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Establishment of a femoral critical-size bone defect model in immunodeficient mice

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Abstract

Background—The development of innovative therapies for bone regeneration requires the use of advanced site-specific bone defect small-animal models. The achievement of proper fixation with a murine model is challenging due to the small dimensions of the murine femur. The aim of this investigation was to find the optimal defect size for a murine critical-size bone defect model using external fixation method.

Methods—An external fixation device was attached to the right femur of 30 mice. Femoral bone defects of 1 mm ($n = 10$), 2 mm ($n = 10$), and 3 mm ($n = 10$) were created. Wounds were closed without any additional treatment. To investigate bone healing during the 12-wk observation period, x-ray analysis, histomorphology, immunohistochemistry, and μ CT scans were performed.

Results—MicroCT analyses after 12 wk showed that 3/8 1-mm defects, 5/8 2-mm defects, and 8/8 3-mm defects remained as nonunions. The defect volumes were $0.36 \pm 0.42 \text{ mm}^3$ (1-mm group), $1.40 \pm 0.88 \text{ mm}^3$ (2-mm group), and $2.88 \pm 0.28 \text{ mm}^3$ (3-mm group; $P < 0.001$, between all groups).

Conclusion—Using external fixation, a defect size of 3 mm is necessary to reliably create a persisting femoral bone defect in nude mice.

Keywords

External fixator; Femoral bone defect; Murine; Immunodeficient

1. Introduction

Localized bone loss associated with trauma, tumor, infection, periprosthetic osteolysis, or congenital musculoskeletal disorders, with all of these conditions requiring surgical intervention, is a major worldwide socioeconomic problem. Immunodeficient small-animal models are of particular interest for translational research strategies, as they allow for the use of human cells—a critical step on the path from bench to bedside.

So far, both tibial and femoral murine segmental bone defect models have been used. The tibial fracture model was first described by Hiltunen *et al.* in 1993 [1]. The disadvantages of this model are the triangular, distally declining caliber of the tibia and the bent longitudinal axis. Additionally, the close proximity of the fibula can influence the fracture repair process [2]. In contrast to the tibia, the murine femur is a tubular bone with a relatively consistent inner and outer diameter and a straight longitudinal axis [2].

In order to develop cell-based tissue engineering strategies for bone repair, there is a need for high-quality bone defect models in small animals. The small dimensions of the murine femur make it difficult to achieve proper stabilization of a critical-size bone defect. The aim of this study was to find the optimal defect size for a murine critical-size bone defect using external bony fixation method. The defect size has to be large enough to get reliable nonunions, while at the same time being small enough to achieve proper stabilization when using an external fixation device. Our hypothesis was that a segmental osseous defect size of a minimum of 2 mm would be required to generate a reliable nonunion.

2. Methods

2.1. Experimental procedure

Mice were randomized to three groups. One surgeon implanted the external fixation device (Fig. 1A, MouseExFix, RISystem; AO Research Institute, Davos, Switzerland) onto the right femur of each mouse. A defect of 1 mm, 2 mm, and 3 mm was created in groups 1 ($n = 10$), 2 ($n = 10$), and 3 ($n = 10$), respectively. After wound closure no additional treatment was provided in an effort to avoid influencing the natural pattern of bone regeneration. All of the operations were performed within a 12-d period. The mean operative time was 40 min, independent of the defect size being created. The weight of the complete external fixator, including the four pins and the body, was measured to be 0.20 g. The postoperative observation period was 12 wk. X-ray films were obtained immediately after surgery and every 2 wk during the 12-wk postoperative period. The animals were then euthanized and histomorphometry, immunohistochemistry, and μ CT analysis was performed on the femura.

2.2. Animals

For the *in vivo* investigation 30 male nu/nu nude mice (40.7 ± 2.8 g, 95 ± 2.6 d old) were used. Mice were bred at the Animal Experimental Center of the Medical Faculty of the Technical University of Dresden, Germany. The animals were kept on a 12-h light-and-dark cycle and were fed a standard diet with food and water *ad libitum*. All experiments were performed in adherence to the National Institutes of Health Guidelines for the Use of

Experimental Animals and were approved by the Local Animal Care Committee (protocol no. 24-9168.11-1/2010-29).

2.3. Surgical procedure

2.3.1. Preoperative care—Animals were anesthetized by inhalation of 2% isoflurane for the surgical procedure. The mice were first placed in an inhalation chamber. Upon adequate anesthesia, the animals were transferred to a warming pad and the head and front paws were placed inside a plastic tube that was connected to the anesthesia apparatus (Fig. 1B).

