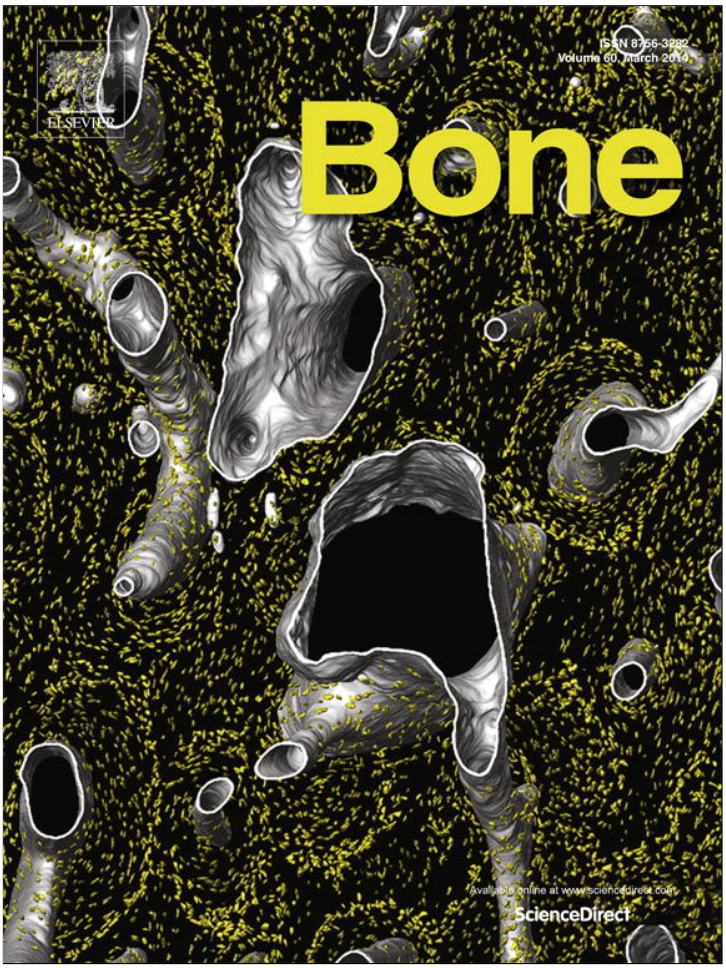

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Original Full Length Article

Elevated serum soluble CD200 and CD200R as surrogate markers of bone loss under bed rest conditions

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ABSTRACT

CD200 is a transmembrane protein that belongs to the immunoglobulin family of proteins and is ubiquitously expressed on a variety of cell types. Upon interaction with its receptors (CD200Rs) expressed on myeloid-derived cells and T lymphocytes, an immunoregulatory signal is delivered to receptor-expressing cells. Previous studies have implicated a role for CD200:CD200R in the regulation of the expression of mRNA markers of osteoclastogenesis/osteoblastogenesis, following interaction of CD200 (on osteoblast precursors) with CD200R1 (on osteoclast precursors). Signaling of CD200R1 is hypothesized to attenuate osteoclastogenesis. We have investigated whether levels of soluble forms of CD200 and/or CD200R1 (sCD200, sCD200R1) are altered in volunteers undergoing 6° head down tilt bed rest to mimic conditions of microgravity known to be associated with preferential osteoclastogenesis and whether countermeasures, reported to be beneficial in attenuation of bone loss under microgravity conditions, would lead to altered sCD200 and sCD200R1 levels. Our data suggest that, as predicted, sCD200 levels fall under bed rest conditions while sCD200R1 levels rise. In subjects undergoing 30-minute per day continuous centrifugation protocols, as a countermeasure to attenuate changes which may lead to bone loss, these alterations in sCD200 and sCD200R1 levels seen under conditions of bed rest were abolished or attenuated. Our results suggest that measurement of sCD200 and/or sCD200R1 may prove a useful and rapid means of monitoring subjects at risk of bone loss and/or accessing the efficacy of treatment regimes designed to counter bone loss.

Introduction

A major concern in health care is osteoporosis and bone loss. Immobilization caused by injury (e.g. spinal cord injury, stroke etc. [1,2]), as well as exposure to microgravity [3,4] reduces forces applied to bone especially to weight bearing bones and leads to reduction of bone mass.

6° head down tilt (HDT) bed rest is a commonly used model to study changes in many physiological systems, including changes in bone mass, occurring during conditions of weightlessness such as in space flight [5,6]. During both bed rest and space flight bone loss has been documented, with the greatest changes noted in weight bearing skeletal sites, such as the hip and pelvis, and with minimal changes in the wrist or arm [4]. The mechanism behind this bone loss is thought to be the uncoupling of bone remodeling, such that in microgravity bone resorption increases while bone formation remains unchanged or changes very little. There are large variations in bone loss between individuals and the sexes, implying that genetic variations are also important in the regulation of bone mass [4,7].

Many countermeasures (exercise, nutrition, pharmaceuticals etc.) have been tested for their efficacy in preventing bone loss during either

Abbreviations: STBR, Short term bed rest; HDT, Head down tilt; AG, Artificial gravity; BDC, Baseline data collection.

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space flight, or under bed rest conditions, with different success rates [3,4]. Nutritional countermeasures tested so far have had either no [8] or only very limited benefits for bone. Flywheel resistive exercise and pamidronate were only partially effective in preventing bone loss as judged by changes in bone mineral density in the lower leg [9]. However some exercise countermeasures have been shown to be more effective and have some protective effect, including whole body vibration in combination with resistive exercise [10,11], and treadmill exercising in a lower body negative pressure (LBNP) chamber [12]. Combining two different types of exercise, namely resistive (on a flywheel) and aerobic (with LBNP) exercises, helped mitigate net bone loss by promoting bone formation (but did not prevent bone resorption) [13].

