Treatment of atrophic nonunions is a challenge to orthopaedic surgeons. Growth factors potentially are valuable factors for improvement of tissue healing. The use of growth factors, however, is limited by their short half-lives. Gene therapy has the potential to improve the treatment. This study aimed to establish and validate an atrophic nonunion model in a rabbit for the use of a percutaneous in vivo gene therapy protocol. An atrophic tibial nonunion was established in 24 New Zealand White rabbits. Radiologic and histologic followup was for 64 weeks. The rabbit tibias showed no radiologic or histologic signs of healing. In addition, an adenoviral vector carrying a marker gene was injected percutaneously into the nonunion site in 12 rabbits. Expression of the marker gene was assessed for as many as 4 weeks. The percutaneous gene delivery resulted in transgene expression in the nonunion site for as many as 4 weeks. The described model reliably leads to an atrophic tibial nonunion in rabbits. Adenoviral percutaneous gene delivery into the nonunion site is feasible and leads to transgene expression locally for at least 1 month. This study provides investigators with a reliable and reproducible model of an atrophic nonunion.
a reliable animal model. No reliable animal model for an atrophic nonunion has yet been reported and validated. We therefore decided to investigate an observation of Oni, who studied fracture healing and observed that some rabbits did not achieve healing of a tibial fracture when the bone was devascularized and revascularization of the fracture was prevented for 4 weeks. The first goal of the current study therefore was to validate an atrophic nonunion model in a rabbit tibia based on the observations of Oni.

In the second part of this study we investigated the feasibility of delivering a marker gene percutaneously into the nonunion site, as a prelude to developing a percutaneous in vivo gene therapy approach to the treatment of atrophic nonunions. Several methods are available for the delivery of transgenes into musculoskeletal tissues. We chose to test the feasibility of a percutaneous gene transfer to the atrophic nonunion site using an adenoviral vector carrying a marker gene.

MATERIALS AND METHODS

We used 24 skeletally mature New Zealand White rabbits weighing 4.5–5 kg. All experimental animals were housed and operated in the Central Animal Facility of the University of Pittsburgh Medical Center, and the treatment protocol for animal subjects was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh Medical Center.

All 24 rabbits included in this study had the same surgical procedure. The rabbits were tranquilized with an intramuscular dose of 4 mg/kg xylazine and 40 mg/kg ketamine preoperatively. Anesthesia was maintained using 1.5–2.5% isoflurane delivered through a mask. The animals were monitored with electrocardiography and pulse oximetry throughout the procedure. Postoperatively, a dose of 0.1 mg/kg Torbugesic® (Wyeth, Philadelphia, PA) was administered subcutaneously twice a day for 2 days. After sterilization and preparation of the right tibia, an anteromedial incision of approximately 7–8 cm was made. The tibia was exposed using a periosteal elevator. In rabbits, the distal fibulotibial insertion usually is located in the upper ½ of the tibial shaft (Fig 1A). This structure was used as a bony landmark, and the tibia was cut using a high-speed dental burr with a 2-mm burr bit 1 cm distal from the fibulotibial insertion. The periosteum was thoroughly stripped 1.5 cm proximal and distal from the fracture site. Then the marrow cavity was reamed using the dental burr and a 2.5-mm drill bit. A hole was drilled distal from the fracture site to hold the silastic tubing in place (Fig 1D). After routine irrigation, the wound was closed. After 4 weeks, the cerclage wires and the Silastic tubings were surgically removed through two small incisions.

For radiologic analysis, anteroposterior and lateral views from each rabbit were immediately obtained postoperatively and consecutively 1, 2, 3, 4, 8, 16, and 64 weeks after surgery. The radiographs were analyzed for nonunion, callus, and ectopic bone formation. At the time the radiograph was obtained, the tibia was clinically assessed for rotational stability.

At 8, 16, and 64 weeks after surgery, four rabbits each were euthanized for histologic analysis. The right tibia was harvested and the muscle tissue was stripped. The fibrous capsule of the nonunion site was left in place. The tibia was cut 2 cm proximal and 2 cm distal from the nonunion site and the intramedullary Steinmann pin was removed. The samples were fixed in 4% paraformaldehyde (pH 7.5) for 7–10 days. After fixation, the tibial samples were decalcified for 4–6 weeks in 50 mL conical tubes containing 20% EDTA. Paraffin sections were cut at a thickness of 5–7 μm and stained with hematoxylin and eosin.