2.3.2. Surgical technique—The operations were performed under surgical aseptic conditions. All mounting pins were prethreaded into the plastic bodies of the external fixation device and removed in order to create a preformed thread, thereby facilitating smooth drilling during the surgical procedure. Each mouse was placed in the prone position. The right rear extremity was extended and rotated inward at the hip joint (Fig. 1B). Exact locations of the hip and knee joints were detected by flexion and extension of the hip and of the knee. A 12-mm incision was then performed along the lateral upper leg, giving exposure to the greater trochanter (Fig. 1C). An incision was then made along the fascia lata, following a line from the greater trochanter to the knee joint. The quadriceps femoris muscle was mobilized anteriorly towards the knee and the hip using two spatulas (Fig. 1D). A sharp elevator was used to expose the distal femur and the first hole (diameter 0.45 mm) was drilled into the distal femur (Fig. 1E). The first pin was put into one of the lateral holes of the plastic body of the external fixation device and was subsequently orthogonally drilled into the distal femur, penetrating both the lateral and the medial cortex (Fig. 1F). Subsequently, the proximal part of the femur was exposed (Fig. 1G). The second hole was drilled through the remaining lateral hole on the plastic body and into the proximal femur or the lateral femoral neck as described above (Fig. 1H). The second pin was then placed through this hole. The inner two pins were then placed after drilling the inner two guide holes (Fig. 1I). After having fixed all four pins, two Gigli wires were placed around the femur and placed into the fixed saw guide on the body of the external fixator (Fig. 1J–M). A defect was cut using two Gigli wires while the body of the fixation device was stabilized with the help of two clamps (Fig. 1M). The clamps were then removed and the femur and surrounding structures were all guided back into their natural anatomic positions (Fig. 1N). Finally, the skin was closed with interrupted sutures (Fig. 1O).

2.3.3. Postoperative care—Two and a half micrograms of buprenorphine in 100 μ L saline per animal was used for postoperative pain control and injected subcutaneously. A single dose of 0.75 mg amoxicillin in 300 μ L saline was injected subcutaneously for postoperative infection prophylaxis. Both of these injections were repeated on the first and second postoperative day. For the x-ray procedures, the animals were anesthetized with single intraperitoneal injections of 100 mg/kg ketamine and 10 mg/kg xylazine.

2.4. Preparation of the specimens for μ CT and histologic analysis

After 12 weeks all mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine and then euthanized by cervical dislocation. Both femora were dissected, freed from soft tissue, and placed in 4% buffered formaldehyde for 8 h at 4°C. MicroCT scanning was done

under these conditions. Specimens were then decalcified and embedded in paraffin. Five-micrometer sections were cut in the sagittal plane and mounted on silane-coated slides. Hematoxylin–eosin, tartrate-specific acidic phosphatase (TRAP), osteocalcin, osteonectin, and osteopontin staining were performed in all specimens.

2.5. Histologic analysis

Femurs stained with hematoxylin–eosin were used to evaluate the histomorphologic features of the tissue that had grown in the defect gap. Microscopy was done by two independent, masked observers using a light microscope (Leica DMRBE and Leica DC 300 digital camera [Leica Camera AG, Solms, Germany]). The histologic grade of fracture healing was classified according to the scale described by Huo *et al.* [3]. The number of cells stained with TRAP was evaluated using an optical magnification of 25-fold and osteocalcin, osteonectin, and osteopontin at 200-fold. Cells were counted in three representative histologic sections per animal using a standard 10 × 10-mm grid.

2.6. Radiographic analysis

Radiographs were obtained using a mobile x-ray device (AMX4-IEC; GE Medical Systems [Little Chalfont, Buckinghamshire]). Images were digitally read and saved. In order to measure the initial size of the bone defect, the x-ray image was scaled using the known distance of the peripheral pins (11.2 mm) and then analyzed by ImageJ v1.44p software (U. S. National Institutes of Health, Bethesda, Maryland).

Using μ CT (SCANCO vivaCT 75; SCANCO Medical AG, Brüttisellen, Switzerland), 20- μ m data sets of all operated and six untreated contralateral femurs were scanned. After determining the region of interest, bone volumes of the operated and the untreated contralateral femurs were calculated. The defect volume reported is the difference of the bone volume of the healthy femurs and the operated femurs.

2.7. Statistical evaluation

Numerical data were statistically analyzed by standard analysis of variance and the Kruskal-Wallis test using Graphpad Prism 4 software (San Diego, California). The Mann-Whitney test was used to detect *post hoc* statistical significance between selected groups. Statistical significance was set at $P < 0.05$.