Previous work from our lab showed that the interaction between the ubiquitously expressed molecule CD200, and its receptor, CD200R1, expressed on myeloid-cells, controls activation of cells of the monocyte/macrophage lineage [14]. Osteoclasts express CD200R1, while osteoblast precursors express the ligand, CD200 [15]. We hypothesized that CD200:CD200R1 interactions suppress osteoclastogenesis, leaving osteoblastogenesis as the permissive pathway [16,17]. In a study conducted as part of a spaceflight project (eOSTEO) [18], we showed that overexpression of CD200 (using cell cultures derived from transgenic mice expressing CD200 under control of a doxycycline-inducible promoter) under microgravity conditions led to attenuation of the suppression of mRNA markers of osteoblastogenesis (bone sialoprotein and osteoprotegerin) normally observed under such conditions. At the same time, in the presence of increased CD200 expression, it was observed that the increase in expression of genes related to osteoclastogenesis (receptor activator of nuclear factor kappa-B ligand (RANKL) and tartrate-resistant acid phosphatase), which are normally seen in such microgravity conditions, was abolished [18].

These observations led to our considering, as an auxiliary hypothesis, that HDT bed rest with no countermeasures would be associated with changes in levels of markers for bone homeostasis, namely CD200 and/or CD200R1. We have documented that both CD200 [19] and CD200R1 (Kos and Gorczynski—unpublished) exist in normal serum in soluble form (sCD200, sCD200R1). Both sCD200 and sCD200R independently attenuate the signals (in ELISA/FACS) triggered by binding mAbs to CD200 (or CD200R) to the cell membrane-expressed molecules. Accordingly we reformulated our study to include assessment of whether bed rest would be associated with decreased levels of sCD200, and increased levels of the soluble form of the receptor, sCD200R1, while countermeasures to attenuate bone loss would lead to the reverse effects on the levels of both of these two molecules.

In the present study, short term 6° head down tilt bed rest was used with two different artificial gravity prescriptions (AG1: continuous 30 min centrifugation per day; and AG2: intermittent 6 × 5 min centrifugation daily) to assess their ability to attenuate markers of bone loss induced by 5 days of bed rest. As surrogate markers for bone loss, we monitored changes in: a serum marker of bone turnover, sRANKL, beta CTX, sCD200 and sCD200R1; and markers of bone formation, bone specific alkaline phosphatase (bALP) and propeptide of type I Collagene (PINP). In urine, we measured changes in the collagen breakdown products DPD, NTX and CTX.

Methods

The short term bed rest (STBR) study was approved by the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, Midi-Pyrenees (France), and the local ethics committees, including the Office of Research Ethics, University of Waterloo. The entire protocol was performed in accordance with the declaration of Helsinki. Each subject provided informed written consent before participation and was aware of the right to withdraw from the study for any reason without prejudice. The study itself was performed at the Institut de Médecine et de Physiologie Spatiales (MEDES), Toulouse, France between April and July 2010.

Subjects

12 healthy male volunteers agreed to participate in the study. The mean age (±SD) was 32 ± 8 yr. The subjects had a mean height of 178 ± 8 cm, and weight 74.6 ± 7.8 kg. Only males were used in the present study to avoid fluctuations in bone metabolism known to occur in cycling females [20–22].

Study design

The STBR study was designed as a randomized crossover trial with one control- and two treatment groups (AG1 and AG2) with a 30 day wash out period between, during which the subjects were released from the facility. The subjects were randomly assigned to one of the three groups in their first campaign then to the other treatments in subsequent campaigns. One participant fell ill midway through the control period and was accordingly assigned to the control group during the third campaign. Data from this subject was not included in the statistical analysis. Each campaign consisted of a 5-day period of 6° head down tilt (HDT) bed rest, with a 5-day data collection period before bed rest, and a 5-day data collection period after bed rest. During the bed rest period the subjects were confined to strict 6° HDT bed rest.

Treatment

During the bed rest phase, control, AG1 and AG2 subjects were transported daily on a 6°HDT gurney to the centrifuge facility, where they were transferred to the centrifuge arm (horizontal) and monitored. AG1 subjects were centrifuged continuously for 30 min at a force equivalent to 1 *g* at the center of the mass, AG2 subjects received 6 × 5 min centrifugation, and control subjects did not receive any centrifugation. Some subjects did not tolerate continuous centrifugation well, and for statistical analysis in the AG1 group only data from the 6 subjects who completed the whole centrifugation protocol was included.

Because, the sample material was in limited supply, only 3 of those 6 subjects were used to assess their response to bed rest and applied countermeasures using measurement of the concentration of collagen breakdown products (namely βCTX) in their serum.

Biological sample collection and processing

Blood samples were collected first thing in the morning; urine samples were continuously collected throughout the day and pooled for 24 h collection period. Table 1 lists the sample collection time points with corresponding bone metabolism markers that were analyzed.

Table 1
Time points of biological sample collection and the corresponding bone metabolism markers analyzed.

Time points of sample collection	Bone marker analyzed in	
	Serum	Urine
BDC-5		uNTx, uCTx, uDPD
BDC-4	sCD200, sCD200R1, sRANKL, βCTX ,	uNTx, uCTx, uDPD
BDC-3	sCD200, sCD200R1, sRANKL, βCTX, Calcium,	uNTx, uCTx, uDPD
BDC-2		uNTx, uCTx, uDPD
BDC-1	bALP, PINP,	uNTx, uCTx, uDPD
HDT1		uNTx, uCTx, uDPD
HDT2		uNTx, uCTx, uDPD
HDT3	sCD200, sCD200R1, sRANKL, βCTX,	uNTx, uCTx, uDPD
HDT4		uNTx, uCTx, uDPD
HDT5	sCD200, sCD200R1, sRANKL, βCTX, bALP, PINP,	uNTx, uCTx, uDPD
R + 1	sCD200, sCD200R1, sRANKL, βCTX,	uNTx, uCTx, uDPD
R + 2		uNTx, uCTx, uDPD
R + 3		uNTx, uCTx, uDPD
R + 4	bALP, PINP, Calcium	
R + 5		

Biochemical analysis

Blood

Blood samples were collected into the appropriate tubes and processed to yield serum.

