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Alteration of noncollagenous bone matrix proteins in distal renal tubular acidosis

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Abstract

Our previous report on bone histomorphometry in patients with distal renal tubular acidosis (dRTA) revealed decreased bone formation rate (BFR) when compared to healthy subjects. The abnormality improved significantly after alkaline therapy. The modest increase in osteoblastic surface, after correction of metabolic acidosis, could not explain the striking improvement in bone formation, suggesting additional influence of metabolic acidosis on osteoblast function and/or bone matrix mineralization. Osteoblasts and, to a lesser extent, osteoclasts synthesize and secrete bone matrix including type I collagen and various noncollagenous proteins (NCPs). Substantial evidence suggested diverse functions of NCPs related to bone formation, resorption, and mineralization. Metabolic acidosis, through its effect on bone cells, may result in an alteration in the production of NCPs. Our study examined bone histomorphometry with detailed analysis on the mineralization parameters and NCPs expression within the bone matrix of patients with dRTA before and after treatment with alkaline. Seven dRTA patients underwent bone biopsy at their initial diagnosis and again 12 months after alkaline therapy. Bone mineral density (BMD) and bone histomorphometry were obtained at baseline and after the treatment. The expression of NCPs was examined by immunohistochemistry, quantitated by digital image analysis, and reported as a percentage of area of positive staining or mineralized trabecular bone area. Alkaline therapy normalized the low serum phosphate and PTH during acidosis. The reduction in BMD at baseline improved significantly by the treatment. Bone histomorphometry demonstrated the increase in osteoid surface and volume without significant alteration after acidosis correction. In comparison to the normal subjects, osteoid thickness was slightly but insignificantly elevated. Osteoblast and osteoclast populations and their activities were suppressed. The reduction in mineral apposition rate and adjusted apposition rate were observed in conjunction with the prolongation of mineralization lag time. Alkaline therapy improved the mineralization parameters considerably. In addition to the increase in BFR relative osteoblast number after acidosis correction, osteocalcin expression in the bone matrix increased significantly from 16.7% to 22.3%. Six of seven patients had decreased osteopontin expression. In conclusion, the abnormal bone remodeling in dRTA is characterized by low turnover bone disease with some degree of defective mineralization. Alteration of NCPs expression suggested the effect of metabolic acidosis on bone cells. Alkaline therapy increased bone mass through the restoration of bone mineral balance and, perhaps, improved osteoblast function.

Keywords: Acidosis; Bone matrix; Osteoblasts; Osteoclasts

Introduction

Distal renal tubular acidosis (dRTA) is a clinical syndrome characterized by impaired ammonium and titrable acid excretion by the kidney resulting in persistent metabolic acidosis while excreting relatively alkaline urine. The presence of chronic metabolic acidosis results in various metabolic consequences, including hypokalemia, hypophosphatemia, and abnormal bone metabolism [1–3]. Recent studies from our laboratory revealed the findings of osteopenia and suppressed bone formation in patients with dRTA when compared to healthy controls. Correction of metabolic acidosis with alkaline improved the abnormality.

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The modest decrease in osteoblastic surface at baseline, which only improved slightly after alkaline treatment, could not explain the striking improvement in bone formation, suggesting the possibility of additional influence of metabolic acidosis on osteoblast function and/or bone matrix mineralization [4,5]. Previously, Bushinsky et al. [6,7] investigated the effect of metabolic acidosis on bone and discovered that metabolic acidosis resulted in skeletal demineralization through physicochemical dissolution of the bone as excess protons were buffered by bone carbonate. Later on, the effect of metabolic acidosis on bone cells was examined. Using cell culture model of neonatal mouse calvariae, the same group discovered that metabolic acidosis inhibited osteoblastic collagen synthesis while enhancing osteoclastic β-glucoronidase activity [8,9]. The opposite findings were observed when the incubation was performed in alkaline medium [10]. This data provided evidence regarding the effect of metabolic acidosis on both cellular and noncellular components of the bone.

Osteoblasts and, to a lesser extent, osteoclasts synthesize and secrete bone matrix, which include type I collagen and various noncollagenous proteins (NCPs). Of these NCPs, osteocalcin and osteonectin secreted mostly by osteoblasts have been known to involve in the process of bone formation and mineralization [11–13], while osteopontin and bone sialoprotein secreted by both osteoblasts and osteoclasts play roles in bone resorption [14–17]. Metabolic acidosis, through its effect on osteoblasts and osteoclasts, may alter the production of NCPs, resulting in the abnormal bone remodeling. Alkaline therapy may restore cellular functions and NCPs composition.