The remaining 12 rabbits were used for the gene transfer feasibility study. The first generation adenoviral vector (ΔE1, ΔE3) used in this study is replication deficient because of a deletion of the E1 gene. The lacZ marker gene is inserted in place of the E1 gene. Gene expression is driven by the human cytomegalovirus early promoter. The Ad/CMV/lacZ virus is grown in 293 cells (ATCC, Bethesda, MD), a human embryonic kidney cell line that constitutively expresses the E1-encoded proteins E1a and E1b. Viral titers were determined by optical density at 260 nm. Eight weeks after the initial surgery, these 12 rabbits were tranquilized using 2 mg/kg xylazine and 20 mg/kg ketamine intramuscularly. The nonunion was observed fluoroscopically. After sterilization of the tibia, a syringe with a 27-gauge needle was inserted percutaneously into the fibrous gap under fluoroscopic control. After the needle was placed correctly, 1 × 10⁷ pfu (plaque forming units) of Ad/CMV V-lacZ virus diluted in 50 μL saline solution was injected into the nonunion site. The rabbits were divided into four groups of three animals each. In the first group, the histologic analysis of the marker gene expression was done 1 week after viral injection; in the second group, the histologic analysis was done 2 weeks after viral injection; in the third group, the histologic analysis was done 3 weeks after viral injection; and in the fourth group, the histologic analysis was done 4 weeks after viral injection. The expression of the lacZ marker gene was detected histologically by X-gal staining.

RESULTS

Twenty-three of the 24 rabbits had a radiologically documented nonunion (Fig 2). One rabbit had a soft tissue infection after the primary procedure and had to be excluded from the study. Radiographic analysis showed no callus formation in the original fracture site for as many as 4 weeks when the silastic tubing was removed. After 2 weeks, in three of the rabbits there was minor callus formation at the interface of the distal edge of the silastic
tubing to the distal tibia. However, this was a minor reaction that did not interfere with percutaneous removal of the silastic tubing. After removal of the silastic tube, no additional callus formation was detected. Radiologic healing was not seen in any of the rabbits at any time for as many as 64 weeks. The bone originally covered by the silastic tubing showed a slightly reduced diameter after 16 weeks in some animals. The clinical examination showed unrestricted rotational instability of the distal part of the right tibia in all 23 rabbits until euthanasia. Because of this obvious clinical instability we did not do a biomechanical analysis on the specimen.

The paraffin sections showed normal eosin staining of the bone with regular bony architecture and fibrous ingrowth into the nonunion site (Fig 3). There was progressive loss of viable osteocytes in their osteons toward the nonunion site. Within 200 μm proximal to and distal from the nonunion site no viable osteocytes were detectable. From the periphery we detected a fibrous ingrowth into the nonunion site, which was intimately associated with the ends of the bone. Although scar tissue was found 8 and 16 weeks after silastic tube removal, very little inflammatory activity was detected in the fibrous scar tissue.

Expression of the lacZ marker gene was detected histologically by X-gal staining at all times. The most striking finding was distribution of the transfected cells in the nonunion (Fig 4). We did not see lacZ + cells in the bony parts of the nonunion. The fibrous scar tissue, however, showed marked expression of β-galactosidase. The transfected cells were distributed throughout the fibrous scar with noticeable accumulation in the fibrous capsule that had formed around the nonunion. We detected marker gene

**Fig 1A–D.** The tibial bone was exposed and the tibia was cut 1 cm distal from the fibulotibial insertion. (A) The proximal stump with the distal fibulotibial insertion is shown after periosteal stripping. (B) The marrow cavity wasreamed and a Steinmann pin was inserted. (C) Silastic tubing was placed over the proximal end of the fracture. The distal fracture end was reduced into the silastic tubing and the Steinmann pin was advanced into the distal marrow cavity. (D) The silastic tubing was fixed and sealed using two cerclage wires.
expression for as many as 28 days. There was no sign of monocyte or neutrophil infiltration, hyperemia, new blood vessel formation, or any other indications of an inflammatory reaction directed against virally infected cells. The samples taken from the lung, liver, and spleen did not show any cells with β-galactosidase activity at any time.