The serum level of total sRANKL was measured using a commercially available ELISA from BioVendor-Laboratorní medicína a.s. (Brno, Czech republic); for measurement of total human TNFα. A Platinum Sandwich ELISA from eBioscience, Inc. (San Diego, CA) was used; the serum concentration of beta-Crosslaps (βCTX) was measured using a competitive ELISA from Uscn Life sciences (Wuhan, P.R. China). Serum levels of calcium were assayed at MEDES using flame photometry method. The basal level of bone specific alkaline phosphatase (bALP) was assessed using Hybritech Tandem-R Ostase immunoradiometric assay (Liége, Belgium). Procollagen-I-N-terminal propeptide (P1NP) levels in the serum were measured using assay from Orion Diagnostica (Finland). Intra- and Inter-Assay CVs for PINP were 1.9% and 2.6% correspondingly.

Urine

To assess changes in the amount of collagen breakdown products excreted by the body, daily urine collections were performed. Urine was continuously collected on a void-by-void basis, kept in the dark at 4 °C, and pooled in the laboratory for 24-h collection periods. Daily urine volumes were measured and small aliquots were frozen at −20 °C. Markers of bone resorption, C-Telopeptide (CTX) and N-Telopeptide (NTX) were analyzed by commercially available assays (NTX: Osteomark, TECOmedical, Bünde, Germany; CTX: Urine crosslaps, IDS, Frankfurt, Germany) and deoxypyridinoline (DPD) was analyzed by HPLC method. Intra-Assay CV for uCTX was 1.4% and Inter-Assay CV—4.4%. Corresponding values for uNTX were 1.4% and 4.9%. All results were normalized against 24 h urine volume and are shown as excretion rate per day. Excretion of urinary bone resorption markers are often normalized against creatinine. However, since creatinine levels change as a result of muscle breakdown, which is one of the hallmarks of bed rest [23], and they also depend on nutrition, we opted to determine the excretions of urinary markers as excretion rates per day and not to normalize the values against creatinine.

The only creatinine values available to us were serum creatinine levels measured 3 days before the start and 4 days after the end of each bed rest campaign as shown in Supplementary Table 1. Interestingly, at the end of the second campaign, increased creatinine levels of all participants were seen independent of the applied countermeasures during this campaign, whereas at the end of the first and the third campaigns creatinine levels did not change substantially from pre bed rest levels.

sCD200 ELISA

Levels of soluble CD200 in serum were measured using an ELISA developed in our laboratory [19,24]. Monoclonal antibody 1B9 (rat anti hCD200) was used as a capture reagent and polyclonal rabbit-anti-human CD200 (V + C) serum was used as the detection antibody. Some modifications to the assay as initially described were made to improve sensitivity in this analysis. All serum samples were diluted 1:4 in blocking buffer before analysis. In addition, the incubation time with antigen and the incubation temperature were changed. Thus, plates to which serum was added were incubated not for 2 h at room temperature, but overnight at 4 °C, followed by warming up at 37 °C for 1.5 h on the following day. This addition of overnight incubation at 4 °C rather than performing the complete assay within 1 day was found to improve both sensitivity (approximately 3–5-fold) and reproducibility in the assay (Kos and Gorczynski—unpublished). Intra-Assay CV was 4.6% and Inter-Assay CV—6.9%.

sCD200R1 ELISA

Serum levels of the decoy receptor, sCD200R1, were measured using a newly developed ELISA as follows. A high binding ELISA plate (Corning Life Sciences) was coated with 100 ng (100 μL/well) of commercially available anti-human CD200R1 antibody (R&D Systems, Inc.; Minneapolis, MN) and kept overnight at 4 °C. Following blocking for 1 h at RT (200 μL blocking buffer/well, 5% FBS in PBS), serum samples, diluted 1:4 in blocking buffer, were added in duplicate and incubated at 4 °C overnight. As a standard, a serial dilution of pure hCD200R1his protein (a kind gift from Trillium Therapeutics Inc., Mississauga, Ontario) was used at concentrations ranging from 7.8 to 250 pg/well. For detection reagent, a 1:2000 dilution of polyclonal rabbit-anti hCD200R1 serum was added to each well, with incubation for 1 h at 37 °C. This antibody was raised against pure human CD200R1his protein, and it shows no cross reactivity with CD200, either in ELISA (with CD200Fc) or on Western Blot (with cell lysate of CD200 overexpressing HEK 293) (see Supplementary Fig. 1). As secondary antibody, a goat anti-rabbit IgG-HRP (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) at 1:15,000 dilution was used (100 μL/well) with incubation for 45 min at 37 °C. At least 5 washes with wash buffer (PBS with 0.01% Tween20) were performed between each step. Finally 100 μL of TBM substrate (Thermo Fisher Scientific Pierce Protein Research, Rockford, IL) was added for 15 min at 37 °C, with color development stopped by addition of 50 μL of stop solution (0.2 M sulfuric acid). Absorbance was read at 450 nm in a Multiskan Ascent 96/384 Plate reader (MTX Lab Systems, Vienna, Austria). Intra- and Inter-assay CVs were determined to be 3.8% and 7.1% correspondingly.

However since all samples from one subject collected during one campaign were analyzed on one ELISA plate, the analysis should not affect the inter-assay CV.