3. Results

All of the animals survived the operation. Twenty-four out of 30 animals reached the 12th postoperative week. Six out of 30 animals ($n = 2$ per each group) died before the end of the observation period due to severe anemia ($n = 1$), infection ($n = 1$), pin loosening necessitating euthanasia ($n = 2$), and anesthetic complications during follow-up radiographs ($n = 2$).

3.1. Radiographs

The postoperative x-rays (Fig. 2, Fig. 3) show a continuous reduction of the gap size within the 12-wk observation period from 1.19 ± 0.25 mm to 0 ± 0 mm (1-mm group), 2.09 ± 0.30

mm to 0.29 ± 0.38 mm (2-mm group), and 2.98 ± 0.18 mm to 0.77 ± 0.56 mm (3-mm group), respectively. At each time point the difference between all groups was statistically significant ($P < 0.05$).

3.2. MicroCT

MicroCT analysis demonstrated that after 12 wk the defect volume was significantly different between the three groups (Fig. 4, Fig. 3, $P < 0.001$). The 1-mm group reached a defect volume of 0.36 ± 0.42 mm³; 2-mm group, 1.40 ± 0.88 mm³; and 3-mm group, 2.88 ± 0.28 mm³, respectively. Furthermore, we observed a statistically significant intergroup difference in the percent reduction of the defect size during the 12-wk postoperative period (Fig. 5, $P = 0.152$). The defect size decreased by 77.6% (1-mm group), 56.8% (2-mm group), and 28.6% (3-mm group). At the end of the observation period, three out of eight, five out of eight, and eight out of eight defects remained as nonunions for the 1-, 2-, and 3-mm groups, respectively.

3.3. Histomorphology

To evaluate the degree of bone healing histologically, we applied a scoring system described by Huo *et al.* [3] (Fig. 6, Fig. 3). The 1-mm group had a median score of 8, meaning that the former defect gap was filled predominantly by immature (woven) bone with a small amount of cartilage. In the 2-mm group the average score was 4.5, implicating that the defect was filled predominantly with cartilage and small amounts of fibrous tissue. In the 3-mm group the average score was 2.5, denoting that the former defect gap was predominantly filled with fibrous tissue and with a small amount of cartilaginous tissue. *Post hoc* testing showed a significant difference in the histomorphologic pattern between the 1-mm group and the 3-mm group ($P = 0.038$), indicating a significantly lower stage of fracture healing in the 3-mm group.

3.4. Immunohistochemistry

The defect area tissue was evaluated for selected intracellular markers of bone resorption and formation. Osteoclast-specific TRAP (Fig. 7) showed the highest density of osteoclasts per field in the 1-mm group, as an average of 3.3 ± 1.9 cells were counted. The other groups had slightly fewer cells, at 2.8 ± 2.0 cells and 2.6 ± 1.2 cells for the 2-mm and 3-mm groups, respectively. The differences between the three groups were not statistically significant ($P = 0.714$). Cells stained with osteoblast-specific markers resulted in the following means. Osteopontin staining showed 9.9 ± 6.4 cells per field for the 1-mm group, 7.5 ± 4.5 for the 2-mm group, and 13.4 ± 4.9 for the 3-mm group. The differences between the three groups were not statistically significant ($P = 0.89$). Osteonectin staining showed 8.4 ± 5.0 cells per field for the 1-mm group, 8.4 ± 3.9 for the 2-mm group, and 6.5 ± 5.1 for the 3-mm group. The differences between the three groups were not statistically significant ($P = 0.37$). Osteocalcin staining showed 12.4 ± 7.5 cells per field for the 1-mm group, 11.9 ± 4.8 for the 2-mm group, and 11.0 ± 2.1 for the 3-mm group. Again, the differences between the three groups were not statistically significant ($P = 0.19$).

4. Discussion

The aim of this study was to establish a murine femoral critical-size bone defect model using external fixation technique. Our main finding was that a 3-mm defect is needed to achieve a bony segmental nonunion rate of 100% after a 12-wk observation period.

