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Decisive differences in the bone repair processes of the metaphysis and diaphysis in young mice

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ABSTRACT

Fractures are common traumatic injuries that mainly occur in the metaphyses of long bones such as the proximal humerus, distal radius, and proximal femur. However, most studies of fracture repair processes have focused on the diaphyseal region. In this study, we compared the bone repair processes of the metaphysis and the diaphysis of the mouse tibia. Bone apertures were formed in the tibial metaphysis and diaphysis. At indicated times after surgery, samples were collected, and the healing process was investigated using micro-computed tomography, as well as histological, immunohistochemical, and mRNA expression analyses. In the metaphysis, cartilage formation was not detected on the periosteal side. The bone aperture was filled with newly formed bone produced from bone marrow at day 7. In the case of the diaphysis, cartilage was formed around the aperture at day 4 and sequentially replaced by bone on the periosteal side. The bone aperture was filled with newly formed bone at day 14. In the bone marrow, expression of the osteogenic markers such as alkaline phosphatase, osteocalcin, and type I collagen, appeared earlier with metaphyseal injury than with diaphyseal injury. The mRNA expression of chondrogenesis markers was markedly upregulated in the diaphysis compared with that in the metaphysis on the periosteal side. These results indicate differences in the bone repair processes of the two regions, suggesting functional heterogeneity of the periosteum and bone marrow mesenchymal cells in response to bone fractures.

1. Introduction

Long bones are anatomically divided into a cancellous bone-rich metaphysis at each end and the cortical-rich diaphysis in the center (Standring, 2015). In humans, fractures are common traumatic injuries that mainly occur in the metaphyseal regions of long bones such as the proximal humerus, distal radius, and proximal femur (Hedström et al., 2010; Driessen et al., 2016). However, most studies of the bone repair process have focused on the diaphyseal region, in which bone repair has been widely studied (Schindeler et al., 2008; Einhorn and Gerstenfeld, 2015). Experimental models for bone repair in the diaphysis have involved various animals, methods, and ages (Histing et al., 2011; Mills and Simpson, 2012). In the diaphysis, the bone repair process is divided into four histological stages on the periosteum side: inflammation, soft (cartilaginous) callus formation, hard (bony) callus formation, and remodeling (Schindeler et al., 2008; Einhorn and Gerstenfeld, 2015). The initial stage after injury is characterized by hematoma formation and subsequent inflammation. After inflammation, a cartilaginous callus is formed around the fracture site and then is gradually replaced by a bony callus. The bony callus is remodeled to the original bone architecture by osteoclasts. The presence of the bony callus is one of the important criteria for assessment of fracture union (Corrales et al., 2008). As a commonly used model for closed fractures, an intramedullary pin is inserted into the medullary canal of a long bone, and then the bone is bent or cut to produce a stable fracture (Bonnarens and Einhorn, 1984); thus, in this fracture model the medullary callus has been ignored.

In contrast, it has been suggested that stable metaphysis fractures are repaired by direct bone formation within the bone marrow and that cartilaginous and bony callus formation are not observed on the periosteum side (Jarry and Uhthoff, 1971; Uhthoff and Uhthoff and Rahn, 1981; Chen et al., 2015; Han et al., 2015). The histological stages of the metaphyseal repair process are also different from those of the diaphyseal repair process (Chen et al., 2015; Han et al., 2015). Han (Han et al., 2015) reported that the metaphyseal repair process is divided into five histological stages in the bone marrow. The first stage is characterized by a bleeding event, and inflammation is reduced compared to that observed in the diaphysis. The second stage is mesenchymal stem cell activation and differentiation into osteoblasts, and the third stage is the formation of woven bone. The fourth stage is characterized by the transformation of the newly formed woven bone to lamellar bone, followed by a shift to the final stage of continuous bone

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remodeling. In humans, the repair process of the metaphysis also lacks cartilage formation, and direct bone formation has been observed in bone marrow by biopsies of distal radius fractures (Aspenberg and Sandberg, 2012). In the repair processes of the two sites, the effects of several drugs are also different (Sandberg and Aspenberg, 2015a; Sandberg and Aspenberg, 2015b; Sandberg et al., 2016). Non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids inhibit diaphysis healing but not metaphysis healing (Sandberg and Aspenberg, 2015a; Sandberg and Aspenberg, 2015b). Furthermore, alendronate, a bisphosphonate that inhibits osteoclastic bone resorption, increases the amount of medullary callus earlier in the metaphysis than in the diaphysis (Sandberg et al., 2016). These results indicate clear differences in the repair processes and effects of drugs between the two sites.

Mesenchymal stem cells residing close to the bone surface exhibit different activities (Siclari et al., 2014; Guarnerio et al., 2014) and stronger osteogenic potential than those in the central bone marrow (Siclari et al., 2014). The cancellous bone-rich metaphyseal region has more mesenchymal progenitors than the diaphyseal region. Generally, bone repair in the cancellous bone-rich metaphysis is considered to progress faster than that in cortical bone-rich sites such as the diaphysis (Brown et al., 2014), but there is no clear evidence of time-specific kinetic differences in bone repair between these two regions.

In terms of bone repair processes, it is important for fracture management to have a clear understanding of the time course of the appearance of callus and the cells related to bone repair. However, there have not yet been any reports that have chronologically analyzed callus formation and bone repair-related cell differentiation directly in both regions of bone. Fracture in long bone healing in humans is quite similar, but not completely identical to that described for laboratory animals (Postacchini et al., 1995). In this study, we used adolescent mice as experimental animals, and compared the repair processes under mechanically stabilized conditions of the metaphysis and diaphysis of tibia.