Statistical analysis

The results for all sandwich ELISAs (sRANKL, sCD200, sCD200R1 and TNFα) were calculated applying a linear standard curve method. βCTX data obtained using a competitive ELISA were calculated with a 5 point logistic curve created by MasterPlex ReaderFit software.

Statistical analyses were performed using GraphPad Prism software (version 3.02) with the data in their original form. Changes in blood levels of sCD200, sCD200R1, βCTX and sRANKL among all four phases (pre bed rest (average of BDC results), head down tilt bed rest day 3 (HDT3), and day 5 (HDT5) and one day after bed rest (R + 1)) were tested for statistical significance using repeated measures analysis of variance (ANOVA). Significance was assumed when $p < 0.05$. To analyze differences from control values (measured before bed rest) and head down tilt samples or post bed rest samples, Dunnett's multiple comparison tests were used. The same methods were used for statistical analysis of collagen breakdown product data, namely uNTX, uCTX and uDPD, as well as both bone formation markers bALP and P1NP.

We elected not to use two way ANOVA, which would have allowed us to take into account the day and time of the intervention in addition to other variables, since we did not anticipate changes at early time points. As an alternative we compared individual area under the curve (AUC) values using a paired *t* test. This analysis revealed no statistical significance except for a trend observed (sCD200 levels) in AUC in AG1 campaign compared to control campaign ($p = 0.0554$) (data not shown).

A paired *t* test was used to determine statistical significance in serum concentrations of Ca++ before (BDC) and 4 days after (R + 4) bed rest.

Results

Graphic representations of the changes in serum levels for markers of bone remodeling during the study are shown in Figs. 1–5 and Supplementary Fig. 2. Some raw data are shown in Supplementary Tables 1–2.

Urine bone resorption markers uCTX, uNTX and uDPD

As seen in Fig. 1, under control conditions, the levels of all urine bone resorption markers significantly increased at day 4 of HDT bed rest when compared to a corresponding averaged pre bed rest levels.

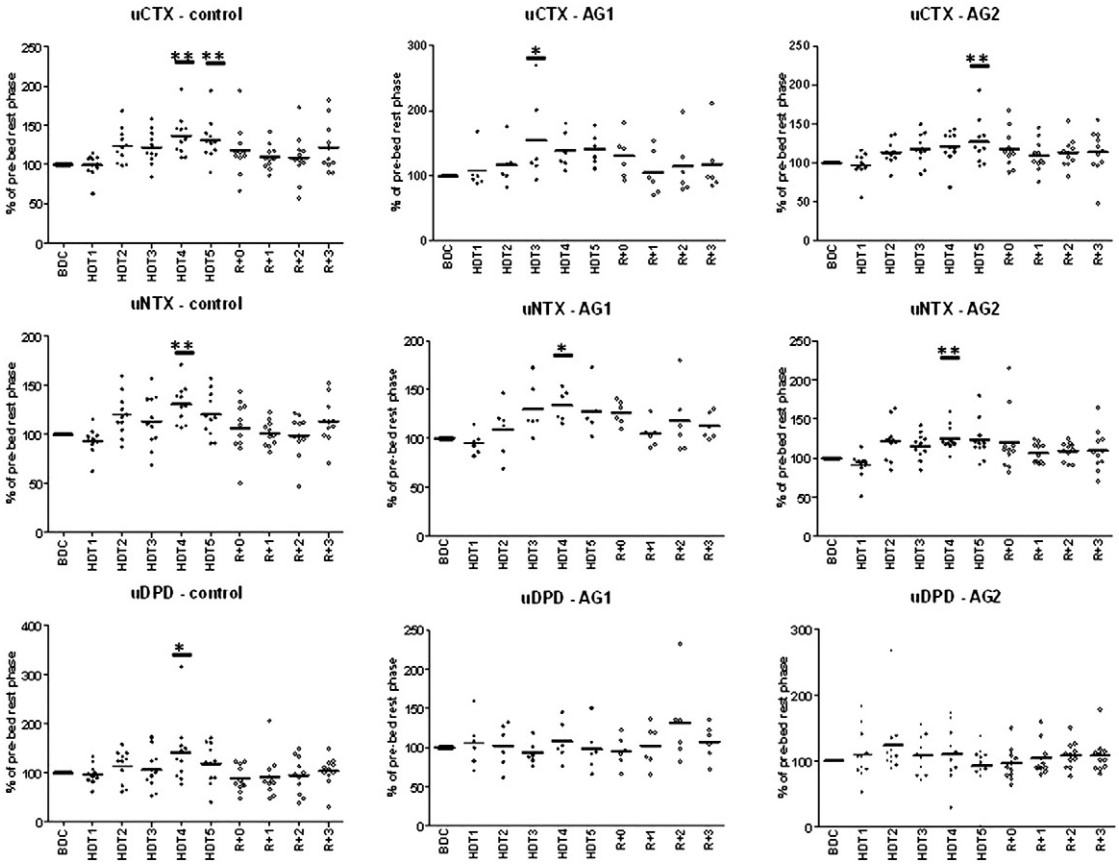

Fig. 1. Percent changes in urine level of collagen type I C-Telopeptide (CTX), and N-Telopeptide (NTX) as well as deoxypyridinoline (DPD) during 5 days head down tilt bed rest study in control and two countermeasure groups (AG1, AG2). Data are expressed as percent changes from pre bed rest calculated for each subject, with mean calculated for each group. *Significant difference as compared with pre bed rest; $^{*}p < 0.05$, $^{**}p < 0.01$, calculated using repeated measures analysis of variance (ANOVA) followed by Dunnett's test with data in their original form. BDC—basal data collection; HDT3 and HDT5—head down Tilt bed rest days 3 and 5 accordingly; R + 1—one day of recovery.

