Increased Sclerostin Serum Levels Associated with Bone Formation and Resorption Markers in Patients with Immobilization-Induced Bone Loss

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Context: Sclerostin, a Wnt signaling antagonist on the osteoblasts produced by osteocytes, is regulated by mechanical strain and is implicated in the pathogenesis of disuse bone loss. There are no data on sclerostin in humans.

Objective: The aim of the study was to evaluate sclerostin in patients immobilized after stroke, compared with control subjects, and to analyze its relationship with markers of bone formation and resorption.

Design: This was a cross-sectional study.

Setting and patients: We studied 40 postmenopausal women immobilized after a single episode of stroke 6 months or longer after onset, and 40 postmenopausal women from the general community. Bone status was assessed by quantitative ultrasound measurements at the calcaneus. Bone alkaline phosphatase (b-AP), carboxy-terminal telopeptide of type I collagen (CrossLaps), and sclerostin were evaluated by ELISA. We also used ELISA to measure serum levels of Dickkopf-1, another soluble inhibitor of Wnt/β-catenin signaling, highly expressed by osteocytes.

Results: Immobilized patients had higher sclerostin serum levels (median 0.975 ng/ml; 25th to 75th percentiles 0.662–1.490) than controls (median 0.300 ng/ml; 25th to 75th percentiles 0.165–0.400: $P < 0.0001$) and an increased bone turnover with a more significant rise in bone resorption (CrossLaps) than formation (b-AP) markers. Sclerostin correlated negatively with b-AP ($r = -0.911$; $P < 0.0001$) and positively with CrossLaps ($r = 0.391$; $P = 0.012$). Dickkopf-1 did not significantly differ between the groups. Patients also had quantitative ultrasound measurements index lower than controls ($P < 0.001$).

Conclusions: This study shows for the first time that long-term immobilized patients present hypersclerostinemia associated with reduced bone formation, and suggests that sclerostin could be a link between mechanical unloading and disuse osteoporosis in humans. **(*J Clin Endocrinol Metab* 95: 2248–2253, 2010)**

Bone loss associated with skeleton unloading is a critical issue for bed-ridden patients (1–5). Histomorphometric studies have shown an increase in the number of osteoclasts and an enlargement of resorption cavities in patients immobilized for more or less prolonged periods (6). The results on biochemical parameters show that markers of bone resorption are dramatically increased in these patients, whereas bone formation markers are only

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Abbreviations: b-AP, Bone-specific alkaline phosphatase; BMD, bone mineral density; BMI, body mass index; BUA, broadband ultrasound attenuation; CrossLaps, carboxy-terminal telopeptide of type-1 collagen; CV, coefficient of variation; DKK-1, Dickkopf-1; LRP, lipoprotein receptor-related protein; QUS, quantitative ultrasound; SI, stiffness index; SOST, sclerostin gene.

slightly elevated or within the reference range, regardless of the length of immobilization, suggesting an uncoupling between bone resorption and formation (2, 3). On the other hand, pharmacological intervention with bisphosphonates (which dramatically reduce bone resorption) shows limited efficacy in restoring bone mass (7). The discovery of the Wnt signaling pathway and its relevance to bone homeostasis has allowed rapid progress in the understanding of the cellular and molecular mechanisms responsible for the adaptation of bone to unloading (8, 9). According to a fairly well-established model of action for the Wnt pathway in bone cell function, the binding of the appropriate Wnt to a coreceptor complex involving Frizzled receptor and low-density lipoprotein receptor-related protein (LRP)-5 or -6 on the osteoblast stabilizes cytoplasmic β-catenin protein, which translocates into the nucleus and activates the transcription of target genes important for the differentiation, proliferation, and functionality of osteoblastic cells.

Wnt signaling can be antagonized by secreted or intracellular inhibitors. Secreted Wnt signaling inhibitors are secreted frizzled-related proteins, the Wnt inhibitor factor, and Cerberus, which block Wnt function by binding and sequestering the molecule; Dickkopfs (DKK-1) and sclerostin, the protein product of the sclerostin gene (SOST) expressed mainly by osteocytes, which prevent the formation of the Wnt-Frizzled-LRP5 complex by internalization of the LRP5/6 coreceptor and competitive binding to LRP5, respectively (10, 11).

