Bone 75 (2015) 32–39

Contents lists available at ScienceDirect

Bone

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

Original Full Length Article

A reversal phase arrest uncoupling the bone formation and resorption contributes to the bone loss in glucocorticoid treated ovariectomised aged sheep

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ARTICLE INFO

Article history:
Received 5 December 2014
Accepted 9 February 2015
Available online 14 February 2015

Edited by Robert Recker

Keywords:
Glucocorticoid-induced osteoporosis
Bone remodelling
Reversal phase
Coupling
Bone markers
Sheep model

ABSTRACT

Large animals as sheep are often used as models for human osteoporosis. Our aim was therefore to determine how glucocorticoid treatment of ovariectomised sheep affects the cancellous bone, determining the cellular events within the bone remodelling process that contributes to their bone loss. Twenty female sheep were assigned for two groups; an untreated control group and an ovariectomised group treated with glucocorticoids (0.6 mg/kg/day, 5 times weekly) for 7 months.

At 7 months the glucocorticoid-treated ovariectomised sheep showed a significant change in the bone microstructure revealed by a decreased trabecular bone volume and thickness compared to the control sheep. The treatment led to a temporary elevation of the bone resorption marker CTX (c-terminal collagen telopeptide), while the bone formation marker osteocalcin remained suppressed all 7 months. Histomorphometrically, the treated sheep had a complete absence of osteoid surfaces, and a 5-fold increase in the extent of eroded/reversal surfaces after 7 months. Most of these reversal surfaces were actually arrested reversal surfaces, defined as reversal surfaces without the presence of neighbouring osteoid surfaces or osteoclasts, which is classically observed next to active reversal surfaces. As in humans, these arrested reversal surfaces had compared to active reversal surfaces a reduced canopy coverage, a significantly decreased cell density, and a decreased immunoreactivity for the osteoblastic markers osterix, runx2 and smooth muscle actin in the mononuclear reversal cells colonising the surfaces.

In conclusion, glucocorticoid treatment of ovariectomised sheep induced a significant bone loss, caused by an arrest of the reversal phase, resulting in an uncoupling of the bone formation and resorption during the reversal phase, as recently demonstrated in postmenopausal women with glucocorticoid-induced osteoporosis. This supports the relevance of the sheep model to the pathophysiology of glucocorticoid-induced osteoporosis in postmenopausal women, making it a relevant preclinical model for orthopaedic implant and biomaterial research.

Introduction

Osteoporosis is a common metabolic disorder characterised by a systemic loss of bone mass and structure, increasing the risk of fractures [1,2]. Osteoporotic fractures are often problematic because the poor bone quality complicates the fixation of the fracture and implants [3]. Efforts to improve the fixation of implants under osteoporotic conditions have resulted in an increasing demand for a suitable large animal model, and here sheep have become a preferred experimental animal due to their adequate bone size for research within biomaterials, prosthetic components and medical devices [4,5]. In an attempt to mimic the clinical situation in osteoporotic patients researchers have used sheep models in which osteoporosis was induced by a combination of ovariectomy (OVX) and glucocorticoid treatment [6–8]. Nevertheless, the underlying cellular mechanisms responsible for the bone loss in those sheep models have been considered different from cellular mechanism responsible for osteoporotic bone loss in humans, questioning its relevance to human osteoporosis [6,9,10]. Furthermore, most of these studies focused, mainly on microarchitectural changes such as bone mineral density, trabecular thickness (Tb.Th.)

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http://dx.doi.org/10.1016/j.bone.2015.02.014
8756-3282/

and biomechanical properties [6–9], while only a few of these studies assessed the cellular mechanisms regulating the bone remodelling of sheep [11,12].

The bone remodelling process is conducted by numerous microscopic units, which includes three tightly coordinated sequential events: bone resorption by osteoclasts (OCs), an intermediate reversal phase and bone formation by osteoblasts (OBs) [13,14]. The cancellous bone remodelling process is separated from the marrow cavity by a canopy of flattened osteoblastic cells, forming an enclosed bone remodelling compartment (BRC) [15,16]. The reversal phase is recognised as eroded/reversal surfaces colonised by mononuclear osteoblastic reversal cells (Rv.Cs), which are believed to prepare the eroded surfaces for the subsequent bone formation [17–19]. Both the reversal phase and the canopies have been reported to be key players in the coupling of bone formation to resorption [20–22]. The present notion is that the osteoporotic bone loss either results from an unbalanced bone remodelling process, where the resorbed bone matrix is not completely substituted with a new bone matrix by bone forming OBs [13], or from an uncoupled bone remodelling process, where the initiation of the bone formation following the bone resorption is prevented during the reversal phase [21,23,24]. In both postmenopausal and glucocorticoid-induced osteoporosis an uncoupled bone remodelling process has been shown to result in a decreased extent of the formative osteoid surfaces (OS) and in an accumulation of the reversal surfaces (Rv.S) [20,23,24]. Here the accumulating reversal surfaces appeared arrested without the neighbouring OCs and OS, which is usually observed next to the active Rv.S. The Rv.Cs on arrested Rv.S were reported less immunoreactive for OB-lineage markers, indicating that the Rv.Cs differentiation into bone forming OBs is inhibited. This highlights the reversal phase as a potential target for the treatment and prevention of osteoporosis. From that perspective, it appeared relevant to investigate whether the reversal phase and an uncoupled bone formation and resorption contributes to the bone loss in the sheep model as well.