Osteomalacia has been reported in 20–30% of patients with renal tubular acidosis [3,18]. The diagnosis was mostly based on clinical and roentgenographic findings. Bone pain was relieved and osteomalacia improved after alkaline therapy [3,19]. Previously, we reported significant increase in osteoid volume and surface in bone of patients with dRTA, while there was no major increase in osteoid thickness. A detailed analysis on the parameters associated with mineralization was not performed. In a present study, we examined bone histomorphometry with detailed analysis on the mineralization parameters and osteoblastic activity in patients with dRTA before and after alkaline treatment. Composition of NCPs in the bone matrix was examined by immunohistochemistry.

Materials and methods

Patients

Subgroup of dRTA patients who completed 1 year of alkaline therapy with adequate bone specimens available for further examination by immunohistochemistry from our previous studies were included [4,5]. These were idiopathic dRTA patients who were residents of Khon Kaen province, Thailand, where a very high incidence of dRTA has been reported [18]. Seven patients, three males, and four females, who were diagnosed with dRTA on the presence of (1) persistent hyperchloremic metabolic acidosis with serum bicarbonate less than 18 mmol/l found in at least two occasions, 1 month apart, (2) failure to acidify urine (with urine pH > 5.5) or urinary excretion of ammonia less than 50 mEq/day in the presence of systemic acidosis, (3) absence of bicarboturia exceeding 15% of that filtered at normal plasma bicarbonate concentration, serum creatinine of less than 190 μmol/l, and absence of proteinuria, Fanconi syndrome, chronic diarrhea, current usage of diuretics, carbonic anhydrase inhibitors, and all kinds of alkaline therapy were included in this study. The calcium intake of dRTA patients was 9.45 ± 3.35 mmol/day. All patients were then treated for 1 year with 60 mEq/day of potassium citrate in two divided doses to keep the serum bicarbonate above 20 mmol/l throughout the study. For patients who initially failed to achieve the target serum bicarbonate level, the dosages of potassium citrate were increased in a stepwise fashion until reaching the desired serum bicarbonate level. No medication that might affect calcium and bone metabolism, for example, diuretics, vitamin D, estrogen, bisphosphonate, and calcium supplements, was allowed throughout the study.

Biochemical analysis

Serum electrolytes, calcium, phosphate, intact PTH (iPTH), and 24-h urine collections for sodium, potassium, calcium, phosphate, and creatinine were obtained at the time of bone biopsy. Hypercalciuria was defined as urinary calcium excretion > 4.75 mmol/day in either sex [1,20]. Serum iPTH was determined by an immunoradiometric assay (ELSA-PTH; CIS BioInternational, GIF-sur-Yvette Cedex, France). The normal serum iPTH was 10–60 pg/ml.

Bone mineral density

Bone mineral density (BMD; g/cm^2) was determined at vertebral (L2–L4), femoral neck, trochanter, and Wards' triangle by dual energy X-ray absorptiometry (Lunar Expert XL, Lunar Corp., USA). Precision of the BMD measurement in our laboratory at L2–L4, and neck of femur was 1.2 and 0.6%, respectively. The control values of BMD were obtained from 28 normal farmers who were permanent residents of Khon Kaen province, age, 32.9 ± 11.2 years, weight, 54.3 ± 8.3 kg, height, 1.55 ± 0.05 m, and male–female ratio, 22:6.

Bone biopsy and histomorphometry

At the beginning of the study, transiliac crest bone biopsy was taken from the anterior superior iliac spine after tetracycline double labeling and again on the opposite side after 1 year of alkaline therapy using protocol reported