Other investigators have previously used custom-made femoral external fixators for similar purposes. Cheung *et al.* developed an external fixation device composed of two aluminum blocks that were interconnected by two rods [4]. The femur was stabilized by four nonthreaded stainless steel pins. Closed fractures were created and healed after 14 to 21 d. The postoperative observation period was 60 d. Srouji *et al.* described another custom-fabricated external fixation device that uses four 27G needles connected by acrylic dental cement to stabilize the femur [5]. This fixation device penetrated the skin on the lateral and the medial side of the upper leg. Bone defects of up to 3 mm were created with this device. Two-millimeter defects using this device were reported to be of adequate size to achieve a critical-size defect and larger defects of 2.5 and 3 mm were found to be excessively unstable. The postoperative observation period reported by Srouji *et al.* [5] was 8 wk. The external fixator presented in this paper has several advantages compared to the previously described systems. With a weight of 0.20 g, the external fixator used in the present study was three to five times lighter in comparison to the previously described systems' weights of 0.6 to 0.8 g [5] and 1.1 g [4], respectively. The weight of the implant used in our study is thought to have minimal interference with murine biological movement as it comprises only 0.5% of their body weight. Furthermore, the double-threaded pins we used provide increased stability in comparison to the previously described systems. One end of the pin is threaded and locked into the connecting plastic body and the other end is threaded into the femur in accordance with the principles of a locked plate. The pins are made of TAN (medical grade titanium alloy), which is believed to provide favorable conditions for bone healing due to its osteoconductivity [6].

Between the 8th and the 10th postoperative week, we euthanized one animal from the 2-mm group and one from the 3-mm group due to pin loosening. We hypothesize that a chronic pin infection with subsequent osteolysis may have been the cause. Another animal died due to a probable systemically induced abscess on a cubital joint. This finding underscores a disadvantage of external fixation systems when compared to internal fixation systems—the skin-penetrating pins provide an ongoing potential risk of infection [7]. One may be able to reduce this risk with frequent cleaning and disinfection of the device, as is done for humans.

We found μ CT to be the best imaging modality to evaluate the bone morphology of mouse femurs. Artifacts resulting from metal implants currently present a challenging imaging issue. The use of an intramedullary nail or an internal metal-based plate makes it very difficult to obtain high-quality images of the defect area. The described external fixation device allowed us to get an undisturbed scan of the defect area, as the plastic body and the TAN pins do not adversely affect the imaging scan.

Several technical and genetic reasons may explain why different studies have found various critical defect sizes in mice. Beamer *et al.* showed that the adult bone density of different

inbred strains of mice can be significantly different and that none of the parameters (femur length, density, bone mineral content, volume) was related to the body weight of the mouse [8]. The fact that the length of a mouse femur increases up until the age of 8 to 12 wk argues strongly for our opinion that mice used for critical-size bone defect models should be at least 12 wk old. Lu *et al.* demonstrated a sharp decline in fracture healing ability between juvenile and middle-aged animals, and a more subtle decrease in healing ability was observed between middle-aged and elderly mice [9].

Immunodeficient murine animals offer enormous possibilities to investigate xenograft tissues and cells; however, the results of these investigations should be carefully interpreted in relation to the immunocompetent state and the relevance to human bone healing. Generally, mice and rats do not have a Haversian system like that of higher mammals or humans [10]. Furthermore, adult nude mice have a marked lack of T lymphocytes [11]. Barbul *et al.* found that T lymphocytes play a dual role in wound healing [12]. They described an early stimulatory role on macrophages, endothelial cells, and fibroblasts, and a later counter-regulatory role, which may be responsible for physiological wound healing. This may impact regenerative capacity and thereby affect bone healing. However, Gan compared the bone healing of immunodeficient nude mice with immunocompetent mice and detected no difference [13].

We did not detect statistically significant differences between the three groups with respect to cell numbers stained positively with both bone resorption (TRAP) and bone formation (osteocalcin, osteopontin, and osteonectin) markers. This is not surprising, as no attempts were made to influence fracture healing. The principal mechanism of bone repair is the same at the bone ends irrespective of the defect size. However, these numbers may serve as a baseline for future experiments. It is conceivable that, with modification of the materials used to bridge the defect, the cellular reaction will be different if bone healing can be successfully influenced [14].