Applied countermeasures were shown to have a relatively weak effect in attenuating those increases. In fact, only increases in uDPD measured under the control campaign at HDT4 (41.2%), were abolished by the applied artificial gravity protocols. In the case of uCTX and uNTX, countermeasures were unable to mitigate those changes and in both the AG1 and AG2 campaigns, similar increases in urine levels of uCTX and uNTX were measured during HDT bed rest.

Serum bone resorption marker βCTX

Levels of one of the collagen breakdown products (βCTX) measured in serum were elevated during the control and AG2 campaigns, with the AG1 protocol more effective. As shown in Fig. 2, under control conditions the levels of βCTX in the serum of the 3 subjects tested were increased (mean 18.1%) by day 3 of bed rest, and remained elevated on day 5. These increases became significant only one day after the end of bed rest (26.4%, $p < 0.05$). The same pattern was seen in the AG2 group, where the mean increase in βCTX level was 20% ($p = 0.063$) at HDT3 and 23% ($p = 0.093$) at HDT5. One day after the end of bed rest the 63% increase in βCTX reached statistical significance ($p < 0.05$). In a campaign where continuous centrifugation as a countermeasure was applied, some minor but nonsignificant elevations in βCTX levels (12.1% at HDT3 and 8.7% at HDT5) were detected, but at R + 1 the levels fell close to the pre bed rest values.

sCD200R1

Both centrifugation protocols were effective in attenuating changes in sCD200R1 level. Even though countermeasures applied during this study were unable to prevent the increases in sCD200R1 levels at the beginning of bed rest (mean of 37.7% increase on day 3, $p < 0.001$ in AG1 and 40.5% $p < 0.05$ in AG2), they did lead to normalization of sCD200R1 levels more quickly than with subjects under control conditions and already by day 5 of bed rest the levels of sCD200R1 were not significantly different from pre bed rest values (Fig. 3, and Supplementary Table 2). In contrast, under control conditions the levels of sCD200R1 were significantly ($p < 0.001$) increased throughout bed rest (mean of 35.0% on day 3 ($p < 0.001$) and 28.3% on day 5 ($p < 0.05$) of HDT) and remained elevated one day after bed rest (30.3%, $p < 0.001$).

Comparing the changes in sCD200R1 and the urinary bone resorption markers (uCTX and uNTX) occurring during bed rest (Supplementary Figs. 2 and 3), it is clear that bed rest caused an increase in all those markers. However, as indicated in Supplementary Fig. 2, where the X-axis indicates the times at which sample collections were made, changes in the levels of urinary bone resorption markers occur at later times than changes in the levels of sCD200R1. In Supplementary Fig. 3 the X-axis shows levels of serum sCD200R1, and the figure compares changes with changes in urinary bone resorption markers. In control and AG2 campaigns no statistically significant correlations between those markers were found. In the AG1 campaign changes in sCD200R1 and uNTX were found to be statistically significant but changes between sCD200R1 and the two others urinary bone resorption markers weren't. Given that changes in urinary markers usually occur at the same time, the correlation between sCD200R1 and uNTX may be more apparent than real.

sCD200

Serum levels of sCD200 (Fig. 3 and Supplementary Table 3) were significantly decreased during head down tilt bed rest. Under control conditions, the sCD200 levels dropped approximately 25% below pre bed

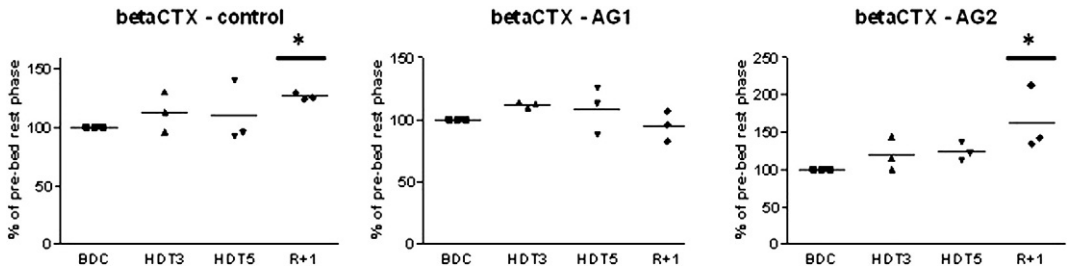

Fig. 2. Percent changes in serum level of betaCTX during 5 days head down tilt bed rest study in control and two countermeasure groups (AG1, AG2). Data are expressed as percent changes from pre bed rest calculated for each subject, with mean calculated for each group. *Significant difference as compared with pre bed rest; *$p < 0.05$, calculated using repeated measures analysis of variance (ANOVA) followed by Dunnett's test with data in their original form. BDC—basal data collection; HDT3 and HDT5—head down Tilt bed rest days 3 and 5 accordingly; R + 1—one day of recovery.

rest values (mean of 27.7%, $p < 0.001$ on day 3 and 22%, $p < 0.001$ on day 5 of bed rest). After one day of recovery, CD200 levels still remained significantly lower ($p < 0.001$) compared to pre bed rest levels. Even though levels of sCD200 at R + 1 showed some tendency to increase when compared to levels measured at HDT3 and HDT5, those changes were not statistically significant, indicating that one day of recovery was not sufficient to attenuate changes induced by bed rest.

Countermeasures applied during this study had some effect on attenuating decreases in sCD200 levels seen under control conditions (Fig. 3). Continuous centrifugation, despite being not well tolerated, produced a more marked effect in the subset (6) of subjects who completed all 5 days of the centrifugation protocol, with the decreases in sCD200 levels from baseline no longer significant. Intermittent centrifugation was much better tolerated, but was not as effective at preventing alterations to sCD200 levels. Thus, the decreases in sCD200 levels were significantly lower on day 3 of head down tilt bed rest (19.8%, $p < 0.001$) and after bed rest (17.2%, $p < 0.05$) in this group, although changes in sCD200 levels on day 5 of bed rest were no longer significant.

