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Biochemical consequences of serum sclerostin elevation in systemic lupus erythematosus patients with persistent proteinuria

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ABSTRACT

Sclerostin, a 190 amino acid glycoprotein synthesized in osteocytes, is a potent downregulator of bone metabolism in patients with chronic kidney diseases. In this work, the prevalence of serum sclerostin in 50 systemic lupus erythematosus (SLE) patients with persistent proteinuria, 35 SLE patients without proteinuria and 42 normal subjects was studied. The relationships of serum sclerostin with estimated glomerular filtration rate (eGFR), bone specific alkaline phosphatase (BAP), calcium, protein/creatinine ratio (P/C), phosphorus, parathyroid hormone (PTH), vitamin D were also tested. The mean level of serum sclerostin in SLE patients with and without proteinuria and normal subjects were, 106.1, 14.3 and 13.8 ng/ml, respectively. It was significantly higher in SLE patients with proteinuria than normal subjects and SLE patients without proteinuria ($P<0.0001$). No significant change was observed in the mean level of sclerostin between normal subjects and SLE patients without proteinuria. PTH and phosphorus were significantly increased while calcium, BAP, eGFR and vitamin D significantly decreased in SLE patients with proteinuria when compared with normal subjects and SLE patients without proteinuria. In SLE patients with proteinuria, Sclerostin correlated positively with P/C ratio and phosphorus and negatively with calcium, BAP, eGFR, and vitamin D. Serum sclerostin, in patients with proteinuria, was not correlating with PTH, though it was significantly elevated compared to patients without proteinuria and normal subjects. Circulating levels of sclerostin were elevated in SLE patients with persistent proteinuria and it appeared to be due to lowered eGFR and hyperphosphatemia. Considering that sclerostin is an inhibitor of bone formation, the observed negative correlations of serum sclerostin with calcium, BAF, and vitamin D supported this hypothesis. Further biochemical and clinical studies on a larger patient population are needed to completely clarify the role of elevated sclerostin in such patients with a special concern to the value of its early elevation in anticipating biochemical abnormalities.

Introduction

The Wingless and Integration (Wnt) signaling pathways are a group of signal transduction pathways made of proteins that pass signals into a cell through cell surface receptors. These Wnt proteins are a family of secreted proteins which regulate many aspects of cell growth, differentiation, function, and death [1]. Considerable progress has been made in the understanding of the molecular links between Wnt signaling and bone development and remodeling since initial reports that mutations in the Wnt coreceptor low density lipoprotein receptor–related protein 5 (LRP5) are causally linked to alterations in human bone mass [2].

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Signaling through the Wnt/β-catenin pathway results in increases of bone mass through a number of mechanisms including renewal of stem cells, stimulation of preosteoblast replication, induction of osteoblastogenesis, and inhibition of osteoblast and osteocyte apoptosis. β-catenin, which is a protein that acts as an intracellular signal transducer in the Wnt signaling pathway, is required for the early stages of osteoblastogenesis, and indeed its absence steers the fate of mesenchymal precursors toward chondrogenesis [3].
The Wnt/β-catenin signaling pathway is controlled by endogenous regulators that are largely divided into extracellular and intracellular antagonists based on their sites of action. sclerostin is an example of the extracellular inhibitors [4]. Sclerostin, which was first discovered in 2001

from the genetic and molecular study of two rare sclerosing dysplasias, is a glycoprotein with 190 amino-acids found nearly exclusively in osteocytes [5]. Sclerostin was found to be a potent inhibitor of bone formation since it acts as an inhibitor of the Wnt coreceptor, LRP 5. By competitively inhibiting LRP 5, sclerostin inhibit the binding of Wnt ligands to LRP5 and as a result glycogen synthase kinase 3β is activated. This enzyme constitutively phosphorylates β catenin which leads to its ubiquitination and proteosomal degradation and as a result, the process of osteoblastogenesis is inhibited [6,7].

Chronic kidney disease (CKD) is a kind of kidney disease in which there is a progressive loss in kidney function over a period of months or years. CKD is initially without specific symptoms and is generally only detected as an increase in serum creatinine or protein in the urine [8]. Chronic kidney disease-mineral bone disorder is a clinical syndrome that develops as a result of systemic disorder of mineral and bone metabolism due to CKD manifested by abnormal serum levels of calcium, phosphorus, parathyroid hormone, and vitamin D [9]. In many CKD patients, previous kidney disease or other underlying diseases are already known. One of the most common causes is glomerular disease which is classified as: **primary** such as focal segmental glomerulosclerosis and IgA nephropathy and **secondary** glomerular disease as in diabetic nephropathy and lupus nephritis (LN) [8,10].