DISCUSSION

Throughout the last decade, numerous animal studies have been done which assessed the healing potential of nonunions after certain operative and nonoperative procedures. All of these studies used a critical-size defect in long bones of different animal species (segmental defect) to mimic a nonunion. The segmental defect model creates a gap in a long bone, which cannot be bridged by fracture callus and therefore the fracture cannot heal. At the fracture ends, however, a normal fracture healing response showing granulation tissue and formation of a fracture callus, with spontaneous filling of 15–20% of the defect can be observed. In addition, there is no increased micromotion between the bone ends and the surrounding healthy soft tissue usually is highly vascularized providing an ideal environment for bone healing. In contrast, an osseous nonunion in humans usually is caused by hypermobility of the fracture or decreased blood supply to the fracture. From the biologic standpoint, the segmental defect mimics a different biologic situation than a common hypertrophic or atrophic nonunion. Therefore, we tested a new approach to establish an atrophic tibial nonunion in a rabbit model. Our procedure was based on the observations of Oni, who reported that tibial fractures in a rabbit eventually fail to heal in a devascularized situation. An additional focus of this study was to test the feasibility of a novel therapeutic approach to promote healing of atrophic nonunions using a percutaneous gene transfer technique. Atrophic nonunions usually are treated with techniques involving extensive surgery. However, atrophic nonunions frequently are the result of an unfavorable soft tissue envelope which may complicate additional invasive procedures. The available nonoperative or minimally invasive techniques have not produced reliable results in the treatment of atrophic nonunions. Bone growth factors, such as BMP-2, have been shown to increase fracture healing significantly in animal models and in human clinical trials. One of the major
drawbacks of single delivery of growth factors is the short biologic half-life of most growth factors. We know from work on chronic skin wounds that one application of growth factors in a fibrous scar tissue is not sufficient to reestablish a healing response. Sustained delivery of growth factors, such as BMP-2, may be necessary for treatment of atrophic nonunions, and a delivery system that can provide a continuous local release of growth factors is desirable. The suggested delivery of BMP-2 or OP-1 in a collagen sponge still requires an extensive debridement and surgical approach for growth factor delivery. Using adenoviral vectors, gene expression has been seen for as many as 6 weeks in various soft tissues. In addition, it was shown that gene delivery to bone and fractures using viral vectors is feasible and leads to an accelerated healing response. Therefore, we also investigated the feasibility of percutaneous gene delivery into the nonunion site, using an adenoviral vector carrying the lacZ marker gene.

The current study has strengths and limitations. Our operative technique created an atrophic nonunion as determined by radiologic and histologic criteria. The opposing ends of the bone seemed atrophic with focal necrosis and no radiologic signs of healing were recorded. However, the validity of our data is limited since the radiologic and histologic evaluations were qualitative and therefore subject to interobserver and intraobserver variations. Moreover, no detailed investigation of the immunologic response to the adenoviral gene transfer was done. Although the histologic sections did not show any significant neutrophil or lymphocyte infiltration, we cannot completely rule out an early inflammatory response because we did not do immunohistochemical analysis for CD4/CD8 or antibody titer measurements for specific neutralizing antibodies against the adenoviral vector or the transgene. At distant organ sites, we did not find any cells with β-galactosidase activity at any time. However, a real-time PCR was not done and therefore we are unable to determine whether adenoviral particles were present at distant organ sites. In addition, no quantification of new vessel formation has been done in the current study. We attempted to establish an atrophic nonunion model using several steps including periosteal stripping, intramedullary reaming, and application of a silastic tube for 4 weeks. Because no control groups were used, we are unable to determine whether it was the periosteal stripping, the intramedullary reaming, the silastic tube, or the combination of these agents that led to the atrophic nonunion. Using this suggested model, it was shown that healing of the fracture did not occur in any animal in our study, therefore, study provides investigators with a reliable animal model of an atrophic nonunion. Experimental methods for healing of atrophic nonunions can be tested, using our suggested nonunion model. The study showed that percutaneous in vivo gene delivery into a nonunion site is feasible. Additional studies should focus on gene transfer of bone growth factors or angiogenic factors to test their healing potential.

In contrast to segmental defect models described earlier, this model is based on periosteal stripping, intramedullary reaming, and decreased vascular supply to the fractured bone. These etiologic factors are consistent with the clinical risk factors for development of an atrophic nonunion.

Novel treatment approaches in orthopaedic surgery include application of bone growth factors. Several growth factors, such as TGF-β, BMP-2, and BMP-7 have been tested in different animal models and have been reported to enhance fracture healing. The treatment of
Atrophic fracture nonunions may benefit from such approaches. We suggest a reliable and reproducible atrophic nonunion model in a rabbit. Novel treatment approaches, such as injection of bone growth factors or gene transfer, can be tested using our suggested nonunion model.

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References


