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Full Length Article

Healing of fractures in osteoporotic bones in mice treated with bisphosphonates – A transcriptome analysis☆

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ABSTRACT

Bisphosphonates (BP) are inhibitors of bone resorption and are used to treat postmenopausal osteoporosis. Long-term treatment with BP attenuates bone remodeling, possibly leading to detrimental consequences for the bones' ability to repair defects. To test this hypothesis, an animal model was established. Twelve week old mice were ovariectomized (*OVX*). Following confirmation of bone loss 8 weeks after *OVX*, the animals were treated with Alendronate (ALN) until sacrifice. After 5 weeks of ALN injections, the femoral bones were osteotomized and the osteotomies were either rigidly or non-rigidly stabilized. In rigidly fixed defects, no callus developed between 1 and 5 weeks after osteotomy, whereas after non-rigid fixation, callus development occurred. The administration of ALN resulted in an increase in newly formed bone at the defect site 5 weeks after osteotomy, irrespective of the estrogen status or fixation system. Transcriptome analysis demonstrated that both rigid and non-rigid fixation affected gene expression primarily during the middle phase of bone repair. Furthermore, the number of differentially expressed genes in tissues from non-rigidly fixed defect sites increased in animals treated with ALN over the course of bone repair. This indicates that ALN-dependent repair processes become increasingly dominant in the late phases of the healing process. Ranking of the factors affecting the composition of the transcriptome and their impact on the healing process revealed fixation at the defect site to be the strongest causative factor, followed by bisphosphonate treatment and estrogen deficiency. The present study suggests that the continuous administration of ALN is detrimental to bone repair, eventually causing a delay in healing in mechanically compromised situations. Consequently, rigid fixation may prove essential for a successful intervention.

1. Introduction

Bisphosphonates (BP) are widely used for the treatment of postmenopausal osteoporosis [1,2]. Treatment of osteoporotic patients with alendronate (ALN), the most frequently prescribed BP, results in a reduction of fractures of the hip, wrist and spine [3,4]. The beneficial effects are achieved by the suppression of bone resorption, followed, through a coupling mechanism, by a reduction in bone formation [3–9]. Due to this mechanism of action, the widespread use of BP and the fact that elderly osteoporotic patients are prone to fractures, there exists a vivid interest in understanding the effects of BP on bone repair.

The process of bone repair is characterized by a sequence of distinct phases that, to a large extent, parallel wound healing. An initial phase of inflammation and removal of debris and blood clots is followed by primary stabilization of the defect site through membranous or endochondral bone formation. The repair process is concluded by removal of the primary woven bone, which is replaced by lamellar bone. The pathway of repair depends on the mechanical stability at the defect site. Non-rigid fixation of a defect, allowing for inter-fragmentary motion, favors endochondral bone formation and is characterized by the formation of a cartilaginous callus to enhance primary stability. This process is followed by subsequent remodeling and restoration of the original shape [10,11]. In rigidly fixed defect sites and in the absence of inter-fragmentary motion, intramembranous bone formation will occur. The healing process is characterized by the absence of a callus and a direct remodeling of the Haversian canals [12]. Elucidation of the molecular events in bone repair is essential for the prediction and treatment of the aberrant healing of bone defects.

The potentially adverse effects of BP on fracture healing have been investigated in animal models. Most notably, studies have demonstrated that rats [13–15] and mice [16,17] under treatment with BP experienced a delay in the remodeling of the cartilaginous callus in non-

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vertebral fractures. The elucidation of signaling pathways activated during defect repair has revealed crucial involvements of the TGF-β [18,19], Wnt [20] and TNF-α pathways [21,22]. Transcriptome analyses have been performed on different animal models of fracture repair [23,24]. Detailed knowledge on the molecular events involved in normal and impaired bone repair processes, however, remains scarce. Moreover, the interplay between the mechanical environment, estrogen depletion and BP therapy and their respective effects on bone repair are relatively unexplored. The aim of the present study was to elucidate gene expression during fracture healing in a femoral defect model in dependence of rigidity of fixation, treatment with BP and estrogen depletion and to combine the data with histology and MicroCT analysis. The data provides evidence as to whether treatment with BP exerts detrimental effects on the repair of osteoporotic bone and also demonstrates which of the three parameters, has the strongest modulatory effects on bone healing.

2. Methods

2.1. Reagents

Alendronate was purchased from Sigma-Aldrich (Buchs, Switzerland). The anesthetic mix for surgery and peripheral Quantitative Computer Tomography (pQCT) measurements contained fentanyl dihydrogen citrate (Sintetica, Switzerland), Dorbene® (Dr. E. Graeub AG, Bern, Switzerland) and Dormicum® (Roche, Basel, Switzerland). The antidote mixture was composed of Alzane (Dr. E. Graeub AG, Bern, Switzerland), Anexate (Roche, Basel, Switzerland) and Temgesic (Reckitt Benckiser Healthcare Ltd., Berkshire, UK). The antidote mix to end anesthesia after pQCT measurements was the same, except for Temgesic, which was replaced by Naloxon (Orpha Swiss, Küsnacht, Switzerland).

