Increased Osteoblastic Activity and Expression of Receptor Activator of NF-κB Ligand in Nonuremic Nephrotic Syndrome

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Patients with nephrotic syndrome (NS), even with normal GFR, often display altered mineral homeostasis and abnormal bone histology. However, the latter, mostly osteomalacia and increased bone resorption, cannot be readily explained by the prevalent concentrations of parathyroid hormone and vitamin D metabolites. The transmembrane receptor activator of NF-κB ligand (RANKL) of osteoblasts is essential for osteoclast formation and differentiation. Osteoblasts activity and the expression of RANKL were tested in cultures of normal human osteoblasts with sera obtained from patients with NS and normal GFR (129 ± 26 ml/min per 1.73 m^2) during relapse and remission of their NS. Osteoblasts that were cultured *in vitro* with sera during relapse displayed elevated concentrations of alkaline phosphatase (AP) and increased expression of RANKL. By contrast, during remission, AP concentrations were significantly lower ($P < 0.05$) and RANKL expression notably attenuated or absent. AP correlated with the proteinuria ($r = 0.5$, $P < 0.05$) and was not significantly affected by the therapeutic administration of corticosteroids. Whereas parathyroid hormone levels were normal (35 ± 21 pg/ml), the serum markers of bone formation (osteocalcin and bone-specific alkaline phosphatase) were lower during relapse compared with remission. Thus, sera from patients with NS and normal GFR stimulate the activity of osteoblasts and upregulate their expression of RANKL. These alterations, more prominent during clinically active NS, are transient and reversible upon remission. These disturbances of bone biology may play an important pathogenic role in the abnormal bone histology observed in patients with NS even before a decline in GFR occurs.

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Although most patients with idiopathic nephrotic syndrome (NS) present with normal GFR, alterations of mineral homeostasis similar to those encountered with chronic renal failure are frequently identified, including hypocalcemia, reduced serum vitamin D metabolites, and elevated levels of immunoreactive parathyroid hormone (PTH) (1–6). Metabolic balance studies have demonstrated intestinal malabsorption of calcium (5) as well as excessive urinary losses of various vitamin D metabolites and their binding proteins (2,7). Furthermore, important biologic consequences such as reduced bone mineral density (BMD) (3,8) and abnormal bone histology (9,10) have been documented. The latter include osteomalacia of varying degree (10) as well as excessive bone resorption resembling secondary hyperparathyroidism (9). Early identification and management of these abnormalities, even before the decline of GFR, could ameliorate the growth retardation and renal osteodystrophy that invariably affects children with more advanced chronic kidney disease (CKD) (11). This is particularly relevant to patients with NS because a growing incidence of corticosteroid-resistant NS secondary to focal segmental glomerulosclerosis (12,13) is a leading cause of CKD stages 4 to 5 in children (14).

Nevertheless, bone histologic changes that are identified in patients with NS do not correlate consistently with the prevailing serum concentrations of divalent ions or calciotropic hormones. Serum phosphorus concentration is usually normal (1,3,5), calcitriol remains normal in most patients (3,15), and despite low serum levels of ionized calcium, PTH levels are not consistently elevated (1,3,5,6,15). Thus, the mechanisms that result in altered BMD and bone histology in patients with NS before the reduction in GFR are still unclear.

Other circulating and local factors, in particular cytokines and growth factor members of the TNF superfamily, have been identified as important modulators of bone remodeling (16,17). The interaction of the receptor activator of NF-κB ligand (RANKL) produced by osteoblasts lineage cells with its specific receptor RANK triggers a signaling pathway in the osteoclasts, inducing osteoclast formation, fusion, activation, and survival, leading to bone resorption and bone loss (18). Cytokines such as TNF-α and several interleukins (IL-2, IL-4, and IFN-γ) are increased in serum of patients with active NS, whereas remissions are characterized by downregulation of these cytokines (19–21). Therefore, other factors that are present in the sera of patients with NS besides vitamin D and PTH may affect osteoblast activity and contribute to the abnormalities observed in

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bone histology. To test this hypothesis, we decided to evaluate the ability of sera from patients with NS to activate *in vitro* osteoblasts and to determine the possible role of RANKL in the osteoblast activation in patients with normal GFR during different clinical stages of the NS.