previously [4]. In brief, bone specimens of 5 mm in diameter and 20–30 mm in length were fixed for 24 h in 70% ethanol, dehydrated in graded ethanol, and impregnated and embedded in the mixture of methylmethacrylate, dibutylphthalate, and benzoyl peroxide at room temperature for 5 days and subsequently in 42°C oven for 3 days. After polymerization, bone sections of 6-μm thickness were cut using Reichert–Jung Polycut S (Cambridge Instruments, NuBloch, Germany) equipped with tungsten carbide-edge knife (Leica, Germany), mounted on coated slides and incubated at 42°C for 2 weeks. Undecalcified sections were stained with modified Masson–Goldner trichrome, aurin tricarboxylic acid (Aluminon), Von–Kossa, and hematoxylin–eosin stain. If the specimen had a positive stain for aluminum, a further stain with Perls stain to exclude the cross-reaction with iron deposit was done. Unstained sections of 15-μm thickness were prepared for examinations by a fluorescent light microscopy. All sections, both pre- and post-potassium citrate therapy, were studied qualitatively and quantitatively for static and dynamic parameters of bone formation and bone resorption by the same pathologist and technician who had no knowledge of the patients' clinical presentations and treatments. Histomorphometric measurements were carried out with a semiautomatic image analyzer (Osteomeasure; Osteometric Inc, Atlanta, USA). At least 30 different fields of the same bone biopsy specimen were analyzed. Histomorphometric parameters were expressed according to Parfitt et al.'s [21] standardized nomenclature. The reference values for normal histomorphometric parameters were obtained from 17 normal Thai adults without bone disease, eight men and nine women, age, 35.1 ± 2.8 years (range, 19–58), height, 1.61 ± 0.06 m, and weight, 59.2 ± 7.8 kg.

Protocol of the study has been approved by ethical committee on research involving human subjects of Ramathibodi hospital, Mahidol University. Written informed consents were obtained from all subjects.

Antibodies

Rabbit polyclonal antibodies LF-32 (osteocalcin), LF-120 (bone sialoprotein), BON-I (osteonectin), and mouse monoclonal antibody LFMb-14 (osteopontin) were generous gifts from Dr. Larry W. Fisher, National Institutes of Health (NIH), Bethesda, MD [22].

Immunohistology

Immunohistochemistry was performed on the bone sections as described previously by Derkx et al. [23]. Briefly, plasticized bone sections were deacrylated in three changes of 2-Ethoxyethylacetate (BDH, Poole, England) overnight, rinsed in xylene, rehydrated, decalcified with 1% acetic acid for 2 days, and rinsed with distilled water for 30 min. Sections were stained using Universal LSAB2 Kits (Dako, CA, USA) according to manufacturer's recommendations with modifications. All steps were carried out at room temperature. Endogenous peroxidase activity was inhibited by 3% H_2O_2 in PBS for 30 min followed by 5-min wash in PBS. Subsequently, slides were blocked with 10% normal goat serum (Dako, CA, USA) in PBS for 1 h. Excessive serum was removed. Sections were then incubated with primary antibodies including osteocalcin 1:1600, bone sialoprotein 1:800, osteonectin 1:800, and osteopontin 1:3200 diluted in goat serum for 2 h and 30 min. The washings were carried out in PBS containing 0.05% tween (tween-PBS) for 10 min and PBS for additional 5 min. Primary antibodies were detected by incubation with ready-to-use biotinylated goat anti-immunoglobulin second antibody (detecting both mouse and rabbit antibodies) for 10 min and washed for 5 min each in tween-PBS and PBS. Next, peroxidase-conjugated biotin–streptavidin complex was allowed to react with second antibody for 10 min and sections were washed for 5 min in tween-PBS and 30 min in PBS. Antibody complexes were visualized by incubation with diaminobenzidine obtained from Dako liquid DAB substrate-chromogen system. Sections were rinsed in distilled water for 5 min, counterstained with Mayer's hematoxylin, rinsed in tap water, dehydrated with ascending alcohols, cleared with xylene, and mounted on glass slides with cover slips using Permount (Fisher Scientific, New Jersey, USA) mounting medium. Bone biopsy sections from pre- and post-alkaline treatment of the same patients were stained at the same time. Negative control

Table 1
Patient characteristics at presentation

Patient no.	Age years	Height (cm)	Weight (kg)	Sex	Blood/urine pH	Urine ammonium (mEq/day)	Duration[a] (months)
1	16	157	44	F	7.31/7.0	36	8
2	35	145	46.7	F	7.30/6.0	29	18
3	30	170	65	M	7.29/6.2	30	36
4	50	146	50	F	7.31/6.8	30	60
5	42	158	53	M	7.28/6.4	32	120
6	20	136	32.3	M	7.30/6.9	24	144
7	25	148	39.5	F	7.32/6.5	31	24
Mean	31.1	151.4	47.2		7.3/6.5	30.3	58.6
SD	12.1	11.1	10.4		0.01/0.4	3.6	53.2

[a] Duration of symptoms, for example, muscle weakness, renal stone, or fracture.