In addition to the external fixation device used in our study, there are also alternative concepts to create a femoral bone defect in mice. Stabilization of a femoral defect site can be achieved by either intramedullary or extramedullary fixation, each with its own unique set of advantages and disadvantages. The advantages of intramedullary fixation are its ability to minimize soft tissue irritation while providing high axial stability. A disadvantage of this method is that the intramedullary nail causes irritation of the endosteum and the bone marrow. Alternatively, an external fixator [4,5] or a plate [15] can be applied to stabilize the defect site. A lateral approach must be used for this method, as the femur must be exposed from knee to femoral head and at least four pins or screws must be drilled directly into the femoral shaft. The disadvantage of this method is a potentially higher rate of periosteal and soft tissue compromise as compared with intramedullary fixation. The advantages of this method, however, are the ability to protect the endosteum and the bone marrow. Using a femoral bone graft model, Zhang *et al.* demonstrated that 70% of newly formed bone mass is attributed to the expansion and differentiation of donor periosteal progenitor cells [16]. This explains why bone healing is negatively affected when the periosteum is disrupted [17]. Recent findings that bone marrow [18], blood vessels [19], and the soft tissue [20] around the fracture are sources of osteoprogenitor cells explain why loss of these tissues has a

negative impact on bone healing. These findings demonstrate that the plates used in extramedullary fixation can have a detrimental effect on bone healing, as they have a large periosteal contact area. External fixators have the advantage of preserving the soft tissue and the periosteum at the fracture site, thereby avoiding a distorting influence on bone healing.

5. Conclusions

A segmental osseous nonunion was reliably produced in nude mice by creating a 3-mm segmental femoral bone defect maintained by external fixation. This mouse model allows translational evaluation of tissue engineering concepts for site-specific bone regeneration, including strategies using allogeneic and xenogeneic cell types and tissues.

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Fig. 1.

Surgical procedure: implantation of the femoral external fixation device (A) in nude mice. Each mouse was placed in the prone position (B). A 12 mm incision was performed (C). The quadriceps femoris muscle was mobilized anteriorly towards the knee and the hip using 2 spatulas (D). A sharp elevator was used to expose the distal femur and the first hole was drilled (E). The first pin was put into one of the lateral holes of the plastic body of the MouseExFix and drilled into the distal femur (F). The proximal part of the femur was exposed (G). The second hole was drilled into the proximal femur or the lateral femoral neck (H). At last the inner two pins were placed (I). Two Gigli wires were placed around the femur and into the fixed saw guide on the MouseExFix body. (J–M). The defect was cut while the body of the fixation device was stabilized with two clamps (M). The femur and surrounding structures were guided back into their natural anatomic positions (N). Skin was closed with interrupted sutures (O).

Fig. 2.

Defect size progress throughout 12 wk as evaluated by x-ray. The defect sizes (mean and SD) of all groups (1-mm, 2-mm, and 3-mm) decreased until 12 wk postoperatively. At each time point the difference between all groups was statistically significant (* $P < 0.05$).

Fig. 3.

X-ray images, μ CT scans, and hematoxylin–eosin-stained micrographs of the murine femora. (A–C) A, B, and C show postoperative x-ray images of 1-, 2-, and 3-mm femoral bone defects stabilized by an external fixation method, corresponding to columns 1, 2, and 3. (D–F) X-rays after 12 wk. (D) and (E) correspond to osseous unions, (F) to a nonunion. (G–I) MicroCT-based reconstructions of the initial defect area at 12 wk. (G) and (H) denote bony unions, (I) a nonunion. (J–L) Hematoxylin–eosin-stained micrographs defined according to Huo *et al.* [3] 12 wk postoperatively scored 10, 9, and 2, respectively, thereby indicating union of fracture fragments by mature bone (J), union of fracture fragments by immature bone (K), and nonunion with predominantly fibrous tissue with a small amount of cartilage (L).

Fig. 4.

Defect volumes after 12 wk as evaluated by μ CT. The asterisk denotes significant intergroup differences in all combinations (* $P < 0.001$).

Fig. 5.

Percent reduction of the defect size after 12 wk evaluated by combined x-ray and μ CT. The initial (day 0) cylindrical defect area was analyzed by x-ray and compared to a three-dimensional CT reconstruction of the defect at 12 wk. The 1-mm and 3-mm groups demonstrated the highest and lowest defect reduction rate, respectively. A significant difference between all groups was shown at the end of the observation period (* $P = 0.0152$).

Fig. 6. Histologic analysis of hematoxylin–eosin-stained defect areas scored according to Huo *et al.* [3]. The median scores for fracture healing were 8, 4.5, and 2.5 for the 1-, 2-, and 3-mm group, respectively. *Post hoc* testing showed a significant difference in the histomorphologic pattern between the 1-mm group and the 3-mm group (* $P = 0.038$), indicating a significantly lower stage of fracture healing in the 3-mm group.

Fig. 7.

Immunohistologic analysis of TRAP-stained osteoclasts present in the regenerated bone tissue. The highest density of osteoclasts was observed for the 1-mm group; a slightly lower amount of cells were counted for the 2-mm group and the 3-mm group. No statistically significant intergroup differences were observed.