Materials and Methods

Patients

Twenty-nine patients (17 boys, 12 girls) were studied at an average age of 8.5 ± 4.7 yr. All patients had normal GFR (129 ± 26 ml/min per 1.73 m^2), normal PTH concentrations (35 ± 21 pg/ml), and no evidence of metabolic acidosis (serum CO_2 concentrations 22.5 ± 2.0 mEq/L). The original diagnosis of NS was based on the presence of nephrotic-range proteinuria, hypoalbuminemia, edema, and hypercholesterolemia (22). Nephrotic-range proteinuria was defined by a protein excretion in excess of 40 mg/m^2 per d and/or a urinary protein/creatinine ratio (UP/C) >1.0 mg/mg (normal <0.2 mg/mg) on random urine specimens obtained at the time of routine clinical visits (23). Patients who received vitamin D supplements or loop diuretics during the preceding 3 mo were excluded from the study. Diagnostic renal biopsies were performed in 20 patients, and the histopathology consisted of minimal-change disease (n = 16) or focal segmental glomerulosclerosis (n = 4). The remaining nine patients did not undergo a renal biopsy and were presumed to have minimal-change disease on the basis of their clinical course. After informed parental consent, urine and blood sampling were obtained during both stages of clinical activity of the NS (relapse and remission). Relapse was defined by a UP/C >0.2 and a serum albumin concentration <3.0 g/dl. Remission was defined by a UP/C <0.2, a serum albumin concentration >3.0 g/dl, and the absence of edema.

Blood and urine sampling during episodes of relapse were obtained before the initiation of corticosteroid or any other therapy in most patients, whereas only a few patients were analyzed during glucocorticoid (n = 3) or other immunosuppressive therapy (n = 3). During remission, five samples were obtained while the patients were receiving cyclosporine therapy, and three samples were obtained while the patients were receiving glucocorticoids; most samples were obtained off all medications, including glucocorticoid therapy, for at least 6 wk. In four patients, blood sampling was obtained on two separate occasions during remission, and the reported results represent average values.

Laboratory Methods

Biochemical Determinations. Urinary protein and serum creatinine, albumin, and cholesterol concentrations were determined by routine chemical analysis. Serum bone-specific alkaline phosphatase (BSAP) concentrations were measured by an immunoradiometric assay and osteocalcin by an immunochemiluminometric assay (24,25). Serum N-telopeptide cross-links of type I collagen (NTx) concentrations were determined using an immunoenzymatic assay (26), and the results were compared with normal control children who attended the local pediatric clinic for causes not affecting renal function or bone-mineral metabolism (n = 52; age 8 ± 3 yr; range 3 to 17 yr). Of these, samples from six patients were used as controls for the assays performed on the osteoblast cultures described in the following section. Serum PTH was measured by a two-site immunoradiometric assay that detects intact PTH (1–84) and the amino-terminally truncated PTH (7–84) fragments (27).

***In Vitro* Osteoblast Cultures.** Normal human osteoblasts (NHOst, lot number 1 F2254; BioWhittaker, San Diego, CA) were obtained from a single 16-yr-old male white donor. The cells were obtained in the third passage and were cryopreserved and stored in liquid nitrogen until their use. These adherent fibroblast-like cells grow as monolayer. The routine characterization of NHOst includes immunofluorescent staining positive for alkaline phosphatase and for bone mineralization (von Kossa stain) in differentiation medium with ascorbic acid and b-glycerophosphate after 10 to 20 d. Cell viability was assessed using Trypan Blue, and the total number of viable cells was determined by the following equation: Total cell count × % viability/100. Cells were grown at 37°C, 5% CO_2 in DMEM-F12 medium (Sigma, St. Louis, MO) that contained 10% FBS (Life Technologies, Inc., Gaithersburg, MD) supplemented with 2 mM L-glutamine (40 mM) and 1% penicillin/streptomycin. Cells were cultured for 24 and 48 h in the presence of increasing concentrations of sera (0, 10, and 20%) obtained from patients during different stages of NS and healthy control subjects. Activation of osteoblasts was determined by the measurement of alkaline phosphatase (AP) levels in cell lysates using *p*-nitrophenyl phosphate as the substrate. Briefly, cells were cultured in 12-multiwell dishes and incubated with the different sera for 24 and 48 h, washed twice with ice-cold PBS, scraped and lysed in RIPA buffer (1× PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% SDS) without phosphatase inhibitors, and incubated on ice for 30 min. Cell extracts were centrifuged at 14,000 × g for 15 min at 4°C, and the supernatant was recollected, 50 μl of the cell extracts was assayed with 200 μl of assay buffer that contained 10 mM *p*-nitrophenyl phosphate in 0.1 M sodium carbonate buffer at a pH of 10, supplemented with 6 mM $MgCl_2$ and 4 mM mannitol, and followed by incubations and readings at 37°C for 1 and 5 min at 410 nm. Total protein in cell lysates was measured by the Bradford method. The AP activity was calculated as Δabsorption per minute × 2.5 × 10^5 and was expressed in IU/L per mg protein (28).