Table 2
Blood (per liter) and urine (per day) chemistries of dRTA patients

	Baseline		After treatment		Normal[a]	
	Serum	Urine	Serum	Urine	Serum	Urine
Creatinine (μmol)	99 ± 18	974 ± 160	93.7±11.2	700 ± 100	91.5 ± 38	1061 ± 71
Sodium (mmol)	139.9 ± 2.4	97.9 ± 44.9	141.6 ± 2.6	113.2 ± 26	138 ± 5.1	110.6 ± 90
Bicarbonate (mmol)	16.5 ± 3.3	–	22.6 ± 2.4[b]	–	25 ± 1.5	–
Calcium (mmol)	2.1 ± 0.1	2.6 ± 1.6	2.3 ± 0.2	2.9 ± 1.7	2.3 ± 0.5	2.75 ± 1.7
Phosphate (mmol)	0.8 ± 0.2	11.3 ± 4.2	1.1 ± 0.2[b]	10.2 ± 2.9	1.2 ± 0.1	10.6 ± 5.5
iPTH (pg/ml)	12.9 ± 5.6	–	24.1 ± 10[c]	–	–	–

[a] Data obtained from the 28 normal farmers who were permanent residents of Khon Kaen province.
[b] Significant difference when compared to the corresponding baseline value ($P < 0.05$).
[c] Significant difference when compared to the corresponding baseline value ($P < 0.01$).

sections were stained in the same fashion with omission of primary antibody.

Quantitative analysis of the NCPs

We performed quantitative analysis of the NCPs using similar protocol described previously by Derkx et al. [23]. Briefly, a CCD color video camera (Sony, Japan) mounted on a microscope (Zeiss, Germany) with a 10× objective was used to transfer images of the immunostained samples to the computer. The KS-300 (version 2.00) digital image analysis system (Kontron, Munchen, Germany) was used to analyze at least 40 microscopic fields of trabecular bone area in two 6-μm sections cut at steps of 50–100 μm in the same bone biopsy. This has been shown previously to be sufficient to obtain representative data with a small confidence interval [23]. The threshold of positive staining (brown) was determined interactively and the determined threshold was used to automatically analyze the images of the section. The mineralized bone matrix area (purple) was determined by first, manually tracing the perimeter of the mineralized trabecular bone on the computer screen (to exclude the cells in the bone marrow, which also stain purple) and then allowing the analysis system to calculate the area. The ratio of the immunostained and the mineralized bone matrix area was calculated. Bone sections from the same patient obtained pre- and post-alkaline treatment were analyzed at the same time and two separate measurements were performed in all sections.

Statistical analysis

Results were presented as mean ± standard deviation. Comparison between the group means was performed using paired and unpaired Student's t test. Differences between groups were considered significant when $P < 0.05$.

Results

Patient characteristics are shown in Table 1. All the patients were acidemic with impaired urine ammonium excretion (urine ammonium < 50 mEq/day) and relatively high urine pH (>5.5). Blood and urine chemistries for dRTA patients are presented in Table 2. Full details on blood and 24-h urine chemistries before and after potassium citrate therapy have been reported previously [5]. Hypokalemia (3.4 ± 0.8 mmol/l), hypophosphatemia, and low serum iPTH levels were observed at baseline. None of the patients had hypercalciuria. During alkaline therapy with potassium citrate, all dRTA patients could maintain their serum bicarbonate above 20 mmol/l. After treatment, serum potassium (4.1 ± 0.4 mmol/l), bicarbonate, phosphate, and iPTH levels rose significantly above the corresponding baseline values. There were no significant alterations in serum calcium, urine calcium, and urine phosphate after the treatment.

Bone mineral densities at baseline and the end of the study period are shown in Table 3. The basal BMD values of dRTA patients were lower than those of normal controls in all studied areas. After 1 year of alkaline therapy, there were significant elevations in the BMDs of total femur and trochanter of femur ($P < 0.05$). Bone histomorphometric data before and after the treatment are presented in Table 4. At baseline, there were significant elevations in the osteoid volume and surface ($P < 0.05$) compared to the corresponding parameters in normal controls. Osteoid thickness was slightly but insignificantly elevated. Osteoblastic and osteoclastic surfaces were decreased but the differences were not significant. Eroded surface was not different from controls. The reductions in the mineral apposition rate, mineralizing surface or osteoid surface, and adjusted apposition rate were accompanied by the prolongation of mineralization lag time ($P < 0.05$). Bone formation rate (BFR) per bone surface was suppressed at baseline ($P < 0.05$). While

Table 3
Bone mineral density (g/cm^2) of dRTA patients

Area	dRTA patients		Normal
	Baseline	After treatment	
L2–L4	1.05 ± 0.23	1.08 ± 0.17	1.15 ± 0.25
Total femur	0.89 ± 0.16	0.98 ± 0.17[a]	1.05 ± 0.29
Neck	0.85 ± 0.15	0.88 ± 0.18	1.00 ± 0.25
Wards	0.68 ± 0.20	0.72 ± 0.17	0.89 ± 0.30
Trochanter	0.67 ± 0.14	0.75 ± 0.13[a]	0.81 ± 0.27

[a] $P < 0.05$ compared to baseline BMD in dRTA patients.