Bone formation markers: bone specific bALP and PINP

Under control condition a significantly increased level of bALP was detected at HDT5 (16.9%, $p < 0.001$) (Fig. 4). Under the AG1 condition, a 25.6% increase in bone specific ALP was detected at HDT5 ($p < 0.001$), and a 27.6% increase at R + 4 ($p < 0.001$). In the intermittent centrifugation campaign, the level of bALP rose at HDT5 9.6% in comparison to BDC.

As seen in Fig. 4, serum levels for a marker for bone formation – PINP – did not change significantly either under control or AG1 and AG2 bed rest conditions. Unfortunately, the samples for measurement of sCD200, bALP and P1NP were collected at different time points and we were unable to perform a correlational analysis.

sRANKL, TNFα

Relative changes in the levels of sRANKL are shown in Fig. 5. Head down bed rest did not induce any significant changes in this marker of bone remodeling and inflammation. TNFα was not detectable in the serum of any of the 11 subjects that participated in the bed rest study (the limit of detection in this assay was 5.0 pg/mL).

Calcium

Calcium levels (Supplementary Fig. 4) were significantly elevated 4 days after the end of the control and both countermeasure campaigns, when compared with corresponding pre bed rest values, measured 3 days before the start of each bed rest.

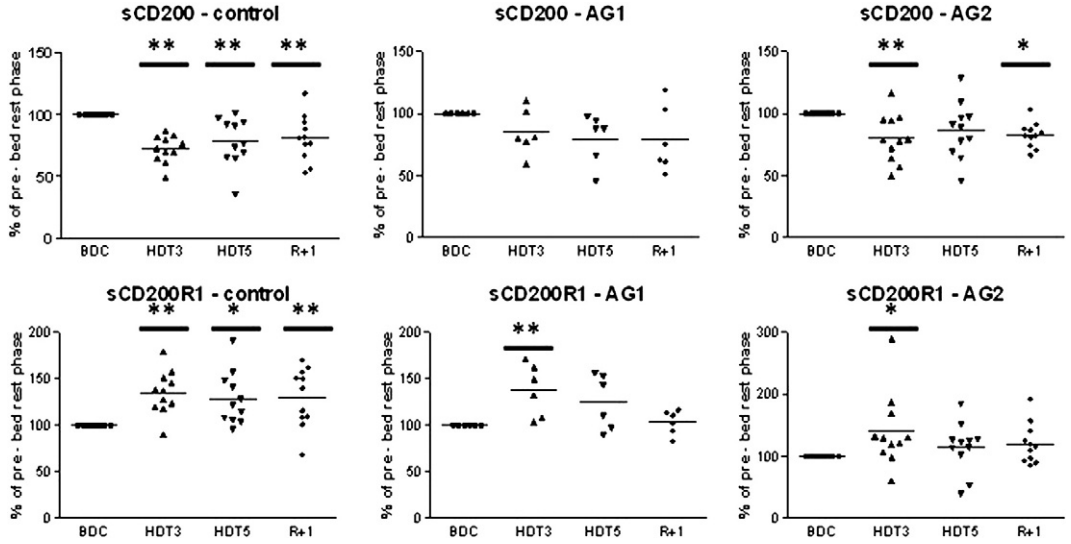

Fig. 3. Percent changes in serum level of sCD200 and sCD200R1 during 5 days head down tilt bed rest study in control and two countermeasure groups (AG1, AG2). Data are expressed as percent changes from pre bed rest calculated for each subject, with mean calculated for each group. *Significant difference as compared with pre bed rest; *$p < 0.05$, **$p < 0.01$, calculated using repeated measures analysis of variance (ANOVA) followed by Dunnett's test with data in their original form. BDC—basal data collection; HDT3 and HDT5—head down Tilt bed rest days 3 and 5 accordingly; R + 1—one day of recovery.

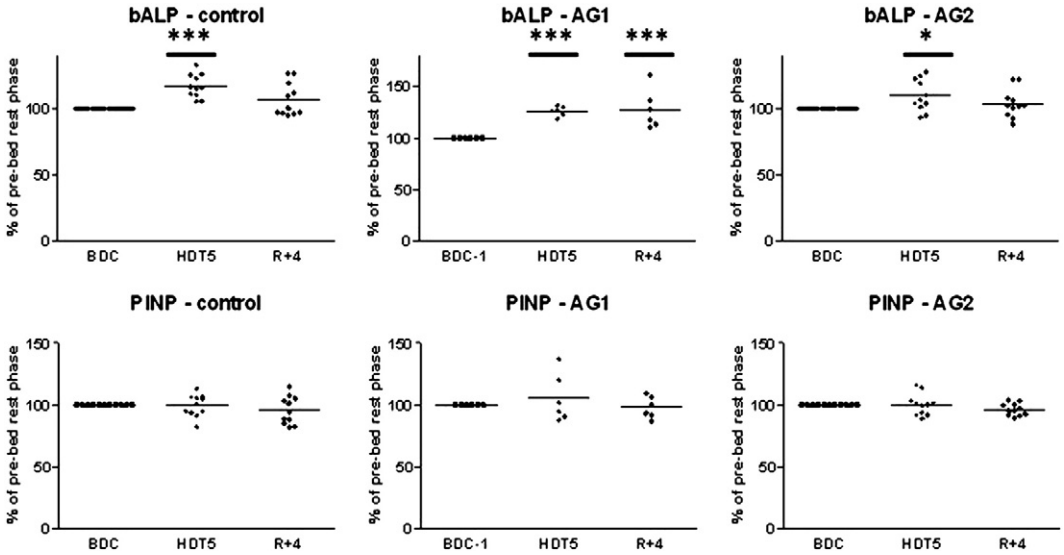

Fig. 4. Percent changes in serum level of bone specific Alkaline phosphatase (bALP) and procollagen-I-N-terminal propeptide (P1NP) during 5 days head down tilt bed rest study in control and two countermeasure groups (AG1, AG2). Data are expressed as percent changes from pre bed rest calculated for each subject, with mean calculated for each group. *Significant difference as compared with pre bed rest; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, calculated using repeated measures analysis of variance (ANOVA) followed by Dunnett's test with data in their original form. BDC—basal data collection; HDT3 and HDT5—head down Tilt bed rest days 3 and 5 accordingly; R + 1—one day of recovery.