RANKL Expression Assay. RANKL expression was determined by immunoblotting in cell lysates from cultured stimulated human osteoblasts using a rabbit polyclonal antibody (anti-RANKL; Santa Cruz Biotechnology, Santa Cruz, CA) following the instructions of the manufacturer. Briefly, NHOst were incubated for 24 and 48 h with sera from patients with NS and normal control subjects; washed with ice-cold PBS; lysed in 100 μl of RIPA buffer (1× PBS, 1% Igepal, 0.5% sodium deoxycholate, and 0.1% SDS) with 0.10 mg/ml PMSF, 10 mM Aprotinin, and 1 mM sodium orthovanadate; and incubated on ice for 30 min. Cell lysates were disrupted and homogenized with a 21-G needle, and the cell extracts were centrifuged at 10,000 × g for 20 min at 4°C. The supernatant cell lysate was collected, and its total protein concentration was measured by the Bradford method. Between 40 to 60 μg (approximately 40 μl) of whole-cell lysate was transferred to a nitrocellulose membrane in a slot blot system by vacuum. The filters were blocked for nonspecific binding with Blotto (TBS, 5% skim milk, and 0.05% Tween-20) for 1 h. Then, filters were incubated with anti-rabbit RANKL antibody (SBC) 1 μg/ml in Blotto for 1 h at room temperature by agitation. After the membranes were washed three times for 5 min each with TBS and 0.05% Tween-20, they were incubated for 1 h at room temperature with horseradish peroxidase–conjugated secondary antibody and diluted to 1:5000 in Blotto. The membranes were washed three times for 5 min each with TBS and 0.05% Tween-20 and once for 5 min with TBS. Thereafter, they were incubated in a chemiluminescence reagent (Pierce Chemical Co., Rockport, IL) according to the data sheet and exposed to a K-Omat x-ray film (Kodak, New Haven, CT) for 15 min. The densities of the bands were determined with the Quality One software in a G-800 Densitometer (Bio-Rad, Hercules, CA).

Statistical Analyses

Results are presented as mean ± SD, except where otherwise indicated. Comparisons between groups were done by the t test for unpaired observations and the Mann-Whitney test, as appropriate. For

patients who were evaluated prospectively, paired *t* tests were applied. Correlations between two variables were obtained by the Pearson linear regression analysis or by the Spearman rank correlation coefficient. $P < 0.05$ was considered significant.

Results

The urinary and serum biochemical data of the patients obtained during both stages of activity of the NS are depicted on Table 1. As expected, during relapse compared with remission, the UP/C ratio (11.4 ± 8.3 *versus* 0.05 ± 0.05 mg/mg), serum albumin (2.38 ± 0.6 *versus* 4.0 ± 0.36 g/dl), and serum cholesterol concentrations (310 ± 100 *versus* 192 ± 83 mg/dl) were significantly different (all differences, $P < 0.0005$). Compared with relapse, during remission, the concentrations of the markers of bone formation osteocalcin (54 ± 22 *versus* 85 ± 32 ng/ml; $P < 0.05$) and BSAP (60 ± 17 *versus* 70 ± 30 μg/L) and those of the bone resorption NTx (47 ± 23 *versus* 70 ± 41 nmol bone collagen equivalent/L; $P < 0.05$) all were higher. The concentrations of osteocalcin and BSAP during remission were not significantly different in patients with or without cyclosporine therapy (95 ± 26 *versus* 79 ± 34 ng/ml, and 86 ± 38 *versus* 62 ± 23 μg/L, respectively). Nevertheless, patients who received cyclosporine ($n = 5$) had significantly higher concentrations of NTx than those who were not receiving the drug (104 ± 58 *versus* 55 ± 18 nmol bone collagen equivalent/L; $P < 0.05$). In addition, BSAP levels during remission were significantly higher in patients who were off corticosteroids compared with those who were still on glucocorticoid therapy (69 ± 19 *versus* 48 ± 15 μg/L, respectively; $P < 0.05$). AP activity obtained from NHOst cultures that were incubated with sera of patients with NS was similar at serum concentrations of 10 or 20%. Therefore, all results reported represent incubations with 10% serum. AP activity from osteoblast cell lysates in the presence of sera from patients with NS during both stages of clinical activity is depicted in Figure 1. AP activity was markedly increased during relapse compared with remission (13,978 ± 16,323 *versus* 4033 ± 5183 IU/L per mg protein, respectively; $P < 0.05$) and with normal control subjects (218 ± 132 IU/L per mg protein; $P < 0.05$). Neither in relapse nor in remission did the AP activity exhibit significant differences while patients were on or off corticosteroid therapy.