Table 4
Bone histomorphometry of dRTA patients at baseline and after alkaline treatment

Histomorphometric parameters	dRTA		Reference value (range)
	Baseline	After treatment	
Bone volume (BV/TV), %	20.32 ± 3.54 (15.63–25.56)	24.91 ± 2.80 (20.64–28.00)	26.44 ± 7.21 (12.72–36.88)
Osteoid volume (OV/TV), %	2.50 ± 1.65[a] (0.18–4.53)	1.83 ± 1.30 (0.42–4.2)	0.92 ± 1.05 (0.2–3.06)
Osteoid surface (OS/BS), %	25.10 ± 21.4[a] (3.27–39.8)	24.02 ± 20.5[a] (7.12–32.16)	5.79 ± 4.39 (0.30–15.86)
Osteoid thickness (O.th), μm	10.11 ± 3.2 (5.29–14.34)	10.16 ± 4.09 (6.12–17.5)	8.69 ± 2.14 (5.53–15.87)
Osteoblastic surface (Ob.S/BS), %	1.05 ± 0.93 (0–2.43)	2.03 ± 1.92 (0.15–5.45)	2.6 ± 1.1 (0.51–4.80)
Osteoclastic surface (Oc.S/BS), %	0.04 ± 0.33 (0–0.08)	0.06 ± 0.06 (0–0.16)	0.13 ± 0.23 (0.01–0.59)
Osteoclast number (N.Oc/T.Ar), mm^2	0.14 ± 0.11 (0–0.32)	0.15 ± 0.14 (0–0.32)	0.24 ± 0.31 (0.01–0.83)
Eroded surface (ES/BS), %	5.79 ± 3.02 (1.78–9.71)	4.32 ± 3.07 (1.69–7.94)	5.68 ± 2.32 (2.08–10.06)
Mineral apposition rate (MAR), μm/day	0.74 ± 0.39[a] (0.30–1.27)	1.20 ± 0.42[b] (0.43–1.63)	1.32 ± 0.69 (0.48–2.94)
Mineralizing surface/osteoid surface (MS/OS), %	20.59 ± 26.7[a] (3.51–71.82)	39.83 ± 31.33[a,b] (8.41–89.79)	81.15 ± 23.66 (36.84–97.79)
Adjusted apposition rate[c] (Aj.AR), μm/day	0.13 ± 0.17[a] (0.03–0.49)	0.52 ± 0.46[b] (0.04–1.24)	0.78 ± 0.59 (0.35–1.93)
Mineralization lag time[d] (Mlt), days	240.93 ± 167.55[a] (13.89–424.3)	90.68 ± 172.67[b] (5.63–479.94)	15.42 ± 11.64 (1.77–30.94)
Bone formation rate per bone surface[e] (BFR/BS), $\mu m^3/\mu m^2$/year	5.97 ± 5.51[a] (2.15–17.92)	28.05 ± 15.96[b] (4.28–44.9)	29.83 ± 20.42 (8.35–44.47)
Bone formation rate per osteoblast number[f] (BFR/N.Ob), μm^2/cell/day	55.57 ± 41.82 (10.89–120.76)	91.46 ± 30.98[b] (39.22–127.48)	88.64 ± 93.29 (21.12–272.35)

[a] Significant difference when compared to the corresponding normal value ($P < 0.05$).
[b] Significant difference when compared to the corresponding baseline value ($P < 0.05$).
[c] Aj.AR was calculated from Aj.AR = [(MS/OS) × MAR]/100.
[d] Mlt was calculated from Mlt = O.th/Aj.AR.
[e] BFR/BS was calculated from BFR/BS = [(MS/BS) × MAR]/100, where MS/BS (mineralizing surface, %) was the extent of tetracycline labeled surface (double plus half single labeled surface) as a percentage of total trabecular bone surface.
[f] BFR/N.Ob was calculated from BFR/N.Ob = MAR × [osteoid perimeter (μm)/number of osteoblasts].