2.2. Experimental design

This study was approved by the Local Committee for Animal Experimentation (Bern Committee for the Control of Animal Experimentation, Bern, Switzerland, permit number BE10/14 to WH). According to local regulations, animals were kept in groups in the specific pathogen free (SPF) facility of the Medical Faculty of the University of Bern following FELASA regulations [25]. In this study, 12 week old female *C57Bl/6J* mice (Charles River, Sulzfeld, Germany) were assigned to one of the 8 experimental groups representing the possible combinations of surgical and treatment protocols including the following parameters: *sham/ovariectomy* (*OVX*), vehicle/ALN, and rigid/non-rigid fixation of the osteotomy (MouseFix™ rigid/MouseFix™ non-rigid). The experimental design and the time course of the *in vivo* study are depicted in Fig. 1. Briefly, at the start of the experiment, animals underwent either *OVX* or *sham* surgery. Vehicle (control)/ALN treatment was initiated 8 weeks after *OVX/sham*, and continued until sacrifice of the animals. A femoral defect was introduced 5 weeks after the onset of vehicle/ALN treatment and was stabilized either with a rigid (MouseFix™ rigid; RISystem AG, Davos, Switzerland) or non-rigid (MouseFix™ flexible; RISystem AG, Davos, Switzerland) osteosynthesis system. For surgical procedures, the mice were anaesthetized by subcutaneous injections (2 ml/kg body weight) of fentanyl dihydrogen citrate (0.05 mg/kg body weight; 0.02 mg/ml)/medetomidine hydrochloride (0.5 mg/kg body weight; 0.2 mg/ml)/climazolame (5 mg/kg body weight; 2 mg/ml). Post-operatively, an antidote of Alzane (1.1 mg/kg; 0.22 mg/ml)/Anexate (0.45 mg/kg; 0.09 mg/ml)/Temgesic (0.075 mg/kg; 0.015 mg/ml) was injected *s.c.* (2.5 ml/kg body weight). The antidote used after pQCT measurements was composed of Alzane (1.02 mg/kg; 0.17 mg/ml)/Anexate (0.42 mg/kg; 0.07 mg/ml)/Naloxon (0.6 mg/kg; 0.1 mg/ml) and was injected *s.c.* (3 ml/kg body weight). Group sizes were n = 6 for histological and MicroCT analyses and n = 3 for RNA sequencing. The mice were sacrificed at 3, 7, 14 and 28 days after osteotomy for the purpose of RNA sequencing. All animals (192) were subjected to pQCT analysis.

2.3. Ovariectomy

For *OVX*, ovaries were approached through two 0.5 cm flank incisions at the mid-dorsum. The ovaries were located, clamped and removed (gently pulled through the incisions and a hemostat was placed between the oviduct and the ovaries). The oviducts were ligated and a cut was made between the hemostat and the ovaries. Hemostasis was controlled before replacing the ligated oviducts into the abdomen. The muscle layer was closed with absorbable sutures, and the skin was sutured with a non-absorbable thread. *Sham* operated animals underwent the identical surgical procedure, but without ligation of the oviducts and removal of the ovaries.

2.4. Treatment with alendronate

ALN in 0.9% NaCl (1.61 μmol/kg body weight; 2 ml/kg bodyweight) or vehicle (0.9% NaCl; 2 ml/kg bodyweight) was administered 8 weeks after *OVX* by *s.c.* injection twice weekly until sacrifice of the animals [28].

2.5. Femoral osteotomy

At 13 weeks after *OVX*, and 5 weeks after start of the ALN treatment, the left femora were surgically osteotomized. Briefly, a longitudinal incision in line with the left femur was made into the lateral thigh. The interval between the *vastus lateralis* and the *biceps femoris* was developed to expose the bone, and the *gluteus superficialis* tendon was detached from the *trochanter tertius*. Either a MouseFix™ rigid or MouseFix™ flexible system, manufactured from pure Titanium, was mounted onto the intact femora. Subsequently, by using a Gigli saw, a mid-femur osteotomy of 0.22 mm was created. Care was taken to avoid

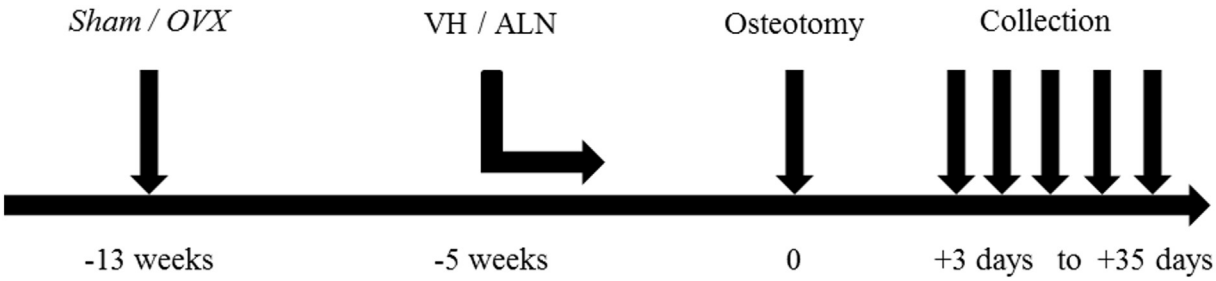

Fig. 1. Experimental design of the ALN-treated osteoporotic femoral defect model. At 13 weeks before the femoral osteotomy, animals were *OVX*- or *sham*-operated. Vehicle or ALN was applied twice/week *s.c.* for 5 weeks prior to osteotomy until sacrifice of the animals. A 0.22 mm femoral mid-diaphysis osteotomy was stabilized with a rigid or non-rigid fixation plate. RNA sequencing was performed on the total RNA isolated from tissues collected at the defect site 3,7,14 and 28 days post-osteotomy. MicroCT and histological evaluations were performed at 7 and 35 days post-osteotomy.