Table 1. Biochemical parameters of patients with nephrotic syndrome[a]

	Relapse	Remission
GFR (ml/min per 1.73 m^2)	132 ± 30	130 ± 21
Serum CO_2 (mEq/L)	23 ± 1.8	22 ± 2.2
UP/C (normal <0.2 mg/mg)	11.4 ± 8.3	0.05 ± 0.05[b]
Serum albumin (g/dl)	2.4 ± 0.6	4.0 ± 0.36[b]
Serum cholesterol (mg/dl)	310 ± 100	192 ± 83[b]
Serum osteocalcin (ng/ml)	54 ± 22	85 ± 32[c]
Serum bone-specific alkaline phosphatase (μg/L)	60 ± 17	70 ± 30
Serum N-telopeptide (nmol BCE/L)	47 ± 23	70 ± 41[c]

[a]UP/C, urinary protein/creatinine ratio; BCE, bone collagen equivalent.
[b]$P = 0.0001$, [c]$P < 0.05$ *versus* relapse.

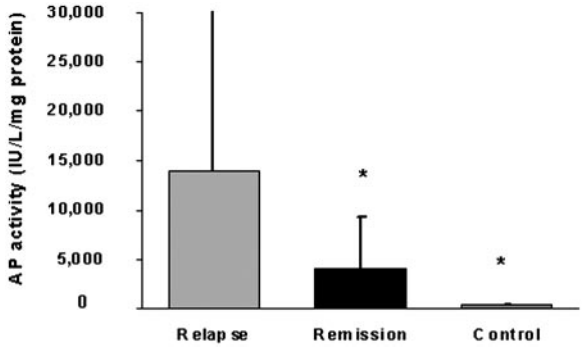

Figure 1. Alkaline phosphatase (AP) activity of normal human osteoblast cultures that were incubated with sera from patients with nephrotic syndrome (NS). AP activity (IU/L per mg protein) of cell lysates is displayed prominently during relapse and declines significantly during remission. Values during remission and from control samples are significantly lower compared with relapse (*$P < 0.05$).

As shown in Figure 2, in which four representative patients are depicted, RANKL expression from NHOst cell lysates during both stages of clinical activity was markedly different. In the presence of sera from patients during clinical relapse, RANKL expression was displayed prominently, whereas during remission, it was absent or barely detectable. The increased expression of RANKL during relapse was similar in samples that were obtained from patients who were on or off corticosteroid therapy. The expression of RANKL during remission was similarly attenuated in samples from patients who did or did no receive cyclosporine therapy.

The specificity of the RANKL binding was demonstrated using 10 μg of RANKL recombinant protein as positive control and an irrelevant antibody as a negative control (anti-rabbit EPO receptor; data not shown).

The degree of proteinuria correlated with the AP activity ($r = 0.5$, $P < 0.05$), but its correlation with the serum concentration of osteocalcin ($r = -0.3$) did not reach statistical significance.