bone formation rate per osteoblast number was lower than that of normal controls, the difference did not reach statistical significance. After potassium citrate therapy, bone volume and osteoblastic and osteoclastic surfaces were modestly increased, but the differences were not significant. A slight decrease in osteoid volume was observed. Osteoid surface and thickness were not significantly altered. Dynamic parameters showed significant improvement in the mineral apposition rate, mineralizing surface, and adjusted apposition rate compared to the baseline values ($P < 0.05$). Mineralization lag time also declined significantly ($P < 0.05$). Bone formation rate per bone surface and bone formation rate per osteoblast number increased significantly after the treatment ($P < 0.05$). On regression analysis, a tendency toward negative correlation between the osteoid thickness and adjusted apposition rate was observed ($r = -0.717$, $P = 0.07$; Fig. 1). No positive staining for the aluminum was found in any of the bone specimens.

All the NCPs strongly stained the mineralized bone matrix compartment but variably stained osteoid and cellular components, including osteoblasts, osteoclasts, osteocytes, and lining cells (Fig. 2). Negative controls were devoid of staining. Each protein had a specific pattern of distribution, but an overlap in localization

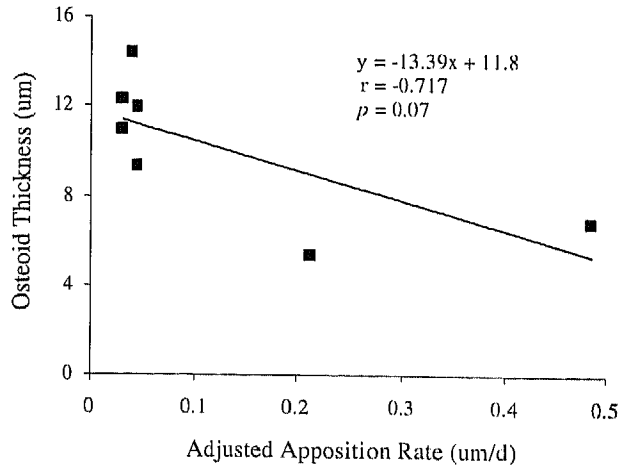

Fig. 1. Relationship between osteoid thickness (μm) and adjusted apposition rate (μm/day) of dRTA patients before treatment with alkaline.

