Determination of Serum and Plasma Sclerostin Concentrations by Enzyme-Linked Immunoassays

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Background: Sclerostin alters bone formation. The precise and reproducible measurement of sclerostin concentrations in biological samples is important for assessment of metabolic bone disease. We determined sclerostin concentrations in serum and plasma using two commercially available ELISA.

Methods: We measured sclerostin concentrations in serum or heparin-plasma obtained from 25 normal human subjects using two commercial ELISA available from Biomedica Medizinprodukte GmbH and TECO medical AG.

Results: With the Biomedica assay, serum sclerostin concentrations were $0.99 \pm 0.12$ ng/ml (mean $\pm$ SEM), and plasma concentrations were $1.47 \pm 0.13$ ng/ml (paired $t$ test, $P < 0.001$). With the TECO assay, serum sclerostin levels were $0.71 \pm 0.05$ ng/ml, and plasma sclerostin concentrations were $0.80 \pm 0.06$ ng/ml (paired $t$ test, $P < 0.001$). Serum and plasma sclerostin concentrations were significantly different when determined by the two assays (serum, $P = 0.015$; plasma, $P < 0.001$). Recovery of added recombinant sclerostin to serum was less than expected with both Biomedica and TECO assays ($P < 0.001$, paired $t$ test).

Conclusions: The concentrations of sclerostin in serum and plasma are different when determined by the two assays. Serum or plasma sclerostin concentrations with current assays should be interpreted with caution. The data suggest that the same assay should be used for comparing groups of patients or patients being followed longitudinally. Standardization of sclerostin assays is required before being introduced into general clinical laboratory use. (J Clin Endocrinol Metab 96: E1159–E1162, 2011)
women with low free estrogen index compared with premenopausal women (17). The expression of sclerostin in bone is related to hip bone mineral density (18), and immobilization is associated with increased sclerostin serum concentrations (19).

In these studies, the assays for sclerostin were performed using various commercially available kits. It is likely that sclerostin measurements will be used for clinical diagnosis of various bone disorders and that more studies will be performed using sclerostin assays. The similarities and differences between different kits are not known. So far, no clinical reference laboratory has performed an independent validation of the sclerostin ELISA. In anticipation of the demand for the sclerostin measurements, we examined the performance of two commercially available immunoassay kits using serum and plasma from healthy normal individuals.

Materials and Methods

Serum and heparin plasma samples were collected from 25 fasted individuals (14 premenopausal females and one postmenopausal female, age range 20–59 yr, average age 33 ± 11 yr; and 10 males, age range 20–59 yr, average age 40 ± 15 yr). The samples were centrifuged at 2500 rpm for 15 min at 4 C. ELISA were performed immediately or an aliquot of the sample was stored at −80 C until time of assay. Immediately before assay, all samples were brought to room temperature. No sample was thawed more than once. When necessary, all samples were diluted before loading the plates. Recombinant human sclerostin (rhSclerostin) produced in murine NSO cells was obtained from R&D Systems (Minneapolis, MN) lyophilized carrier-free and lyophilized in the presence of BSA. Sclerostin, in lyophilized carrier-free format, was reconstituted to 200 μg/ml in 1 mM NaCl, 20 mM Na₂HPO₄, whereas the rhSclerostin/BSA sample was reconstituted in PBS (1× PBS). Reconstituted sclerostin was stored in 10-μl aliquots and either immediately used or stored at −80 C until time of assay. Spike recovery tests were performed using a blank (sample diluent buffer) and serum samples, at a high standard point (3 ng/ml) of rhSclerostin, spiked into each of the 25 samples. The spike volume made up no more than 2.5% of the applied sample, and the sclerostin sample was always prepared in the appropriate sample buffer before spiking. Briefly, 10 μl rhSclerostin was spiked into 490 or 390 μl serum (depending on available sample volume) and was mixed thoroughly by vortexing. Serum samples were diluted 2-fold into the respective sample diluents, using 50 μl serum and 50 μl buffer, and were mixed thoroughly by vortexing.