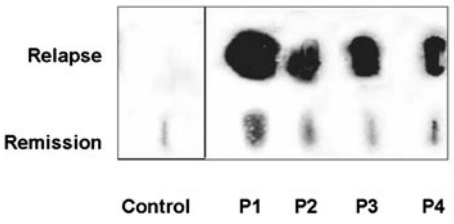

Figure 2. Receptor activator of NF-κB ligand (RANKL) expression from osteoblast cultures that were incubated with sera from patients with NS. Cell cultures after 24 h of incubation with sera of four representative patients (P) during relapse (top) display marked expression of RANKL. During remission (bottom), RANKL expression is attenuated substantially or absent and similar to the control.

The UP/C did not correlate with any other marker of bone remodeling.

Discussion

This study demonstrates that sera from patients with clinically active nonazotemic NS increase the *in vitro* activity of normal human osteoblasts. This augmented osteoblastic activity during clinical relapse is substantiated by increased AP concentration and elevated RANKL expression. In contrast, AP activity was significantly reduced and RANKL expression was markedly diminished during clinical remission. These alterations of bone cellular and molecular activity occurred without a concurrent decline in GFR and in the absence of other overt biochemical derangements of mineral homeostasis.

Disturbances of mineral metabolism that potentially affect bone integrity have been recognized in patients with NS before the development of renal insufficiency and include hypocalcemia, reduced intestinal calcium absorption (5,6), low circulating levels of vitamin D metabolites (2,3,6,7), and occasional elevation of PTH (6,9). Furthermore, altered BMD (3,8) and abnormal bone histology (9,10) have also been documented in patients with NS and normal GFR. However, the abnormalities in BMD and bone histology do not correlate consistently with the prevailing concentrations of PTH or vitamin D metabolites (3,9,10). Similarly, reports on the degree and the type of bone histologic abnormalities still remain contentious (9,10,29,30).

Most studies in which bone histology is available have reported increased bone resorption (9,10) and reduced mineralization (9,29) resembling those of patients with chronic kidney disease stages 1 to 3 (10). However, in nonazotemic NS, contrary to patients with diminished GFR, serum phosphorus concentrations are rarely elevated (9,15,30), metabolic acidosis is not observed, and PTH is mostly normal (15,29,30). Whereas serum levels of 25OH vitamin D3 are frequently reduced during active NS as a result of urinary loses (2), those of calcitriol often remain normal even in patients with osteomalacia (3,10,29,30). Furthermore, despite histologic evidence of secondary hyperparathyroidism in some patients, serum concentrations of PTH are frequently normal (10,29). In contrast, some patients with elevated PTH and/or reduced vitamin D metabolites may display normal or near-normal bone histology (10,29,30). Therefore, the role of other factors that affect bone cellular activity and thereby bone histology need to be considered.

RANKL, secreted mainly by the osteoblasts, is necessary for osteoclast formation from its committed precursor that bears its receptor, RANK (16–18). Thus, osteoblast activity is an essential requisite for osteoclast formation and bone resorption. In this study, osteoblast activity was initially evaluated by measurements of its phenotypic marker AP (16). The concentrations of AP attained the highest concentrations in osteoblast lysates that were incubated with sera of patients with NS during relapse and sustained a significant decline ($71 \pm 68\%$) during clinical remission. The expression of AP is associated with the stabilization of bone matrix and occurs in the maturational phase of osteoblast differentiation (31), and their elevated concentrations in the cultures indicate that the osteoblast cell lines used in these experiments were developed appropriately and capable of responding under the conditions used in our studies.

Coinciding with the elevated AP activity were the measurable serum concentrations of bone formation markers (osteocalcin and BSAP) and bone resorption (NTx), indicating active bone remodeling (32). The significantly lower concentrations of osteocalcin during relapse compared with those during remission and the inverse correlation of osteocalcin levels with the degree of proteinuria suggest a relative diminished bone formation during relapse.

