an aseptic preparation. The sample was returned to the rotary shaker at 200 rpm at 37°C for twenty-four hours. The DMEM-FBS solution was then discarded, 500 mL of DNase-free and RNase-free distilled water was added, and the sample was placed on the rotary shaker at 200 rpm at 37°C for twenty-four hours. The water wash was discarded, 500 mL of a solution containing 1.5% peracetic acid (Sigma) and 2.0% Triton X-100 (Sigma) in distilled, deionized water (diH$_2$O) was added, and the sample was placed on the rotary shaker at 200 rpm at 37°C for four hours. The solution was discarded, and three 500-mL washes with distilled water were performed, each for twenty-four hours at 37°C and 200 rpm on the rotary shaker. At the end of the third wash, the resulting scaffold was removed and placed in a clean, sterile plastic storage bag and frozen for twenty-four hours at −80°C. The scaffold then was freeze-dried (Freeze Dry System; Labconco, Kansas City, Missouri) for twenty-four hours before being returned to the freezer for storage at −80°C until further use.

In Vitro Tensile Testing

Allografts (n = 10) and scaffolds (n = 10) were equilibrated in sterile Dulbecco phosphate-buffered saline solution (DPBS; Gibco) at 37°C for thirty minutes. Specimens were cut using a custom "dog-bone" type punch (Freeman Company), approximating ASTM standards. The punch measurements included an outer length of 40 mm, inner length of 20 mm × outer width of 8 mm, and inner width of 4 mm × measured thickness (determined with digital calipers for each specimen). The outer width (8 mm) tapered at the sample neck to the inner width (4 mm) to form the desired dog-bone specimen shape. Each specimen was measured with digital calipers and the length, width, thickness, and cross-sectional area were recorded.

The specimen was mounted in custom, serrated pneumatic clamps on a uniaxial load frame (Model 5544; Instron) utilizing a 2-kN static load cell (Instron) with a relative accuracy of 0.5% at 0.2% of the maximum load. The tendon was preloaded to 0.1 N, preconditioned ten times to 0.1 mm extension, and loaded to failure at a rate of 10 mm/min (a strain rate of 1.11%/sec). The tendon was kept moist during testing with DPBS mist.

The ultimate tensile load (in N) was the maximal load recorded during each test. The ultimate tensile stress (in MPa) at failure was the maximal load recorded during each test normalized to the specimen cross-sectional area. The percent strain at ultimate tensile stress was calculated using the displacement data and the initial specimen length (measured with digital calipers after placement of the sample in the test grips). The elastic modulus (in MPa) was calculated from the linear portion of the stress-strain curve, and the stiffness (in N/mm) was calculated from the load-elongation data[26-32].

In Vivo Implantation

The Institutional Animal Care and Use Committee approved the animal protocol. Sixteen skeletally mature New Zealand White rabbits (age, twenty-eight to thirty-two weeks; weight, 3.5 to 4 kg) were used for studying the tensile properties and histology of the ACL reconstruction in an established animal model[33]. Rabbits were assigned to ACL reconstruction using either allograft (n = 8) or scaffold (n = 8).

Anesthesia was induced with 35 mg/kg ketamine supplemented by 5% isoflurane and maintained with 1.5% to 2% isoflurane in oxygen. Intramuscular enrofloxacin (10 mg/kg) was used for antibiotic prophylaxis. Surgery was performed using an aseptic technique. A midline skin incision followed by a lateral parapatellar arthrotomy exposed the right knee. The patella was dislocated medially with the knee extended, and the native ACL was resected by sharp dissection. The tibial bone tunnel was created in a retrograde fashion using a MICRO VECTOR Drill Guide System (Smith & Nephew, Andover, Massachusetts) with a 3.2-mm cannulated bullet and a 3.2-mm drill necked down to 3.0 mm distally; the transtibial angle was set at 55°. The femoral tunnel was created from the insertion site of the native ACL in the femoral notch to the lateral femoral condyle at the 10-o'clock position using a freehand surgical technique.

The final graft was prepared by trimming the midportion of either an allograft or a scaffold using a number-15 blade. The midportion was prepared to a diameter of 3 mm and a length of 50 mm. Both ends of the graft were secured with a Vicryl stitch (Ethicon). The proximal Vicryl stitch was passed through the lumen of a large straight needle, and the graft was passed through the tibial and femoral bone tunnels, leaving the proximal stitch within the femoral tunnel. The proximal Vicryl stitch was used to pull the graft into its final position and to ensure a snug fit within the bone tunnels. Adequate exposure for placement of the femoral holding stitch was obtained by dissection to expose the lateral femoral condyle through the existing midline incision. After passing the graft, the knee

joint was moved through five to ten cycles of full motion before both ends of the graft were secured to the periosteum under slight tension in 30° to 35° of flexion using 3-0 Prolene suture (Ethicon).

The deep layers of the wound were closed using 4-0 Vicryl suture, and the skin was closed using a 4-0 nylon subcuticular suture (Ethicon) and tissue glue. Postoperatively, the rabbits moved freely in individual cages and were assessed daily for pain. The rabbits were given 0.05 mg/kg of buprenorphine intramuscularly every six to eight hours for three to five days after surgery to control pain. At twelve weeks, the animals were killed by intracardiac injection of sodium pentobarbital.

In Vivo Tensile Testing

Ten rabbit knees implanted with either allograft (n = 5) or scaffold (n = 5) were used for tensile analysis[33,34]. The right hindlimb was disarticulated at the hip and stored at −20°C until testing. Before testing, the limb was thawed and all extraneous soft tissues were removed. The femur-ACL graft-tibia complex was potted using Bondo epoxy (3M). This complex was then fixed in clamps with the femur and tibia oriented at angles of 45° and 30°, respectively. This allowed tensile loading along the long axis of the graft in the material testing system (Instron model 5544 with a 2-kN static load cell). A preload of 0.5 N was applied for sixty seconds to determine the resting gauge length of the graft and the cross-sectional area of the midsubstance using digital calipers, followed by cyclic preconditioning of the construct from 0 to 0.3 mm elongation (at 10 mm/min) to achieve a steady state. Load-to-failure testing was performed by increasing the tensile load continuously at a rate of 10 mm/min[33,34]. The ultimate tensile load, ultimate tensile stress at failure, percent strain at ultimate tensile stress, elastic modulus, and stiffness were calculated as described previously[26-32].