Systemic lupus erythematosus (SLE)is a highly complex autoimmune disorder involving in multisystem injuries, which usually occurs in females of childbearing age. LN accounts for significant morbidity and mortality inSLE patients. CKD, which occurs in SLE secondary to LN may result, at least in part, to osteoporosis and secondary bone fractures. Indeed, they are the two of the major causes of irreparable injury in patients with SLE [11].

The purpose of the present study was to test the circulating levels of sclerostin in SLE patients with persistent proteinuria and to describe the relationship between its elevation and different biochemical parameters in comparison with normal subjects and SLE patients without proteinuria.

Subjects and methods

Eighty-five SLE patients (50 with proteinuria and 35 without proteinuria) were enrolled in the present work. All patients were attending the outpatient clinic of the Rheumatology Unit, Ain Shams University Hospitals. SLE patients were diagnosed according to the revised classification criteria of the American College of Rheumatology and the 2012 Systemic Lupus International Collaborating Clinics Criteria [12,13]. All patients gave informed permission before entering the study. Forty-two normal subjects were also included.

All subjects were recruited according to the following criteria: **1)** patients with proteinuria had persistent elevated protein/creatinine (P/C) ratio ≥0.5 for at least one year; **2)** patients without proteinuria had normal P/C (<0.2) [14]; **3)** all patients and normal subjects had female gender (to avoid statistical bias since serum sclerostin is higher in males than females [15]); **4)** all patients were non-smokers and non-diabetic; **5)** no administration of calcium and vitamin D.

Exclusion criteria included: **1)** age ≤ 60 and ≥18 years; **2)** the presence of other autoimmune disease; **3)** hemodialysis patients.

Biochemical analysis

Blood samples were collected after overnight fast then sera were stored at -20 ^{0}C for later analysis. Random urine samples were collected for the detection of P/C ratio.

Sclerostin was detected in the sera by an enzyme linked immunosorbent assay (ELISA) Kit obtained from PELOBIOTEC GmbH (Germany). The kit uses a double-antibody sandwich ELISA. Diluted sera were added to the ELISA wells which were pre-coated with human monoclonal antibodies to sclerostin. Incubation; then, sclerostin antibodies labeled with biotin were added and combined with streptavidin –HRP to form enzyme complex; then incubation was carried out and washing to remove the uncombined enzyme. In the next step the chromogen solution was added. The chroma of the color formed and the concentration (ng/ml) of the human sclerostin in sera were positively correlated.

An ELISA kit (Ostase, USA) was used for the determination of Bone Alkaline Phosphatase (BAP). Samples containing BAP are reacted with a solution containing a biotin-labeled, BAP-specific monoclonal antibody. The reaction takes place in plastic well strips (solid phase) coated with streptavidin and enclosed in aplastic frame. Following the formation of a solid phase/capture antibody/BAP complex, the microplates were washed to remove unbound BAP and is then incubated with an enzyme substrate. The amount of substrate turnover was determined calorimetrically as Mcg/l.

Twenty-five vitamin D2/D3 was determined by an ELISA technique (Orgentec, Germany). Antibodies detecting 25-OH Vitamin D2 and 25-OH Vitamin D3 are bound onto microwells. In a first step 25-OH vitamin D contained in the sample were released from its vitamin D binding protein: The undiluted sample was placed in a test tube and mixed with sample buffer containing 25-OH vitamin D tracer reagent and then with vitamin D release reagent. Then 25-OH vitamin D was determined with the 25-OH vitamin D3/D2 ELISA assay. The determination is based on a competitive enzyme linked immunosorbent assay with the following steps: The released 25-OH vitamin D sample is transferred to reaction wells of the microtiter plate. Twenty-five-OH vitamin D in the sample competes with the 25-OH vitamin D tracer reagent for binding to the 25-OH vitamin D antibodies coated onto the microwells. Complexes are formed between antibody and 25-OH vitamin D or antibody and 25-OH vitamin D tracer. After incubation, a first washing step removes unbound and unspecifically bound molecules. Subsequently added enzyme conjugate binds to the immobilized tracer-antibody complexes. After incubation, a second washing step removes unbound enzyme conjugate. Addition of

enzyme substrate solution results in blue color development during incubation. Addition of an acid stops the reaction generating a yellow end-product. The intensity of the yellow color correlates inversely with the concentration of vitamin D reported as ng/ml.

