Acta Biomaterialia 10 (2014) 4009–4019

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Mechanical and structural properties of bone in non-critical and critical healing in rat ☆

Rebecca M. Hoerth [a,b], Britta M. Seidt [a], Miheer Shah [a], Carolin Schwarz [b,c], Bettina M. Willie [c], Georg N. Duda [c], Peter Fratzl [a], Wolfgang Wagermaier [a,*]

[a] Max Planck Institute of Colloids and Interfaces, Department of Biomaterials, Research Campus Golm, 14424 Potsdam, Germany
[b] Berlin-Brandenburg School for Regenerative Therapies (BSRT), Charité – Universitätsmedizin Berlin, Campus Virchow Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany
[c] Julius Wolff Institute & Center for Musculoskeletal Surgery, Charité – Universitätsmedizin Berlin, Campus Virchow Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany

ARTICLE INFO

Article history:
Received 18 March 2014
Received in revised form 19 May 2014
Accepted 3 June 2014
Available online 12 June 2014

Keywords:
Bone healing
Nanostructure
Critical size defect
Mechanical properties
X-ray scattering

ABSTRACT

A fracture in bone results in a dramatic change of mechanical loading conditions at the site of injury. Usually, bone injuries heal normally but with increasing fracture gaps, healing is retarded, eventually leading to non-unions. The clinical situation of these two processes with different outcomes is well described. However, the exact relation between the mechanical environment and characteristics of the tissues at all levels of structural hierarchy remains unclear. Here we studied the differences in material formation of non-critical (1 mm) and critical (5 mm gap) healing. We employed a rat osteotomy model to explore bone material structure depending upon the different mechanical conditions. In both cases, primary bone formation was followed by secondary bone deposition with mineral particle sizes changing from on average short and thick to long and thin particles. Bony bridging occurred at first in the endosteal callus and the nanostructure and microstructure developed towards cortical ordered material organization. In contrast, in critical healing, instead of bridging, a marrow cavity closure was formed endosteal, exhibiting tissue structure oriented along the curvature and a periosteal callus with less mature material structure. The two healing processes separated between 4 and 6 weeks post-osteotomy. The outcome of healing was determined by the varied geometrical conditions in critical and non-critical healing, inducing completely different mechanical situations.

1. Introduction

The capability of bone to structurally adapt to changes of the mechanical environment is based on remodeling of the material [1,2]. Bone is deposited wherever mechanically needed and is resorbed in regions without mechanical need. Bone remodeling can be investigated by changing the mechanical situation of a selected bone and studying the resulting changes in bone mass and structure [1]. Bone regeneration after injury is an extreme situation from a medical perspective which may also change the mechanical environment drastically. In particular, critical defect healing, i.e. the regeneration of a large bone defect, is a well-known and still challenging problem in human orthopedic medicine. Such large defects can result, for example, from resection of large bone parts during cancer treatment or from high-energetic traumatic events [3]. They often end up in a delayed union or a non-union situation, where a pseudarthrosis is established instead of normally healed bone with restored shape and function.

Injured bone is able to recover from damage [4–6] in a very complex, but also highly coordinated process [6], undergoing changes in composition, structure and mechanical properties. Several successive stages can be distinguished in secondary bone healing [5–7]: an inflammatory phase is followed by intramembranous woven bone deposition far away from the fracture site and cartilage callus formation near the fracture, which later ossifies [4,5,7]. This primary bone gets remodeled and replaced by lamellar bone and redundant callus tissue gets resorbed [4], leading to restoration of shape, functionality and properties. This two-wave process was, for example, shown in a sheep osteotomy model [8]. From a mechano-biological point of view, healing depends especially on the mechanical stimulation of the tissue [9] and of the cells [10] around the fracture gap. The establishment of a bony bridge protects the still fragile tissue within the fracture gap from high stresses by transferring load across the gap, enabling further endochondral ossification [11]. Hence, the formation and

☆ Part of the Biomineralization Special Issue, organized by Professor Hermann Ehrlich.

* Corresponding author. Tel.: +49 331 567 9459; fax: +49 331 567 9402.
E-mail address: Wolfgang.Wagermaier@mpikg.mpg.de (W. Wagermaier).

http://dx.doi.org/10.1016/j.actbio.2014.06.003
1742-7061/

differentiation of different callus tissues strongly depends on the loading history [12].

Indeed, bone and callus are hierarchically structured materials and their properties depend on structural features from the millimeter to the nanometer range. The nanometer range consists of carbonated hydroxyapatite platelets and an organic collagen matrix, building up mineralized collagen fibers, the basic building blocks [13,14]. Only a few studies have taken into account the mechanical and nanostructural properties of the bone material itself. Although the clinical situation of healing is well described, the differences between bone material in critical and non-critical healing with regard to the different loading conditions have not yet been addressed.