Discussion

A 6° head down tilt bed rest study is a useful and reliable simulation model to characterize the physiological effects of space flight [5]. Gravitational forces on the longitudinal skeleton are 83% less under bed rest than in the upright, loaded position [25]. Bone adapts to mechanical stimuli and reduction or absence of the latter leads to bone loss, primarily through a sustained increase in bone resorption with more subtle decreases in bone formation [12,26–29]. Weightlessness causes not only a loss of bone density but also a decrease in muscle mass and red blood cells, cardiovascular and sensory-motor deconditioning, and changes in the immune system [6]. Artificial gravity, since it simulates the natural 1-*g* environment, would challenge all systems of the body (bone, muscle, cardiovascular and vestibular systems) and would be predicted to be the ideal countermeasure [30].

The present study has evaluated the role of sCD200:sCD200R1 as metabolic markers of bone homeostasis. CD200, a ubiquitously expressed transmembrane protein with known immunoregulatory properties can regulate the expression of genes involved in osteoclastogenesis and osteoblastogenesis [17,18]. This regulation is thought to occur through an interaction of CD200 (on osteoblasts) with its receptor CD200R1, expressed on osteoclast precursors [15]. The cell surface availability of many important receptor/ligand pairs involved in bone development, including RANKL [31–33], RANK [34], CSF-1 [35,36], CSF-1R [37], is regulated, at least in part, by ectodomain shedding, and we recently described a soluble variant of CD200 (sCD200) in the serum of healthy individuals, likely present in the serum following a process of ectodomain shedding [38], with demonstrable functional activity, binding and phosphorylating CD200R1 [24].

Since the interaction of CD200 with its cell bound receptor CD200R1 delivers an inhibitory signal for osteoclastogenesis, its level in the serum of bed rest participants under control conditions was predicted to decrease, in keeping with the increased bone resorption seen under these conditions. The prediction was that under bed rest conditions without countermeasures the serum levels of sCD200R1 would increase. The short term bed rest study described above was the first attempt to measure simultaneously changes in these molecules under conditions of reduced skeletal loading.

It is nevertheless important to acknowledge that one limitation of the current study is that direct measurements of bone mass over the 5-day experimental study period were not performed in this analysis. All measures used in the study described are surrogate markers of bone loss, some of which (as noted below) have been accepted as such in multiple previous studies from other groups. Our own subsequent analyses of changes in levels of sCD200 and sCD200R1 have, in turn, been correlated with alterations in these other surrogate markers, but have not been directly correlated with alterations in bone mass per se.

Consistent with the hypothesis, we observed by day 3 of head down bed rest that the levels of sCD200 were significantly decreased and those for sCD200R1 were increased in comparison to pre bed rest

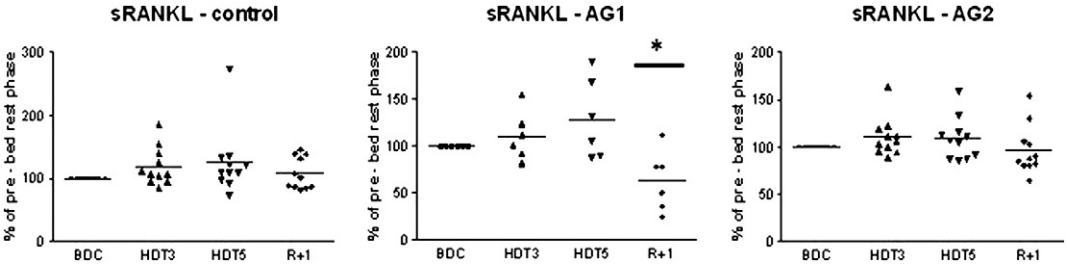

Fig. 5. Percent changes in serum level of sRANKL during 5 days head down tilt bed rest study in control and two countermeasure groups (AG1, AG2). Data are expressed as percent changes from pre bed rest calculated for each subject, with mean calculated for each group. * Significant difference as compared with pre bed rest; $^{*}p < 0.05$, calculated using repeated measures analysis of variance (ANOVA) followed by Dunnett's test with data in their original form. BDC—basal data collection; HDT3 and HDT5—head down Tilt bed rest days 3 and 5 accordingly; R + 1—one day of recovery.

values. Since the levels of sCD200 and sCD200R1 change during bed rest in opposite directions, this may be taken to imply that the molecules regulating shedding release of these two proteins is/are not the same entities.

We used two centrifugation protocols AG1 (continuous centrifugation for 30 min/day) and AG2 (intermittent centrifugation 6 × 5 min) as countermeasures and compared them with each other and with a control group to assess their effectiveness in preventing/attenuating the changes described which we suggest predict bone loss associated with disuse. Both artificial gravity protocols mitigated against the increases in sCD200R1 levels seen with bed rest under control condition, and already at day 5 of HDT changes in sCD200R1 were insignificant when compared with pre bed rest levels. In the case of sCD200, the AG2 protocol appeared to be less effective, with significant increases in sCD200 levels still measured at day 3 of HDT and at R + 1. In contrast, AG1, while not as well tolerated, produced superior attenuation of the decreases in sCD200 levels detected under control conditions. The βCTX and bALP results also point towards the conclusion that continuous centrifugation was a superior countermeasure in this study. In all cases, we observed large variations between subjects, which are perhaps not surprising since bone mechanosensitivity has a significant genetic component [39,40]. As an example of the latter, experiments in C3H/He mice showed significantly less responsiveness to mechanical loading than when two other mouse strains were tested (C57BL/6 and DBA/2) [41].