All samples were brought to room temperature and vortexed for 5 sec before applying to the ELISA plate (Biomedica sclerostin ELISA from Biomedica Medizinprodukte GmbH & Co. KG, Wien, Austria, and TECO sclerostin ELISA from TECOmedical AG, Sissach, Switzerland). Assays were performed following instructions and using reagents and materials provided by the manufacturers.

Briefly, for the Biomedica sclerostin ELISA, 150 μl assay buffer was loaded per well, followed by 20 μl standards or samples, and 50 μl anti-sclerostin antibody. Plates were covered and incubated for 20–22 h at 21 C. The following day, wells were washed five times, and 200 μl conjugate was added to each well and incubated in the dark for 1 h. Wells were washed five times, 200 μl 3',5'-tetramethylbenzidine was added per well, and color was allowed to develop for 30 min at 21 C, followed by the addition of 50 μl stop solution. Absorbance was read within 10 min at 450 nm. With the TECO kit, 25 μl standards or samples were loaded per well, followed by 50 μl matrix and 50 μl antibody solutions. Plates were loaded, incubated on a shaker at 500 rpm for 5 min, and then incubated in the dark at 4 C for 22–23 h. Wells were washed with cold wash buffer four times and then developed in the dark with 100 μl 3',5'-tetramethylbenzidine solution at 21 C for exactly 30 min. The reaction was stopped with 100 μl 1 M HCl. Absorbance was measured at 450 nm. With the Biomedica kit, stated intra and inter-assay coefficients of variation (CV) are 5–6 and 2–6%, respectively. The TECO assay reports intra-assay CV of 1.3–1.6% and inter-assay CV of 1.8–2.7%.

Statistical analysis of the data were carried out using JMP version 8.0 software (SAS Institute, Cary, NC). Differences between groups were assessed using paired t tests.

Results and Discussion

The experimental interassay CV for six Biomedica sclerostin kits across the four highest standards (0.68–5.45 ng/ml) ranged from 4.1–9.8%, whereas the second lowest standard (0.34 ng/ml) had a CV of 35.5%. Interassay CV observed for six TECO kits ranged from 5.6–7.3% for the four highest standards (0.6–4.0 ng/ml) and was 24.4% for the second lowest standard of 0.25 ng/ml (data not shown). Statistically significant differences were noted between the concentrations of sclerostin measured with the Biomedica kit in serum (0.99 ± 0.12 ng/ml) vs. those measured in heparin plasma (1.47 ± 0.13 ng/ml) (Table 1).

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<tr>
<th>TABLE 1. Sclerostin concentrations determined by two ELISA in serum and heparin plasma</th>
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<td>Biomedica assay</td>
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<td>Serum sclerostin (ng/ml)</td>
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<td>Expected serum sclerostin (ng/ml)</td>
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Results are shown as mean ± SEM, n = 25.

* 3 ng recombinant sclerostin was added to samples.
noted, sclerostin concentrations were higher in the presence of heparin than in its absence. With the TECO assay, sclerostin concentrations were lower in serum vs. plasma (serum sclerostin 0.71 ± 0.05 ng/ml, plasma sclerostin 0.80 ± 0.06 ng/ml, paired t test, \( P < 0.001 \)). These data suggest that heparin increases the amount of sclerostin detectable by antibody, most likely as a result of displacement of sclerostin from proteins that mask epitopes recognized by antibody. This observation is consistent with the data showing that the addition of heparin to cells maintained in culture causes a displacement and increase in sclerostin within the cell supernatant, independent of sclerostin synthesis (20), and is supported by the presence of a heparin binding site within the sclerostin molecule. Additional information supporting this hypothesis is the incomplete recovery of added sclerostin to serum samples as a result of protein binding that is observed with both the Biomedica (expected sclerostin concentration, 3.95 ± 0.11 ng/ml, vs. observed sclerostin concentration, 2.93 ± 0.15 ng/ml) and TECO (expected sclerostin concentration, 3.69 ± 0.05 ng/ml, vs. observed sclerostin concentration, 3.21 ± 0.06 ng/ml) ELISA kits (Table 1). An alternative explanation for the lack of recovery of nominal amounts of sclerostin might be the instability of this recombinant form of sclerostin in kit assay buffers. However, rhsclerostin appears stable (as judged by SDS-PAGE) while suspended in PBS.