The role of the Wnt signaling antagonists seems to be crucial in the pathogenesis of disuse osteopenia. Robling *et al.* (12) have shown that production of sclerostin by osteocytes is dramatically reduced by mechanical loading in rats and mice. DKK-1 transcripts were also significantly reduced, although to a lesser degree than sclerostin. A similar experimental model was used by Lin *et al.* (13) to demonstrate that mechanical unloading of wild-type mice caused up-regulation of sclerostin and decreased Wnt/β-catenin signaling activity. These observations provide an attractive explanation for the regulatory mechanism that permits enhanced or reduced Wnt signaling upon mechanical stimulation and unloading, respectively.

The current cross-sectional study was undertaken to examine bone mass and bone metabolic changes in a homogeneous group of 40 postmenopausal women immobilized after an episode of paralytic stroke, trying to evaluate: 1) the serum levels of sclerostin; 2) the serum levels of DKK-1; and 3) their possible relationship with bone mass and bone turnover.

Patients and Methods

A total of 40 consecutive, Caucasian, postmenopausal institutionalized patients aged between 61 and 103 yr were candidates for inclusion in the study. All subjects were recruited on a volunteer basis, according to the following criteria: 1) had a single episode of stroke onset of 6 months or longer, 2) were unable to walk without physical assistance from other people, or 3) had no significant cognitive deficits. Diagnosis of stroke was based on clinical evaluation and computed tomography brain scans. At baseline in each patient the Barthel index, a functional dependence score (14), was evaluated. Exclusion criteria were: 1) neurological disorders apart from stroke, 2) use in the last 12 months of drugs affecting bone metabolism, including bisphosphonates, 3) chronic glucocorticoid use for more than 3 months, 4) known diseases affecting bone (Paget's disease, rheumatoid arthritis, hyperparathyroidism, hypercortisolism, malignant tumors, renal bone disease, chronic liver disease, and posttransplantation bone disease), or 5) history of serious cardiovascular diseases (myocardial infarction, uncontrolled hypertension). Ethical approval was obtained by the hospital ethical committee. All patients gave informed consent before entering the study, which was conducted in accordance with the Declaration of Helsinki. A control group of 40 free-living, age-matched postmenopausal women was formed. The same exclusion criteria as those stated for patients were used for controls. Both patients and controls had a body mass index (BMI) of greater than 20 kg/m^2.

Laboratory analysis

Blood samples were collected in the morning after an overnight fast. In all patients, laboratory analyses were performed either within the 2 h after drawing of blood or thawed serum that had been stored at −30 C before analysis. Serum concentrations of total calcium (corrected for albumin concentration), phosphorus, and creatinine were measured using standard automated laboratory techniques. Serum bone alkaline phosphatase (b-AP), carboxy-terminal telopeptide of type I collagen (CrossLaps), DKK-1, and sclerostin were detected as follows. b-AP and CrossLaps were measured using commercially available immunoenzymetric assays from Immunodiagnostic System Ltd. (Fountain Hills, AZ). Intra- and interassay precision ranged from 1.8 to 10.8% for both biochemical tests. DKK-1 was measured by ELISA using reagents provided by Biomedica Medizinprodukte [Wien, Austria; intraassay coefficient of variation (CV) 7–8%, interassay CV 9–12%].

One aliquot of serum sample was sent on dry ice by courier to the Biomedica Laboratory (Wien, Austria) for the measurement of sclerostin by a sandwich-type ELISA. The sclerostin ELISA uses a biotinylated antibody/horseradish peroxidase-streptovidin for detection of the analyte. The detection limit of the sclerostin ELISA was 0.18 ng/ml and the intraassay CV was between 6 and 10%. In this sclerostin ELISA, 100% of samples of healthy subjects showed detectable values. Serum mean concentration in female blood donors were 0.19 and 0.33 ng/ml in subjects aged younger than or older than 50 yr, respectively (15).

Quantitative ultrasound (QUS) assessment of calcaneus

Bone status was assessed by QUS measurements of the right calcaneus except for the hemiplegic patients, in which the mea-

TABLE 1. Characteristics and biochemical data of study population

	Patients	Controls	Normal values
n	40	40	
Age (yr)	81.4 ± 8.2	79.8 ± 7.7	
BMI (kg/m^2)	24.3 ± 1.3	23.8 ± 1.4	18.5–24.9
Poststroke duration (months)	10.4 ± 3.6		
Paretic side (left/right)	30/10		
Type of stroke (ischemic/hemorrhagic)	28/12		
Barthel index	51.3 ± 11.4		100
Hypertension (n)	15	10	
Coronary artery disease (n)	8	2	
Diabetes (n)	18	8	
Depression (n)	12	4	
Corrected calcium (mg/dl)	9.4 ± 0.8	9.2 ± 0.7	8.6–10.6
Phosphorus (mg/dl)	3.4 ± 0.7	3.6 ± 0.8	2.5–4.5
Creatinine (mg/dl)	0.9 ± 0.3	0.8 ± 0.4	<1.2

Data are expressed as mean ± SD when appropriate.

surements were carried out at the heel of the nonaffected limb, using the Achilles Ultrasound Express device (GE Lunar, Madison, WI), a dry system using a coupling ultrasound gel. It provides three parameters of skeletal status: broadband ultrasound attenuation (BUA), speed of sound, and stiffness index (SI) that derives from the combination of BUA and SOS according to the algorithm SI = 0.67 × BUA + 0.28 × speed of sound − 240.