In this study, we investigate a rat femoral osteotomy model with different gap sizes at different time points during healing. In earlier publications from the underlying study, 1 and 5 mm osteotomies were histologically analyzed (6 and 8 weeks post-osteotomy) by means of differences in tissue composition between both groups [15]. The reproducibility of the atrophic non-union with a prolapse of surrounding soft tissue into the defect and the formation of a bony closure of the marrow cavity was described, for the critical healing model [15]. Furthermore, these osteotomies were analyzed for bony callus formation, patterning and mineralization. The experimental data were combined with finite element analysis, based on micro-computed tomography (μCT) data (2, 4 and 6 weeks post-osteotomy) [16]. It was observed that early mechanical stimuli crucially influence callus patterning [16]. In consequence, with this osteotomy model we found a perfectly suited way to investigate the influence of a bony bridging on the characteristics of the bony tissue and the healing outcome. 1 and 5 mm femur osteotomies were externally fixed and left empty (i.e. no grafts, scaffolds or growth factor treatments) so that the actual gap size is the only difference between the two situations. The bony bridging in the non-critical healing situation alters the mechanical environment dramatically.

In the present study, we aimed at identifying when and to what degree tissue structure and organization diverge between the two healing process. We characterized the changes in compositional, mechanical and structural properties using environmental scanning electron microscopy (ESEM) with backscattered electron imaging (BSE), nanoindentation (NI) and scanning small- and wide-angle X-ray scattering and diffraction (SAXS/WAXD). By comparing the nanoscale architecture of bone material during healing, we propose a model where the same processes at the material scale lead to both scenarios, a regenerated bone in non-critical healing and a non-union in critical healing, just because of different geometric constraints.

2. Materials and methods

2.1. Sample preparation

Externally fixed non-critical healing and critical size femoral osteotomy models, defined by the osteotomy gap size (1 and 5 mm, respectively) were investigated and therefore 16 Sprague Dawley rats (12 weeks old, weight 250–300 g, Charles River Deutschland GmbH, Sulzfeld, Germany) were separated in two groups of eight animals each. The operative procedure has been previously reported [15,17]; for more details see Supplementary Information. All animal experiments were carried out according to the policies and procedures approved by the local legal representative (LAGeSo Berlin, G0071/07). After 2, 3, 4 and 6 weeks of healing, two animals per group were sacrificed by intracardiac potassium chloride injection, while under deep anesthesia. The osteotomized and contralateral femurs were harvested and subsequently fixed in 10% formalin, dehydrated in ascending grades of ethanol and then put in Xylol. The samples were embedded in methylmethacrylate (MMA; Technovit® 9100 new). We denote the non-critical healing samples with N and the critical size defect samples with C, indicate the number of weeks post-osteotomy with a number and specify the first and the second sample with I or II (e.g. the 2 weeks non-critical healing first sample is named N2I; see Supplementary Information, Table S.1).

2.2. ESEM/BSE

For characterizations with an environmental scanning electron microscope, the embedded samples were cut with a low-speed diamond saw (Buehler Isomet, Buehler GmbH, Duesseldorf, Germany) in a longitudinal direction and polished. ESEM (FEI Company, Oregon, USA) with a backscattered electron detector at 200- or 250-fold magnification was performed. An acceleration voltage of 10 or 12.5 kV, a working distance of ~10 mm and a low vacuum environment (0.75 Torr) were used for the measurements. Single images were then put together using the software provided with the device and Photoshop CS5 (Adobe Systems, Munich, Germany). ESEM/BSE images give information about the mineralization of the analyzed tissue. Brighter grey values signify qualitatively higher calcium content as Ca is the element with the highest atomic number present within the tissue [18].

2.3. NI

NI experiments were performed to investigate the mechanical properties of the newly formed bone compared to those of preexisting cortical bone within two C6 samples and one N6 sample, see also Table S.1. Scanning NI measurements were conducted with a NI device (Hysitron Inc., Minneapolis, USA) using a Berkovich diamond indenter tip. The sample was cut as a block and polished to obtain a smooth and plane-parallel surface (PM5, Logitech, Glasgow, UK, using diamond spray, grain size 0.25 μm) and then mounted on the sample stage. To better understand the healing outcome, the mechanical properties were evaluated for several specific regions: the middle and the outer cortex, the callus in the outer, the middle part or adjacent to the cortex, the callus near the osteotomy or in the osteotomy and for the critical healing samples the callus of the closure (Table 1). The evaluated area was ~100 μm × 200 μm per region, consisting of 11 × 21 measurement points, with a step size of 10 μm (for an exact number of indents, see Table 1). The measurement procedure was described previously [19]. Briefly, the starting point of the measurement was specified using a light microscope. The scanning measurement and the approaching of the indenter tip towards the sample were performed using a Triboscanner. The loading of each indentation consisted of a first loading phase (loading rate 1000 $\mu N\,s^{-1}$), a first resting phase (loading 5000 μN for 60 s), a first unloading phase (unloading rate 400 $\mu N\,s^{-1}$), a second resting phase (1000 μN for 10 s) and a second unloading phase (unloading rate 200 $\mu N\,s^{-1}$). Hardness H and indentation modulus E_r were calculated as described before [19] and measurement points with indentation moduli below a threshold of 10 GPa were excluded, since they were attributed to the embedding material or next to holes within the bone. Mean values and standard deviations were determined for all the regions and given as mean values and standard deviations in Table 1.

2.4. SAXS

Longitudinal sample sections were ground down to a thickness of ~200 μm by hand using 1200 silicon carbide grinding paper (grain size 15 μm). The samples were mounted on a motorized