Intact parathyroid hormone (PTH) was quantitatively assayed by an ELISA kit from DIA source (Belgium). Calibrators and samples react with the capture polyclonal antibodies (goat anti 1-34 PTH fragment) coated on the microtiter wells. After incubation, the excess of antigen is removed by washing. Then monoclonal antibodies (mouse anti 44-68 PTH fragment) labelled with horseradish peroxidase (HRP) are added. After an incubation period allowing the formation of a sandwich. Bound enzyme-labelled antibody is measured through a chromogenic reaction. The reaction is stopped with the addition of Stop Solution. The amount of substrate turnover is determined colorimetrically by measuring the absorbance, which is proportional to the PTH concentration (pg/ml).

Serum and urine creatinine were determined by the Jaffe colorimetric method (Egychem, Egypt) [16]. Urine protein was assayed turbidimetrically using tichloroacetic acid according to the method of Henry *et al.* [17]. Serum phosphate and total calcium were determined colorimetrically by kits from Chema (Italy) [18]. Estimated glomerular filtration rate (eGFR) was calculated by chronic kidney disease epidemiology collaboration formula [19].

Statistical analysis

Statistical analysis was performed by SPSS 11.0 program.

Results and discussion

This study included 42 normal subjects, 35 SLE patients without proteinuria and 50 patients with proteinuria (**Table 1**). Members of all groups were females, non-smokers and non-diabetic. The mean of disease onset of SLE patients with and without proteinuria was not statistically significant. The same was observed with the mean of age among all groups. **Table 2** shows the mean and the standard deviation (SD) values of: serum concentrations of Sclerostin, BAP, PTH, creatinine, phosphorus, calcium, and vitamin D in normal subjects, SLE patients without proteinuria and SLE patients with proteinuria. Thee GFR and protein/creatinine ratio are also included. **Table 3** presents the pearson's correlation between serum sclerostin in SLE patients with proteinuria and P/C ratio, BAP, PTH, vitamin D, creatinine, eGFR, calcium and Phosphorus.

The present study showed that the mean concentration of serum sclerostin in SLE patients without proteinuria was not statistically different from normal subjects. As expected, SLE patients with proteinuria, which represents a model of CKD [9], had a significantly higher serum sclerostin compared to normal subjects and SLE patients without proteinuria ($p< 0.0001$).

Out of extensive searches, it was found that Ferna´ndez-Rolda´*et al.* [20] the only paper which studied sclerostin levels in SLE. They found that the mean concentration of serum sclerostin in 38 SLE patients was similar to that in 20 healthy controls. However, it is not possible to compare their results with that of the present study because they did not show whether their SLE patients have nephritis or not. Indeed, the mean serum level of sclerostin in the study of Ferna´ndez-Rolda´*et al.* was 16.0 ng/ml which is nearly similar to those of the current study which were 14.3 and 13.9 ng/ml in normal controls and SLE patients without proteinuria, respectively.

Indeed, serum sclerostin was found to be increased in several cohorts of patients with CKD. Elevated serum sclerostin was first reported in patients with CKD by Cejka*et al.* [21] and their finding has been validated by others [22-24]. The elevated levels of sclerostin in CKD patients are likely to be dependent on accumulation of sclerostin in the serum due to a decline in glomerular filtration rate [25-27]. Moreover, the rapid restoration of serum sclerostin to the normal range in posttransplant also suggests that decreased renal clearance is responsible for the accumulation of sclerostin [28]. In a study comparing sclerostin concentration with the fall in eGFR, it was found that as the eGFR falls the sclerostin levels increase [25]. Recently, two animal model studies confirmed this inverse association between elevated serum sclerostin and eGFR [29,30]. In agreement with previous studies, we also found the eGFR to be significantly lowered in our SLE patients with proteinuria compared with SLE patients without proteinuria and normal subjects. At the same time, serum sclerostin was negatively correlated with eGFR in patients with proteinuria. As a manifestation of lowered eGFR, the correlation between sclerostin and the level of proteinuria (P/C ratio) was examined. A positive correlation between sclerostin and the level of proteinuria was observed ($r = 0.61$ & $p< 0.001$) and indeed, this may support the role of lowered eGFR on sclerostin elevation.