A quality-control procedure using the standard phantom was performed daily before the measurements in the present study. *In vivo* short-term precision, calculated from three repeated measurements by the same operator, with repositioning, on 10 healthy subjects and expressed as the mean square root of the coefficients of variation, was 2.05% for SI. A single ultrasonometer was used throughout the study, and all measurements were carried out by the same specially trained technician. QUS of calcaneus is a radiation-free, low-cost, rapid, and easily used technique for evaluating bone mineral density (BMD) and correlates highly with dual-energy x-ray absorptiometry (16). Moreover, because QUS provides information on not only BMD but also bone quality, a large number of cross-sectional and prospective studies have established it as predictive of osteoporosis-related fractures independently of dual-energy x-ray absorptiometry-BMD (17, 18). In our hands, this procedure has been proven to be a relevant tool for the assessment of bone involvement in patients with multisystemic diseases (19, 20).

Statistical analysis

Descriptive statistics and significance levels were determined using the GraphPad InStat version 4.0 for Windows (GraphPad, San Diego, CA). Results are expressed as means ± SD. The normal distribution of values for different parameters was verified with the Kolmogorov-Smirnov test. Pearson standard linear regression analysis (normal distribution) or Spearman test (nonnormal distribution) was used for correlation studies. For the comparison of two independent data sets, Student's *t* test or Wilcoxon test was performed as appropriate. Multiple regression analysis was used to determine the influence of one independent variable after correcting for others. $P < 0.05$ was considered as statistically significant.

Results

Baseline characteristics of patients and controls

Subject characteristics and biochemical data are described in Table 1. There were no statistically significant differences between patients and controls for age, BMI, calcium, phosphorus and creatinine. Patients spent an average of 22.4 ± 0.6 h/d resting in the bed or sitting in the wheelchair. They did (n = 14) or did not (n = 26) regularly perform passive standing with the aid of a standing device. The mean reported standing time was less than 30 min/d. Patients did not consume alcohol nor did they smoke. Two patients reported a fall from the wheelchair, with no subsequent fracture. Estimated mean calcium intake was 870.2 ± 80.5 mg/d and was considered adequate.

QUS results

Patients had a mean stiffness of 57.89 ± 19.35 (range 42–97), significantly lower than controls (79.40 ± 17.37; $P < 0.001$). We did not find any significant association between SI and time after stroke.

Biochemical parameters

In patients, b-AP serum levels were 38.33 ± 25.21 μg/liter, significantly higher than in controls (14.68 ± 8.75 μg/liter, $P < 0.001$). CrossLaps serum concentrations in patients were higher than in controls (1.05 ± 0.57 *vs.* 0.46 ± 0.18 ng/ml; $P < 0.001$). DKK-1 serum concentrations did not significantly differ between immobilized and free-living subjects (26.51 ± 15.22 *vs.* 30.32 ± 12.40 pmol/liter). Sclerostin serum levels in patients were 1.164 ± 0.654 ng/ml (median 0.975; 25th to 75th percentiles 0.662–1.490). Sclerostin serum levels in controls were 0.298 ± 0.135 ng/ml (median 0.300; 25th to 75th percentiles 0.165–0.400). The difference was significant, with $P < 0.0001$ (Fig. 1).

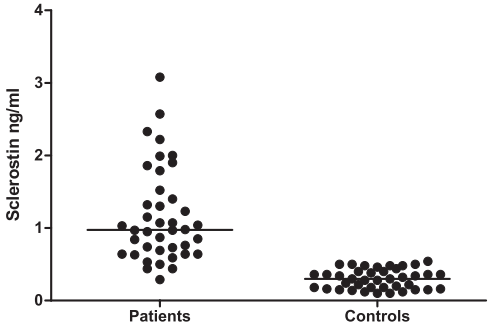

FIG. 1. Sclerostin serum levels (nanograms per milliliter) are higher in immobilized patients *vs.* healthy free-living subjects ($P < 0.0001$). Data are presented as median.

