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Development of a Reliable Non-Union Model in Mice

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Background. **Despite the growing knowledge of the mechanisms of fracture healing, non-unions remain a substantial clinical problem. There is increasing interest in murine fracture models because they would allow studying molecular mechanisms of healing with the help of specific antibodies and gene-targeted animals. However, until now it has not been possible to reproducibly create non-unions in mice. Thus, the aim of this study was to develop a reliable non-union model in mice.**

Methods. **After creating segmental defects of 0.8 mm and 1.8 mm, mice femora were stabilized with a pin-clip fixation. Additionally, the influence of periosteal resection on the development of non-unions was studied. Histological and radiological healing was analyzed 5, 10, and 15 wk after surgery.**

Results. **After 10 wk all animals showed poor healing with predominantly atrophic non-unions. Whereas the 0.8 mm and the 1.8 mm gap with intact periosteum showed radiologically in 4/6 and 3/6 cases possible healing, only the gap of 1.8 mm with additional periosteal resection resulted in 100% (6/6) non-unions. The non-unions were confirmed also after 15 wk and appeared atrophic with typical histological and radiological features. These included lack of fracture bridging with abundant fibrous tissue in the gap, absence of callus formation, and rounded bone ends. Of interest, the non-unions were not avascular, but demonstrated a considerable vascularity within the fibrous tissue.**

Conclusion. **Taken together, we herein demonstrate for the first time a reliable non-union model in mice. This allows us to study molecular aspects of non-union formation and analyze different therapeutical strategies in these animals.**

Key Words: **fracture healing; bone healing; mouse; animal model; musculoskeletal; non-union; delayed union; pseudarthrosis; bone; vascularization.**

INTRODUCTION

Despite the growing knowledge of the mechanisms of fracture healing, delayed healings and non-unions remain a substantial clinical problem. In the United States, approximately 10% of all fractures fail to heal or show a delayed healing [1]. While most hypertrophic non-unions can be successfully treated with stable osteosynthesis, the treatment of atrophic non-unions is often difficult and highly challenging [2]. Many strategies have been developed to treat atrophic non-unions, but there is no consensus on the optimal management [3]. One reason for this is the fact that the pathophysiological mechanisms are not completely understood. Etiologically, non-unions are hypothesized to be multifactorial, like many other diseases. A main causal factor is thought to be a "poor biology" as indicated by a low vascularization. This is in contrast to recent reports by Reed and coworkers, demonstrating that atrophic non-unions are not avascular in nature [4, 5].

To study the complex mechanisms of non-unions, appropriate animal models are needed. These may contribute not only to the understanding of the mechanisms, but also to the development of novel therapeutic strategies. Several animal models of non-unions have been described in rabbits, rats, and other species [6–12]. Little, however, has been reported in mice, although the use of mice should be of increasing interest in trauma research because this species allows the

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study of the molecular mechanisms of fracture healing due to the availability of a large array of antibodies and gene-targeted strains. To our knowledge, it has not been possible yet to reproducibly create non-unions in mice. Choi and coworkers reported on a murine non-union model using distraction osteogenesis [13]. In this model, it is unclear to which extent the manipulation of distraction parameters resembles the clinical situation of non-union formation. Another limitation of this model is that after a time period of only 27 d, already 40% of the animals studied revealed signs of fracture healing. It further remains unclear whether the remaining 60% of animals, which showed lack of healing after 27 d, would indeed have developed non-unions after a time period of 10 to 15 wk, i.e., 70 to 105 d.

Atrophic non-unions in humans have been defined as the absence of progressive repair between 3 and 6 mo after fracture with the typical radiological signs, including lack of bone bridging, rounded bone ends, sclerosis at the fracture ends, and failure of callus formation [2]. Because there exist no murine definition of atrophic non-unions, murine models should orientate on human definitions. Typical radiological and histomorphometric characteristics of non-unions should be observed also in animal non-union models and the observation time should be long enough to exclude progressive repair.

The aim of the present study was to create a reliable and clinical relevant atrophic non-union model in mice. To achieve this, we decided to use segmental bone defects of different sizes in the mouse femur. Because the periosteum strongly contributes to the healing process and mainly fractures with severe soft tissue and periosteal injuries develop non-unions [14], we further analyzed the influence of periosteal stripping on non-union formation.

METHODS

Animals

For the *in vivo* studies, male and female CD-1 mice ($35 \pm 3g$, 9 to 14 wk old) were used. Mice were bred at the Institute of Experimental Surgery, University of Saarland, Germany. The animals were kept at a 12-h light and dark cycle, and were fed a standard diet with water ad libitum. All experiments were performed in adherence to the National Institute of Health guidelines for the use of experimental animals and were approved by the German legislation on the protection of animals.