In this study, conducted on aged sheep, we imitated the clinical situation resembling GC-induced osteoporosis in postmenopausal women, inducing a significant bone loss by treating OVX sheep for 7 months with GCs. Our aim was to address the altered cellular events in the bone remodelling process contributing to the bone loss in GC-treated OVX sheep. Special attention will be given to the reversal phase, which has been reported to play an essential role in the coupling of bone formation to resorption during the bone remodelling process.

Materials and methods

Experimental animals

Twenty skeletal mature female sheep (9 ± 1 years of age) of the *Texel/Gotland* breed were included in the experiment. The sheep were healthy ex-breeders, and were acclimated for one month before the experiment was initiated. They were housed in outdoors paddocks with shelter, fed hay and a calcium-restricted diet (0.55% calcium and 0.35% phosphorus, FAF, DK-5000 Odense C, Denmark). One week prior to surgery and during post-operative recovery, the animals were housed indoors at the central animal facility. The mean body weight of the sheep at the beginning of the experiment was 75 ± 7 kg. All institutional and national guidelines for the care and use of laboratory animals were followed, and the Danish Animal Experiments Inspectorate approved the study (no. 2011/561-1959).

Design and collection of samples

The sheep were randomly allocated into two groups with 10 sheep each. The 10 sheep belonging to the GC-treated group underwent OVX by surgical laparoscopy. Subsequently, they received subcutaneous injections with methylprednisolone at a dose of 0.6 mg/kg/day 5 times weekly (10 mg/ml prednisolone acetate, Intervet, Denmark) for 7 months. The dosage was based on former studies and the treatment was initiated on the same day as the OVX [6,8,9]. Another 10 sheep served as the control group, which were left untreated, but received the same diet as the GC/OVX group.

Blood samples from the GC/OVX group were collected at baseline the same day prior to OVX, and 1 week, 1, 3 and 7 months after OVX and the initiation of GC treatment. Two iliac bone biopsies with a diameter of 6 mm were obtained under general anaesthesia from both control and the GC/OVX group after 1 month and again from the GC/OVX group after 7 months (Fig. 1). One biopsy was used for micro-CT scanning, while the other biopsy was fixed, decalcified and paraffin embedded for the histological analysis. After 1 month (control group) and after 7 months (GC/OVX group) the sheep were euthanized by an overdose of pentobarbital.

Biochemical bone markers

Serum concentrations of CTX were measured with a commercial ELISA kit (Osteometer Bio-Tech A/S, Herlev, Denmark). The bone formation marker osteocalcin was measured with an immunoassay directed against human N-MID osteocalcin (Roche Diagnostics, Hvidovre, Denmark). To avoid diurnal variations all analysis was done on blood samples collected between 08.00 and 10.00 a.m.

Micro-computed tomography (Micro-CT)

One biopsy was used for micro-CT scanning (μCT 50, Scanco Medical AG., Brüttisellen, Switzerland) to quantify the 3D microarchitectural properties of the cancellous bone [25]. Each 3D-image data set consisted of approximately 2000 consecutive 16-bit grey scale micro-CT images with a resolution of 2048 * 2048 pixels. The micro-CT images were segmented using a fixed optimal threshold and reconstructed into 3D-images with a final voxel sizes of 5 * 5 * 5 μm. Based on these 3D-images the trabecular bone volume (BV/TV, %) and trabecular thickness (Tb.Th, μm) were determined [26–29].

Histochemistry and immunohistochemistry

The decalcified paraffin-embedded biopsies were used for downstream histomorphometric and immunohistochemical analysis. 3.5-μm adjacent paraffin sections were either Masson's Trichrome stained [21] or immunostained [20]. The immunostained sections were labelled with mouse anti-tartrate-resistant acid phosphatase (TRAcP) IgG2b (clone 9C5, MABF96; Millipore Cooperation, Billerica, MA, USA), IgG2a smooth muscle actin (SMA) (clone 1A4; Dako, Glostrup, Denmark), anti-osterix (Sp7; Abcam, Cambridge, UK) or anti-Runx2 IgG2b (clone 1D8, ab115899; Abcam) antibodies, which were subsequent detected with alkaline phosphatase-conjugated anti-mouse polymers (Immunologic, Duvien, The Netherlands). As negative control, the primary antibody was omitted. The sections were visualised by liquid permanent red staining (LPR, DAKO) and counter stained with Meyers Haematoxylin before mounting. An immunohistochemical characterisation of the nature of the Rv.Cs was performed by assessing the intensity of the nuclear immunoreactivity of the transcription factors runx2 and osterix, and the cellular reactivity of SMA on both active and arrested Rv.S.

Histomorphometry

The histomorphometric analysis was conducted on a single set of vertical adjacent sections that were either Masson's Trichrome stained or immunostained for the OC marker TRAcP [20]. The extent of eroded surfaces (ES/BS), osteoid surfaces (OS/BS), OC surfaces (Oc.S/BS) and reversal surfaces (Rv.S/BS) were addressed by counting the number of intercepts between a cycloid grid and the respective bone surfaces