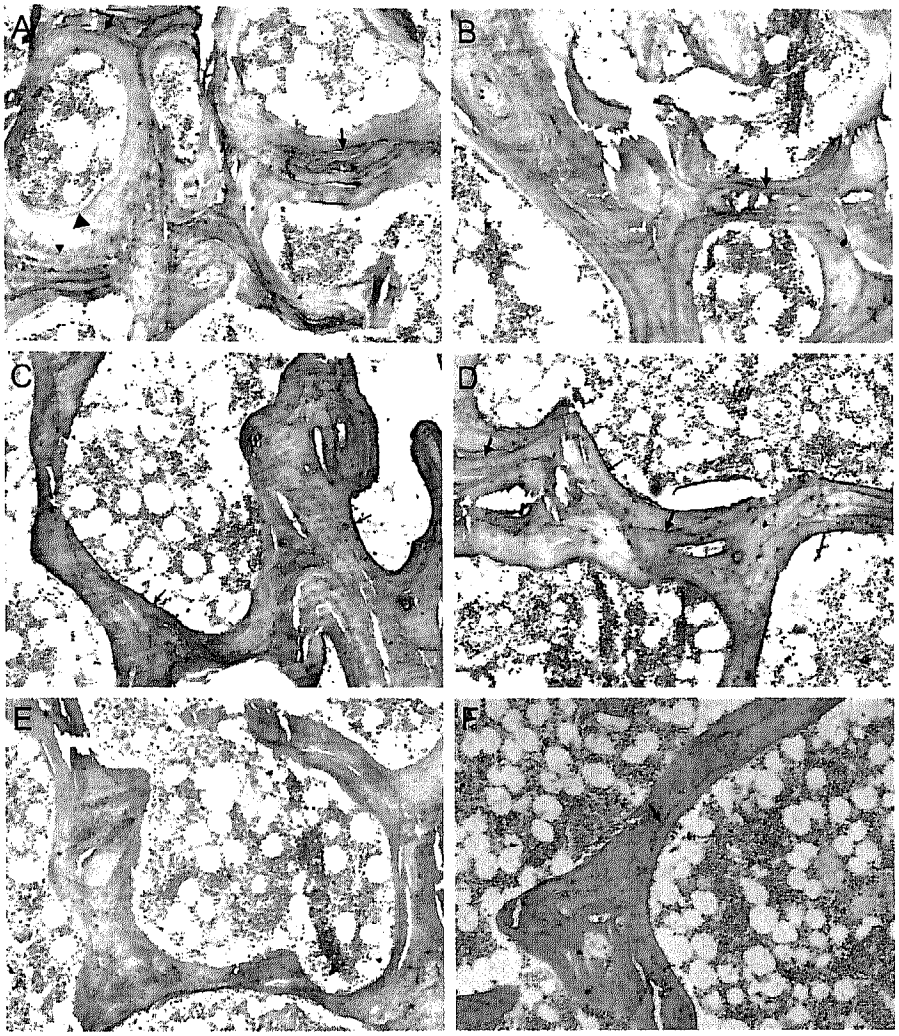

Fig. 2. Immunohistochemistry of NCPs in bone sections of patients with dRTA pre- and post-alkaline treatment. Each protein has specific pattern of distribution but an overlap in localization between the proteins was observed. (A) Osteocalcin pre-alkaline; (B) osteocalcin 1 year post-alkaline treatment. Osteocalcin was detected in the cement lines and more in the outer than the inner lamellae osteon. Note the increase in osteocalcin expression in the bone section after alkaline treatment. (C) Osteopontin pre-alkaline; (D) osteopontin post-alkaline. Osteopontin expressed more diffusely and most prominently in the area of the bone surface adjacent to the bone marrow (lamina limitans). Note the decrease in osteopontin expression in the bone section after alkaline treatment. (E) Osteonectin. Osteonectin diffusely expressed in the bone matrix but minimally in the cement lines. (F) Bone sialoprotein. Bone sialoprotein also diffusely stained the bone matrix and presented in the cement lines. Large arrowhead = inner lamellae osteon; small arrowhead = outer lamellae osteon; large arrow = cement lines; small arrow = lamina limitans. Original magnification, ×100.

between the proteins was observed. As shown in Fig. 2, osteocalcin (Figs. 2A and B) stained more intensely in the outer than inner lamellae osteon, while osteopontin stained diffusely and distinctively in the area of bone surface adjacent to the bone marrow (lamina limitans) (Figs. 2C and D). A significant increase in the area of osteocalcin staining from 16.65 ± 9.25% in bone biopsy at initial diagnosis to 22.26 ± 8.16% after alkaline treatment ($P < 0.04$) was observed (Fig. 3A). Six of seven patients showed decreased expression of osteopontin with an average of 28.91 ± 10.4% at initial biopsy to 22.3 ± 3.76% post-alkaline ($P = 0.16$) (Fig. 3B). Osteonectin and bone sialoprotein diffusely stained the bone matrix and cement lines (Figs. 2E and F). The area of staining of osteonectin (19.46 ± 10.92% and 21.62 ± 9.52%, $P = 0.5$) and bone sialoprotein (11.94 ± 4.37% and 12 ± 3.76%, $P = 0.98$) was not significantly different post-alkaline therapy (Figs. 3C and D). After a careful examination of the sections obtained from initial biopsy and 1 year after alkaline treatment, there was no alteration in the distribution of any of the proteins.

Discussion

These seven patients have compatible characteristics of dRTA as described previously. Successful correction of metabolic acidosis with potassium citrate resulted in the increase in serum potassium, phosphate, and iPTH [4,5]. Earlier studies demonstrated that chronic metabolic acidosis