2. Materials and methods

2.1. Animals

Male ICR mice (8 weeks old) were obtained from Sankyo Laboratories (Tokyo, Japan) and maintained under specific pathogen-free conditions.

2.2. Bone injury model

A total of 78 mice were divided into two groups: the metaphysis injury group (39 mice) and the diaphysis injury group (39 mice). Mice from each group were anesthetized using 2% inhalational isoflurane before surgery. The skin of the right hind limb overlaying the tibia was shaved, and then the skin, muscle, and periosteum were incised along the medial part of the tibia. A bone aperture was formed from medial through both sides cortical bone in the metaphysis or diaphysis, respectively, using a round bur (diameter 0.8 mm; AS ONE, Osaka, Japan). Bone apertures were unilateral in each mouse, and the metaphysis and diaphysis were located about 1.5 mm and 6 mm from the growth plate, respectively (Fig. 1A). The skin was sutured at the end of surgery. This model was stable without external fixation (He et al., 2011). There was no additional injury around the bone aperture during the healing period, and mice exhibited the normal walking activity.

The injured right tibiae were collected day 3, 4, 5, 7, 14, 21, 28, 35, and 42 after surgery (n of 6 at day 3, 4, 5, 7; n of 3 at day14, 21, 28, 35, 42).

2.3. Micro-computed tomography (micro-CT)

Tissue samples were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Newly formed bone was imaged ex vivo using the ScanXmate-L090H (Comscan Techno, Kanagawa, Japan). Imaging conditions were as follows: 87 kV, 37 μA, voxel resolution 14 μm per voxel, and 992 × 992 pixel image matrices. Three-dimensional imaging data were reconstructed by conneCT express software (White rabbit, Tokyo, Japan). Newly formed bone and bone mineral density (BMD) were analyzed using TRI-3D BONE (Ratoc CO, Tokyo, Japan). BMD calibration was performed using phantom materials with known bone mineral densities. Regions/measurements of interest included the periosteal region (periosteal callus, bone volume (BV), mm^3), bone marrow [medullary callus, bone volume/tissue volume (BV/TV), %], and bone defect area (BMD, mg/cm^3), as shown Fig. 1B.

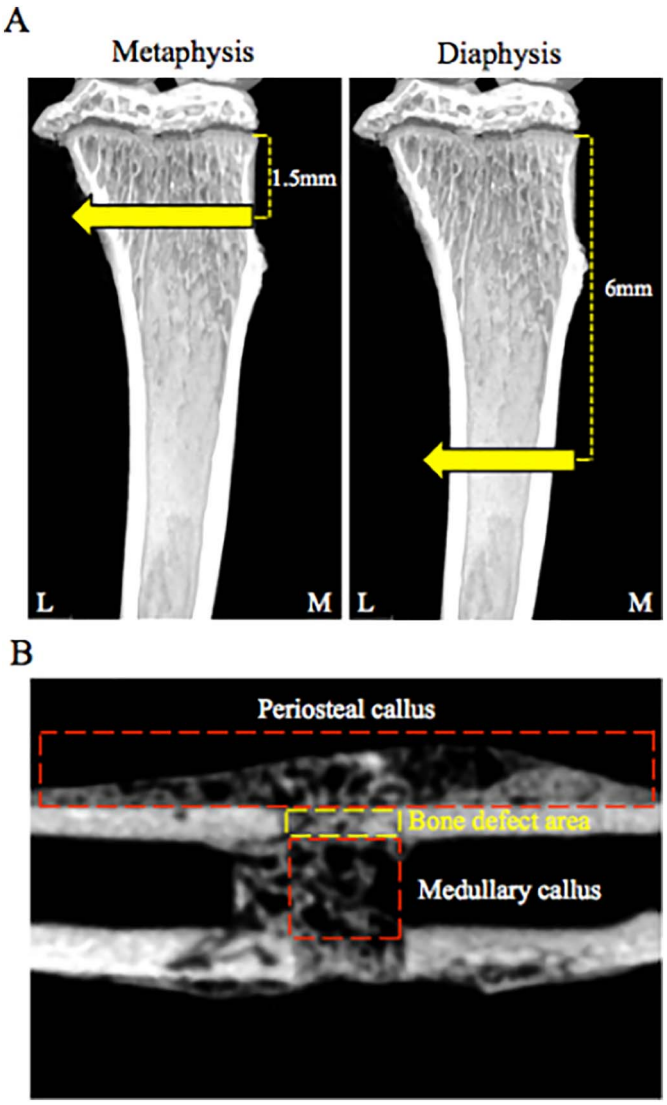

Fig. 1. Schematic diagram of the bone injury site and region of interest (ROI).
A. Bone aperture in the metaphysis and diaphysis were located about 1.5 mm, 6 mm from growth plate, respectively.
B. ROI for analysis periosteal callus, medullary callus, and BMD.

2.4. Histological and immunohistochemical analyses

After analyzed by micro-CT, samples were decalcified with 10% EDTA; some samples were dehydrated in a graded series of ethanol followed by xylene and then embedded in paraffin. To confirm cartilage formation, sections (5 μm in thickness) were stained with toluidine blue at pH 4.1, or with safranin O/fast green, respectively. Toluidine blue-stained images were taken at × 4 magnification using a microscope (Bz-700-All-in-one; KEYENCE, Osaka, Japan), and metachromatic areas