Correlations between sclerostin, DKK-1, and bone turnover markers

A significant negative correlation between sclerostin and b-AP serum levels was observed in immobilized patients (Fig. 2; $P < 0.0001$; r = −0.911; 95% confidence interval −0.953 to −0.835). Moreover, we found a significant positive correlation between sclerostin and CrossLaps serum levels (Fig. 2; $P = 0.0126$; r = 0.391; 95% CI 0.081–0.632). No significant correlation was found between DKK-1 and sclerostin, DKK-1 and b-AP, and DKK-1 and CrossLaps (Fig. 3).

Multiple regression analysis

A subsequent multiple regression analysis was performed to check correlations among the potential determinant variables. Sclerostin was designated as the dependent variable, whereas b-AP, CrossLaps, and DKK-1 were included as independent variables. In this analysis, sclerostin was independently negatively associated with b-AP (β −0.01702; SE 0.002821; 95% CI −0.2275 to −0.01130) and independently positively associated with CrossLaps (β 0.3086; SE 0.1235; 95% CI 0.05798–0.5592). Analysis shows a variance inflation factor, calculated from r^2, not greater than 1, excluding multicollinearity problems in this model.

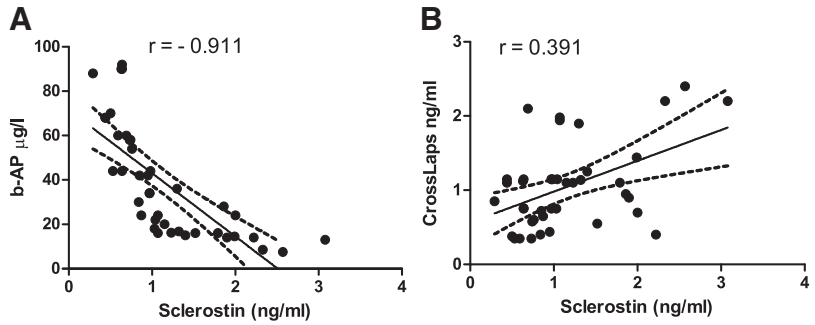

FIG. 2. Univariate correlations between serum sclerostin levels and serum b-AP (A) and serum CrossLaps (B) in 40 immobilized patients. There was a significant negative association between sclerostin and b-AP (r = −0.911, $P < 0.0001$) and a significant positive association between sclerostin and CrossLaps (r = 0.391; $P = 0.012$).

Discussion

This cross-sectional study confirms that sclerostin is detectable in human serum, as observed very recently by Appel *et al.* (21) in patients with ankylosing spondylitis, and documents that patients with immobilization-associated bone loss have sclerostin serum levels higher than controls. We also observed a significant inverse correlation between sclerostin and b-AP, a valuable marker of osteoblast activity. Our findings from QUS measurements were consistent with those reported by Chow *et al.* (22), who used an Achilles device at the calcaneus, and with the data by Zehnder *et al.* (3), who showed that in immobilized patients calcaneal stiffness decreases following a curve that leveled off 6–12 months after the beginning of immobilization. Results obtained with quantitative ultrasound are consistent with BMD results because QUS is partly determined by bone mass (3). The results of biochemical markers of bone turnover show that bone turnover is increased (as indicated by the high levels of CrossLaps and b-AP), although the lack of a significant correlation between CrossLaps and b-AP values suggests an imbalance between resorption and formation, which leads to an uncoupling of the two processes. This appears in line with previous results by Zehnder *et al.* (3) and our own research (2).

The lack of compensatory increase in the bone formation rate contributes to explaining why the severity of bone loss with immobilization is greater than in postmenopausal osteoporosis, accounting for substantially higher fracture rates in these patients (3, 23, 24). The cause of immobilization seems to influence distribution of bone loss: in spinal cord injury, in fact, bone loss is confined to the sublesional areas of the skeleton (25, 26); in stroke patients, bone is rapidly lost on the paretic side, although a loss on the nonparetic side (up to 4% over 1 yr) was also observed at the hip level (27–29). In both stroke and spinal cord injury, the pathogenesis of bone loss is mainly related to loss of mechanical stimulation of the bones. Bones continuously adapt to their mechanical requirements by two distinct mechanisms: modeling and remodeling (30, 31). Whereas the modeling process tends to reshape bone to meet its functional needs during skeletal growth, maintenance of bone tissue once skeletal maturity is achieved depends on the remodeling process. Disuse or lack of loading promotes an acceleration of bone remodeling, with bone resorption exceeding formation. Animal models of disuse have consistently showed that in mature bone, disuse causes bone loss