It is of interest that concentrations of sclerostin in serum and plasma determined by the two assays were different (Table 1) (serum sclerostin Biomedica vs. serum sclerostin TECO, \( P = 0.015 \); and plasma sclerostin Biomedica vs. plasma sclerostin TECO, \( P < 0.001 \)). The correlation between concentrations of serum sclerostin with the two assays is shown in Fig. 1. The \( R^2 \) of 0.135 with a \( P \) value of 0.07 shows a poor correlation between values of sclerostin obtained with two assays. Supplemental Fig. 1 (published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org) is a Bland-Altman plot of the data obtained in the two assays and once again supports a poor correlation between the two assays. In contrast, the correlation between concentrations of plasma sclerostin within the two assays is better with \( R^2 \) of 0.332 and \( P \) value of 0.0026 (Supplemental Figs. 2 and 3). The reason for the observed differences is unclear.

Furthermore, serum sclerostin concentrations upon 2-fold dilution were considerably greater than the expected value in six samples tested with the TECO kit and 14 samples with the Biomedica kits. The differences between the measured values obtained after dilution and subsequent correction for the dilution and the value of the undiluted sample were not statistically different with the Biomedica kit but were statistically different with the TECO kit (\( P = 0.005 \)) (Table 1). Nonlinearity may suggest poor antibody specificity, sample instability, or the presence of interfering substances in the sample matrix. Finally, the relatively high CV observed for the low standards, in both kits, impart uncertainty on values obtained at the lower end of the standard curve.

Sclerostin is bound by and alters the activity of lipoprotein receptor-like protein 5/6 (1–4), bone morphogenetic proteins (5–8), cyr61 (9), and the erbB3 receptor (10). The bone morphogenetic proteins circulate in blood in significant concentrations and are likely bound to sclerostin, given the high affinity of the proteins for one another (9). Structural studies show that the protein contains a heparin-binding site in loops 2 and 3 and that treatment of cells expressing sclerostin with heparin releases sclerostin into the cell culture medium without influencing sclerostin synthesis (20). These data suggest that sclerostin exists in serum as a complex bound to various proteins and that both free and bound forms of the protein might be recognized with different efficiency by antibodies. Furthermore, heparin might influence the binding of sclerostin to these proteins. If this were the case, one would expect that concentrations of measured sclerostin would be different in heparin plasma vs. serum. An alternative explanation for the observed phenomenon is an effect of heparin on antibody binding to sclerostin. Of note, the difference between serum and plasma concentrations is approximately 1.5-fold using the Biomedica kit (0.99 vs. 1.47 ng/ml) and approximately 1.1-fold using the TECO kit (0.71 vs. 0.80 ng/ml). The reason for this difference was not investigated but could be due to the difference between antibodies and differences in antibody binding to sclerostin.

**Conclusions**

Two commercial ELISA give different concentrations of sclerostin in both serum and plasma from normal healthy volunteers. Heparin influences the amount of
sclerostin detected by the two ELISA, suggesting binding of sclerostin to proteins from which the protein is displaced by the oligosaccharide. The incomplete recovery of added sclerostin in serum supports this observation. Caution is required in interpreting the biological importance of changes in sclerostin concentrations in human plasma or serum. We recommend that the same sclerostin assay be used for comparing groups of patients or patients being followed longitudinally. Standardization of sclerostin assays is required before being introduced into general clinical laboratory use.

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