Changes in bone turnover are reported to occur rapidly under bed rest conditions, with increases in levels of bone resorption markers, including N-Telopeptide of type I collagen and type I collagen carboxytelopeptide, indicative of enhanced osteoclast activity, by the 2nd day of immobilization [26,42]. Bone formation markers were not influenced by short term immobilization in the same study [26,42]. The levels of sCD200 and sCD200R1, which we hypothesize are mediators of bone remodeling, were predicted to precede any changes in bone resorption markers. We measured serum levels for carboxy-terminal cross-linking telopeptide of type I collagen (βCTX), which is generated by osteoclastic hydrolysis of collagen I by cathepsin K [43], and recommended by the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine for use as a reference marker for bone turnover in clinical studies [43,44]. The level of this bone resorption marker has been found to be significantly different between osteoporotic and osteopetrotic patients [45] and it also has been shown to have a greater utility for assessing efficacy of antiresorptive treatment than some previously described markers [46].

Changes in levels of sCD200R1 and particularly sCD200 levels did indeed precede changes seen in βCTX. Thus sCD200 was significantly decreased by day 3 of HDT bed rest in both the control and AG2 groups, and did not significantly change in the AG1 group. Similarly βCTX was increased in serum one day after the end of bed rest in the control and AG2 groups, with no appreciable changes in the AG1 group. The changes measured in βCTX on day 3 of HDT bed rest are similar to those reported by Baecker et al. and Heer et al. [26,42], although in our study the changes were not significant, perhaps because of the smaller sample size (n = 3).

The levels of all three collagen breakdown products measured in urine (uCTX, uNTX and uDPD) also increased during HDT bed rest under the control condition to an extent similar to those reported by others [13,42]. Only uDPD was affected by the applied countermeasures, and its level was not significantly elevated during bed rest in AG1 and AG2 campaigns. The increase in uCTX and uNTX was not modulated significantly by any applied artificial gravity protocols.

RANK, RANKL and OPG, are acknowledged mediators of osteoclastogenesis. Extreme skeletal phenotypes (osteoporosis vs. osteopetrosis) in mice are associated with altered expression of these molecules [47]. Denosumab (a human monoclonal antibody to RANKL, which directly inhibits the RANKL/RANK signaling pathway) is an effective antiresorptive agent used for treatment of postmenstrual osteoporosis [48,49]. In contrast to the changes in the levels of CD200 and CD200R1, the levels of sRANKL did not change significantly under control conditions in the course of our short term bed rest study. Measurements of free sRANKL were analyzed in a previous bed rest study, but concentrations were below the detection limit of the assay for most subjects [50]. We have analyzed total sRANKL level and observed a more rapid decrease in sRANKL level at R + 1 when AG1 was applied as countermeasure. Moderate changes in sRANKL during all three campaigns were observed, although only for AG1 was the change significant, perhaps reflecting a homeostatic return of the system to normal after effective stimulation.

Somewhat surprisingly, we observed an increase in the level of bone specific ALP in the control campaign at day 5 of HDT bed rest, in contrast to the results of Smith et al. [50]. However Kim et al. [51] observed similar results in their 14 days bed rest study, namely an increase in bone ALP level at the end of head down tilt. A marker of the later stages of osteoblast differentiation (in the Kim et al. study it was osteocalcin, in our study—PINP) did not change significantly from pre bed rest level.

Applied continuous centrifugation seems to be able to induce bone formation since the level of bone ALP at HDT5 and R + 4 was even higher in the AG1 campaign than in the control one. Other countermeasures tested in different bed rest studies also showed similar results: resistance exercise [52]; a combination of resistive and aerobic exercises [13]; and resistive vibration exercise [53] all led to increased bALP levels when compared to pre bed rest or control group.

Although the current short-duration study did not employ direct measurements of bone loss to confirm the impact of changes in sCD200 and sCD200R1, our data strongly suggest that incorporation of measurements of sCD200, sCD200R1 may provide useful, predictive information for those interested in monitoring bone loss in humans under different conditions. Future bed rest studies of longer duration are planned with measurements of these plasma markers, as well as computed tomographic analysis of bone composition to confirm whether sCD200 and sCD200R1 levels are valid surrogates for subsequent bone alterations. In addition, it will be important to conduct parallel studies with age-matched female participants in the bed rest studies to confirm whether regulation in males and females is similar, or whether hormonal influences can also modify the use of these surrogate markers.

Conclusion

Short term head down bed rest resulted in changes in the serum markers, sCD200 and sCD200R1, that correlate with other markers frequently associated with bone loss. Applied countermeasures (AG) had a positive effect in attenuating the decline in sCD200 with an effect also on sCD200R1. The data suggest that these markers may prove useful in monitoring changes in bone metabolism in such studies in future.

Our data further suggest that fine tuning of the centrifugation protocol is likely required to optimize its impact. At least for sCD200, AG1 produced superior results as a countermeasure but was not well tolerated, with only 6 out of 11 subjects able to complete the required centrifugation. Introducing exercise with centrifugation might eliminate the cardiovascular (hypotension) problems while maintaining the benefits for the skeletal system [54]. Taken together our data suggest that a short term bed rest study may be a useful first step for the development/comparison of countermeasures to prevent bone loss associated with disuse. They argue further that application of artificial gravity may have a beneficial effect in attenuating bone loss, as monitored by the surrogate markers described.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bone.2013.12.004.

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