Table 1: Demographic characteristics of controls and SLE patients

	Controls	SLE patients without proteinuria	SLE patients with proteinuria
Number	42	35	50
Age (years)	34.7 (20-49)	35.4 (22-58)	33.8 (21-52)
Onset (years)	-	3.0 (1-5.5)	2.9 (1-6)

Data of age and disease onset are expressed as mean and range. The mean of age and disease onset were not statistically different among all groups.

Table 2: The laboratory features of controls and SLE patients

Parameters	control	SLE Without proteinuria	SLE With proteinuria	*One way* ANOVA *P*	Tukey Post-hoc (*P value)*		
					Control *vs* SLE -ve	Control *vs* SLE +ve	SLE +ve *vs* SLE -ve
Sclerostin *ng/ml*							
Mean ± SD	13.9 ± 5.0	14.3 ± 4.3	106.1 ± 78.0	0.0001*	NS	0.0001*	0.0001*
Range	7-32	11-28	14 – 290				
eGFR *mL/min/1.73*m²*							
Mean ± SD	96.9 ± 9.0	89.2 ± 6.1	41.9 ± 12.3	0.0001*	0.002*	0.0001*	0.0001*
Range	80 - 111	70 -99	19 - 70				
BAP *Mcg/l*							
Mean ± SD	11.3 ± 3.9	10.4 ± 3.3	6.4 ± 5.5	0.0001*	NS	0.0001*	0.0004*
Range	4.7-19	4.4-17	0.09-18				
P/C ratio							
Mean ± SD	0.06 ± 0.03	0.07 ± 0.04	1.5 ± 0.98	0.0001*	NS	0.0001*	0.0001*
Range	0.01-0.11	0.01-0.17	0.5 - 4.0				
PTH *pg/ml*							
Mean ± SD	44.1 ± 12.4	43.8 ± 13.0	53.5 ± 18.4	0.003*	NS	0.01*	0.01*
Range	22-66	22-70	26-96				
S. Creatinine *mg/dl*							
Mean ± SD	0.88 ± 0.15	0.91 ± 0.17	0.92 ± 0.27	NS	NS	NS	NS
Range	0.6-1.1	0.6-1.2	0.5-1.5				
P *mg/dl*							
Mean ± SD	3.5 ± 0.68	3.4 ± 0.17	3.86 ± 0.93	0.018*	NS	0.08*	0.02*
Range	2.2-4.8	2-4.9	2-6.1				
Ca *mg/dl*							
Mean ± SD	9.2 ± 0.49	9.3 ± 0.5	8.9 ± 0.52	0.001*	NS	0.01*	0.001*
Range	8.5 -10.2	8.6 -10.1	7.9 - 9.9				
Vit. D *ng/ml*							
Mean ± SD	52.7 ± 26.5	43.8 ± 27.1	24.3 ± 20.4	0.001*	NS	0.0001*	0.002*
Range	22 -100	8.0 -100	3.4 - 80				

Abbreviations: SLE –ve and SLE +ve, systemic lupus erythematosus patients without and with proteinuria, respectively; eGFR, estimated glomerular filtration rate; BAP, bone alkaline phosphatase; P/C ratio, protein/creatinine ratio; PTH, parathyroid hormone; S. creatinine, serum creatinine; P, phosphorus; Ca, calcium; Vit. D; vitamin D; NS, non-significant; *, Significant. Values are mean ± standard deviation (*SD). $P < 0.05$* is considered statistically significant for ANOVA and Tukey Post-hoc.

Table 3: Correlation between serum sclerostin in patients with proteinuria and different biochemical petameters

Parameter	*r*	*p*
P/C ratio	0.61	0.001
BAP	- 0.55	0.004
PTH	- 0.05	NS
Vitamin D	-0.32	0.02
Creatinine	0.25	NS
eGFR	- 0.42	0.03
Calcium	- 0.49	0.01
Phosphorus	0.90	0.0001

Abbreviations: P/C, protein/creatinine ratio; BAP, bone alkaline phosphatase; PTH, parathyroid hormone; eGFR, estimated glomerular filtration rate.