To evaluate further osteoblast activity and osteoblasts' ability to support osteoclast formation, we examined the ability of human osteoblast cells to express RANKL when incubated with sera from the patients during different clinical stages of NS activity. The increased expression of RANKL during relapse, along with the higher AP activity and its substantial attenuation or absence during remission, suggests that the enhanced osteoblast activity during the clinical stage of relapse of the NS is transient and reversible. RANKL levels are stimulated primarily by several hormones, including calcitriol and PTH, and are modulated by a variety of cytokines (33). Of the latter, IL-1, -6, -11, -15, and -17, as well as TNF-α, are mainly pro-osteoclastogenic, whereas TGF-β and IL-4, -10, -12, -13, and -18 and calcitonin are inhibitors of osteoclastogenesis (34). Most of these pro- and anti-osteoclastogenic cytokines act primarily through the osteoblasts by altering the levels of RANKL and osteoprotegerin, the balance of which determines overall osteoclast formation. Some cytokines, particularly IL-6, also have direct osteoclastic actions (35). Elevated serum and urinary activity of various cytokines, including IL-1 and IL-6 (19,36), IL-2 and IL-4 (20), and TNF-α (21), have been identified in patients with NS, particularly during clinical activity of their disease. Although not evaluated in this study, elevated serum levels of cytokines in these patients, particularly during relapse of their NS, could have been responsible for the increased expression of RANKL by the osteoblasts. The stimulatory effects on the osteoblasts by these cytokines, independent of PTH and vitamin D, may result in a PTH-like resorptive effect on bone cells (16,17) and thus provide an explanation for the increased bone resorption observed in bone biopsy samples of patients with NS and normal PTH levels (37). Future studies should include the simultaneous determination of serum cytokines, particularly those that promote osteoclastogenesis. This study was not designed to evaluate mechanisms beyond RANKL activation that occur within the osteoclast. However, increased activity of the intracellular regulating NF-κB signaling pathway was demonstrated in the peripheral mononuclear cells of patients with active NS (38). Whether similar abnormalities could take place during active NS in other cell populations, including osteoclasts, and whether elevated serum concentrations of cytokines may have an impact on bone remodeling through direct systemic mechanisms or by disrupting local regulating signals will require further investigation.

Metabolic acidosis, another factor that is capable of stimulating osteoblast activity by upregulating PTH/PTHrP receptors (39) and increasing the expression of RANKL (40), was not present in our patients as judged by the normal serum CO_2 concentrations.

The possible effects of corticosteroid therapy on osteoblast function also need to be considered. Glucocorticoids reduce the total number of osteoblasts by inhibiting osteoblastogenesis and by increasing apoptosis of the mature osteoblasts (41), as has been demonstrated in bone biopsy samples obtained shortly after renal transplantation in patients on high steroid doses (42) and in long-term transplant recipients (43). The significantly reduced BSAP levels in our patients who had NS during remission but still were on glucocorticoid therapy compared with those who were off steroids may reflect reduced osteoblastogenesis and could result in diminished bone mineral mass (8,44,45). Studies have demonstrated defective bone mineralization and an inverse correlation between the administered dose of corticosteroid therapy and bone formation rates in bone biopsies of adults (29) and more recently in children with NS (37). However, children may display preserved bone mineral mass even shortly after the cessation of intermittent high-dose glucocorticoid therapy for clinically active NS, suggesting the capability of the young skeleton to rapidly regain previous steroid-induced bone losses (46). Glucocorticoids may also increase bone resorption by stimulating osteoclastogenesis through the increased expression of RANKL (47). In our study, the few patients who received steroids during remission displayed attenuated RANKL expression similar to patients who were not on corticosteroids. Thus, in the limited number of patients studied, glucocorticoids did not seem to play a role in the increased expression of RANKL.

Finally, the role of cyclosporine needs to be considered because some patients who were evaluated during remission were receiving this drug. Cyclosporine treatment increases bone turnover and is associated with increased plasma concentrations of bone formation and resorption markers (48,49). Indeed, during remission, the concentrations of osteocalcin and NTx were significantly higher compared with those in relapse. At first, the higher NTx values were unexpected because they coincided with the decline in AP levels and RANKL expression. However, one third of the remission samples were obtained in patients while they were on cyclosporine, an agent that is capable of exerting a bone-resorbing effect independent of RANKL activation, with IL-6 being a possible mediator of this action (35).

In summary, our study demonstrates that serum of patients with NS and normal GFR display the ability to elicit increased osteoblast activation, which is proportional to the degree of proteinuria. The increased osteoblast activity during relapse, as indicated by the high enzymatic concentration of AP and elevated RANKL expression in cultures of normal human osteoblasts, is markedly attenuated or absent during remission. These disturbances may represent important contributing factors to the bone histologic abnormalities present in patients with protracted NS before any decline in GFR. Further studies to identify the possible factors that are responsible for the increased osteoblast activity during NS are needed.

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