Surgical Procedure and Experimental Protocol

For the surgical procedure, animals were anesthetized by an intraperitoneal injection of 25 mg/kg xylazine and 75 mg/kg ketamine. Under sterile conditions a 4 mm medial parapatellar incision was performed at the right knee and the patella was dislocated laterally. After drilling a hole (Ø = 0.5 mm) into the intracondylar notch, a distally flattened 24G needle was implanted intramedullary and the wound was closed. The pin was flattened at the distal end to prevent secondary pin loss. After insertion of the pin, the middle of the femur was exposed through a lateral approach and a custom-made clip of 6 mm length was implanted ventro-dorsally into the femur using an operating microscope. The diameter of the pin (0.55 mm) was about half of the diameter of the medullary space (1.0 to 1.2 mm). The clip did not fit into the intramedullar pin. Afterward, osteotomies with a gap size of 0.8 mm and 1.8 mm were created under the metallic clip using size-standardized spherical trephines under permanent saline cooling. The gap sizes were controlled by custom-made templates of 0.8 mm and 1.8 mm. The metallic clip inserted before creation of the gap guaranteed that the gap size could be maintained. All procedures were done under an operating microscope with 25× magnification, which resulted in a high level of precision. In some animals, care was taken to leave the periosteum intact (groups PI). In the other animals the periosteum was stripped (groups PS) 2 mm proximal and distal to the gap along the longitudinal axis. In all animals, wound closure completed the procedure. Animals were killed after 5 and 10 wk by cervical dislocation, and fracture healing was evaluated by histomorphometric, immunohistochemical, and radiological analysis. Additionally, the group with a gap size of 1.8 mm and periosteal resection was analyzed after 15 wk to confirm the lack of progressive repair. Each group consisted of 4 to 6 animals at each time point and each parameter.

Radiological Analysis

At the end of the observation time, mice were anesthetized and ventro-dorsal X-rays of the healed femora were taken. Fracture healing was analyzed according to the classification of Goldberg *et al.* [15] with stage 0 indicating radiological non-union, stage 1 indicating possible union and stage 2 indicating radiological union.

Histomorphometric Analysis

At the end of the experiments, the healed femora were resected and fixed in IHC Zinc fixative (BD Pharmingen, San Diego, CA) for 12–24 h, decalcified in 10% ethylenediamine tetraacetic acid solution for 4 wk, and embedded in paraffin. The implants were removed after the decalcification process to preserve fracture morphology. Longitudinal sections of 5 μm thickness were cut and stained according to the trichrome method. At a magnification of 1.25× (Olympus BX60 Microscope; Olympus, Tokyo, Japan; Zeiss Axio Cam and Axio Vision 3.1; Carl Zeiss, Oberkochen, Germany; ImageJ Analysis System, NIH, Bethesda, MD) structural indices were calculated according to the recommendations of the American Society of Bone and Mineral Research (ASBMR). These included (1) periosteal callus area/femur diameter [Pc.Ar/F.Dm (mm)], (2) maximum callus diameter/femur diameter [Cl.Dm/F.Dm], (3) gap width [mm], and (4) tissue distribution of bone, cartilage, fibrous and fat tissue within the gap area [%]. Periosteal callus area was defined by the outer diameter of the callus in radial direction and 2 mm proximally and distally from the fracture gap in axial direction. The gap area was defined by bone ends in axial direction and 0.25 mm laterally and medially from the center of the gap in radial direction. In addition, a scoring system was used to evaluate the quality of fracture bridging. Both cortices were analyzed for bone bridging (2 points), cartilage bridging (1 point), or bridging with only fibrous tissue (0 point). This score system allows a maximum of 4 points, indicating complete bone bridging, and a minimum of 0 points, indicating complete lack of fracture healing.

Immunohistochemical Analysis

Staining of endothelial cells was done as described previously in detail [16]. In brief, tissue sections were deparaffinized in xylene and rehydrated in a descending, graded series of alcohol. Endogenous peroxidase was blocked by 3% H_2O_2 (10 min). After blocking unspecific binding sites with phosphate-buffered saline and goat normal serum (30 min at room temperature), sections were incubated over night with rat anti-mouse PECAM-1 (CD31) monoclonal antibody (1:25 phosphate-buffered saline; BD Pharmingen) at room temperature. Peroxidase-conjugated goat anti-rat (1:100; Dianova, Hamburg,