Bone alkaline phosphatase (BAP) is the bone-specific isoform of alkaline phosphatase. It is a glycoprotein that is anchored on the surface of osteoblasts, as a by-product of osteoblast activity, BAP became a renowned and very specific marker of bone formation. BAP removes the phosphate (dephosphorylation) from many molecules such as proteins, nucleotides, or pyrophosphates, therefore, BAP is important for bone mineralization [31,32]. Results of our study showed a significant decrease in BAP in SLE patients with proteinuria when compared to patients without proteinuria and controls. The levels of BAP in controls and SLE patients without proteinuria were similar. Sclerostin in SLE patients with proteinuria correlated negatively with BAP ($r = -0.55$, $p < 0.004$). This inverse correlation between sclerostin and BAP confirms the anti-anabolic effect of sclerostin on bone.

A similar negative correlation between sclerostin and BAP was observed in conservative CKD, dialysis patients and kidney transplant recipients [23,33].

The present study showed, as expected, that SLE patients with proteinuria had the typical altered mineral metabolism of CKD. This was manifested by the presence of a significantly lower serum calcium and vitamin D and higher serum phosphate and PTH in SLE patients with proteinuria compared with SLE patients without proteinuria and normal controls. This abnormal mineral metabolism in our SLE patients with proteinuria suggests that these patients as having chronic kidney disease–mineral and bone disorder which is one of the complications accompanying CKD. In correlation analysis, we identified calcium and phosphate to correlate significantly with serum sclerostin levels ($r = -0.49$ & $p<0.01$ for calcium and $r = 0.9$ $p<0.0001$ for phosphorus). Serum sclerostin was negatively correlating with vitamin D (-0.32 & $p< 0.02$). Similar correlations between serum sclerostin and calcium, phosphorus and vitamin D were also reported by others in patients with CKD [34-36]. The effect of serum sclerostin on calcium was studied by Ryan *et al*., [36] and they found that in the absence of sclerostin urinary calcium is diminished, suggesting a direct effect of sclerostin on renal excretion of calcium. Sclerostin also has a similar direct role in lowering vitamin D since it was reported that gene knockout mice had increased concentration of vitamin D [37]. The relationship between sclerostin and phosphorus is different from that of calcium and vitamin D since Oliveira *et al.* reported that phosphate could be a modulator of sclerostin expression in patients with early CKD [38]. Similarly, an animal model study showed that high phosphate diet was associated with an increase in both serum and bone sclerostin [39].

Indeed, alteration in calcium, phosphorus and vitamin D homeostasis occur early in the course of CKD and progress as kidney function decrease. If left untreated, these alterations can result in significant consequences including mainly, osteoporosis, which is the pivotal effect of elevated sclerostin level [25]. In an animal model of osteoporosis, inhibition of sclerostin by monoclonal antibodies induced osteoblast activity and new bone formation, normalized bone mineral metabolism and improved bone architecture and strength [40]. Moreover, sclerostin antibody treatment enhanced bone fracture healing in a rat femoral osteotomy model, as reflected in increased bone formation, bone mass and bone strength [41].

It has been reported in CKD and non-CKD patients, that serum levels of sclerostin are correlated with PTH [42,43]. PTH negatively regulate sclerostin expression by direct binding to osteocytes [26]. However, in contrast with these studies, sclerostin and PTH were not related in the present work. Similar results were reported by Kanbay *et al.* [25] in a cohort of 173 CKD patients. Indeed, there are several potential explanations for this lack of correlation between sclerostin and PTH. First, the level of PTH in patients of the current study is relatively low when compared to other studies. Second, the type of CKD patients is different. Third, sclerostin could be regulated by PTH independent ways.

Lastly, it must be noted that recent clinical studies demonstrated that treatment with anti-sclerostin antibodies resulted in improvement of bone mineral metabolism and osteoporosis in patients with CKD. Romosozumab is a model of humanized monoclonal antibody that targets sclerostin for the treatment of osteoporosis [40,44]. Interestingly, monoclonal antibodies against sclerostin was suggested by Bultink [45] as an attractive therapeutic option for the treatment of osteoporosis in SLE.

Conclusion

The present data showed that serum levels of sclerostin were elevated in SLE patients with persistent proteinuria with low eGFR and hyperphosphatemia as possible causes of its elevation. The negative correlation of sclerostin with BAP, calcium, and vitamin D, as bone related items, greatly confirmed its role as a negative regulator of bone metabolism. Additional biochemical and clinical studies in larger disease populations are recommended for complete understanding of sclerostin's impact in SLE patients, with a special concern to its role in the early prediction of biochemical abnormalities.

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