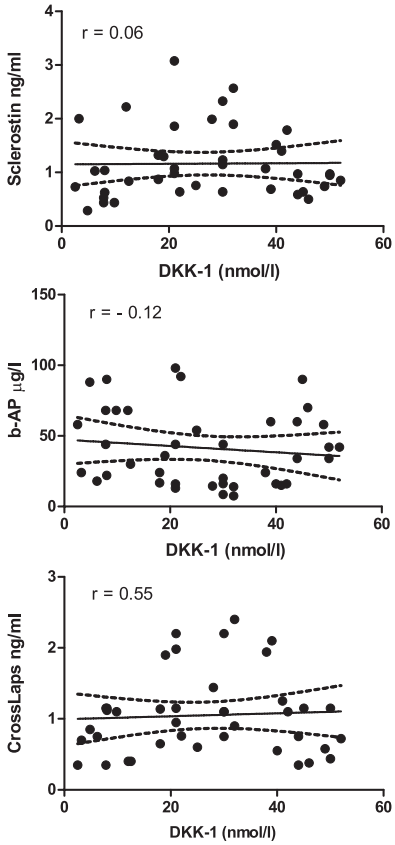

FIG. 3. Lack of significant association between serum DKK-1 levels and serum levels of sclerostin (A; r = 0.06), b-AP (B; r = 0.12), and CrossLaps (C; r = 0.55).

mainly at the endosteal surface of long bones, with rapid trabecular bone loss and increase in cortical porosity (32–34). This pattern is similar in both patients and human volunteers and can be counteracted by appropriate exercise (1, 5).

The molecular mechanism responsible for the decreased bone formation and bone mass during skeletal unloading has been recently addressed by Lin *et al.* (13). According to this author, sclerostin expression is increased in wild-type mouse osteocytes in response to unloading. On the other hand, $SOST^{-/-}$ mice were resistant to unloading-induced bone loss, strongly implying that sclerostin mediates the bone response to unloading through antagonizing Wnt/β-catenin signaling. This supports a previous study by Robling *et al.* (12), who reported that mechanical stimulation of bone reduces the number of $SOST^{+}$ osteocytes, particularly in high-strain regions of the bone. These changes were transcriptionally regulated, as shown by a reduction of sclerostin mRNA levels by 73% in loaded rat ulnar diaphyses. These results indicate that sclerostin levels are tightly regulated by mechanical strain, implying also that sclerostin is a mechanically suppressed signal.

Our finding of increased serum sclerostin levels in immobilized patients suggests that this mechanism also may operate in human beings, at least when unloading of bone is associated with stroke. We found no changes in DKK-1 serum levels in these patients. This was an unexpected finding because DKK-1 is a Wnt signaling inhibitor with direct binding affinity to LRP5/6, whose expression is also decreased by loading, albeit to a lesser degree than sclerostin (12). Reduction in mechanical loading (disuse) has, however, no clear effect on DKK-1 transcript levels, which increased slightly at d 3 of tail suspension, without reaching significance (12). Animal models, like the osteopenic DKK-1 overexpressor mouse (35) and the high bone mass DKK-1 haploinsufficient mouse (36), indicate that DKK-1 is a potent inhibitor of bone formation, although immunohistochemical techniques failed to detect significant amounts of DKK-1 protein in the same sections used for sclerostin immunolocalization (12). This would suggest that SOST/sclerostin regulation represents a more potent mechanism for controlling strain-induced bone modifications.

Our results show that sclerostin plays a role in mediating the bone response to disuse, possibly, as demonstrated in mice, through antagonizing Wnt/β-catenin signaling. This observation may be of some relevance in the long-term therapy of patients immobilized as a consequence of stroke and, possibly, spinal cord injury. In these patients, in fact, the bone-resorption rate is increased, but there is no compensatory increase in the bone-formation rate. Given the exclusive expression of sclerostin in osteoblast/osteocytes in bone, inhibition of sclerostin, which is considered a valid therapeutic target in the treating of patients with low bone mass (13, 37, 38), may prove particularly useful in disuse osteoporosis. The immobilized patients of our study were, however, a small group of subjects, possibly resulting in reduced statistical power. This, together with the cross-sectional nature of our investigation, and the fact that patients were not subjected to any therapeutical intervention may represent a limitation of our study.

Finally, our observation that sclerostin circulates in substantial amounts in healthy subjects and in significantly higher amounts in immobilized patients may lend support to the hypothesis that sclerostin action is not limited to a local bone formation regulator because it might reach LRP5 receptors in tissues other than bone (39).

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