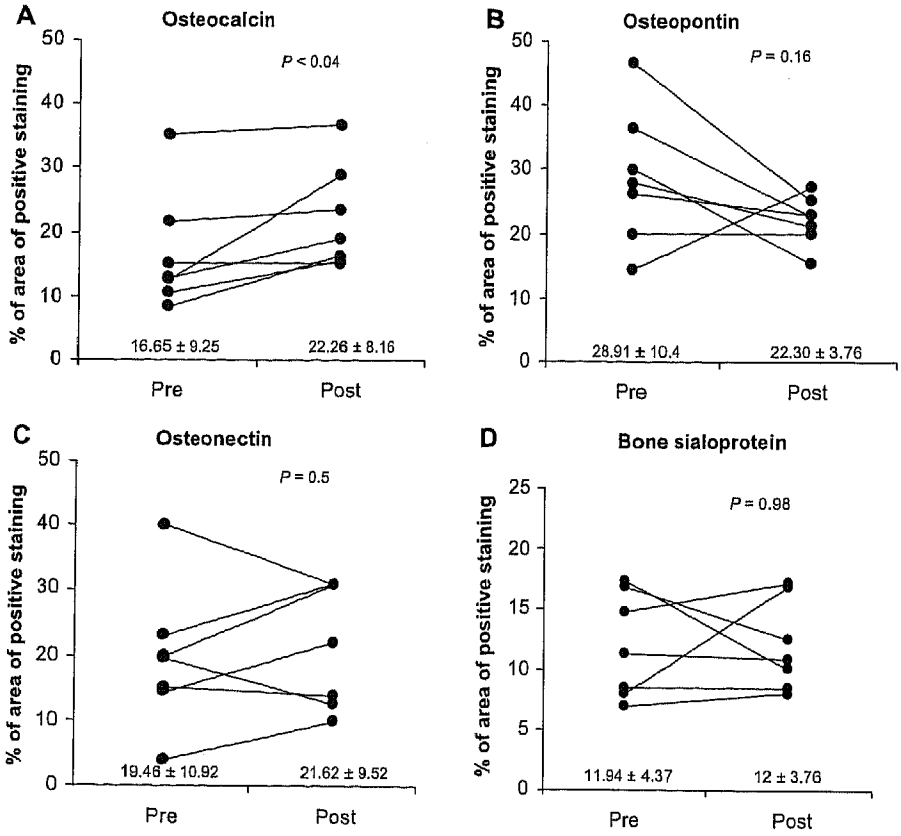

Fig. 3. Quantitation of NCPs expression using digital image analysis pre- and post-alkaline treatment. Data were expressed as percentage of area of positive staining or mineralized bone matrix area. (A) Osteocalcin, (B) osteopontin; (C) osteonectin; (D) bone sialoprotein. Osteocalcin expression significantly increased after alkaline treatment. Six of seven patients had decreased osteopontin expression.

induced by exogenous or endogenous acid loads resulted in physicochemical dissolution of the bone and enhanced osteoclastic bone resorption accompanied by marked elevation in the urinary calcium excretion [24–26]. In our patients, however, hypercalciuria was not observed. The difference in the urinary calcium excretion might be a result of the difference in the chronicity of the disease and the relatively low calcium intake in this group of Thai population. In a study by Lemann et al., all subjects had a rather short period of acidosis of less than a month when compared with our patients, who had suffered from metabolic acidosis for many years. Nevertheless, our findings were consistent with that of Coe and Firpo [27]. In Coe's study, chronic metabolic acidosis produced no hypercalciuria when dietary sodium intake was restricted. When sodium intake was increased, while maintaining the same acid load, hypercalciuria appeared [27]. The urinary sodium excretion in our patients was rather low and approximately half of the amount of urinary sodium excretion associated with hypercalciuria found in Coe's study. Therefore, the low urinary sodium excretion might contribute partly to the relatively low urinary calcium excretion. Another possible explanation is the histomorphometric finding of suppressed osteoclast population and activity, which suggests against a presence of significant process of bone resorption in our patients.

Metabolic acidosis has been known to result in renal phosphate wasting through the reduction in proximal tubular phosphate reabsorption [28,29]. In our patients, serum phosphate was low at baseline; however, the urinary phosphate excretion was unaltered compared to the healthy subjects. The prolonged negative phosphate balance and the chronically suppressed PTH levels might be responsible for the absence of phosphaturia. Correction of metabolic acidosis normalized the phosphate balance and serum phosphate.

The combination of negative calcium balance, phosphate depletion, and suppressed PTH during metabolic acidosis explains the findings of low bone density and the feature of low turnover bone disease, characterized by low bone volume, low osteoblast and osteoclast numbers, decreased mineralizing surface, and normal bone eroded surface, demonstrated by histomorphometry. In addition, the alteration in vitamin D metabolism might play a contributory role. Several studies reported inconsistent results on the levels of 1,25-$(OH)_2$ vitamin D during metabolic acidosis ranged from increased to unchanged to decreased [29–31]. In human, Krapf et al. [29] demonstrated an increase in 1,25-$(OH)_2$D level secondary to phosphate depletion in an experimental NH_4Cl-induced acidosis. The production rate of 1,25-$(OH)_2$ vitamin D was stimulated and PTH decreased

secondarily. We did not measure the 1,25-$(OH)_2$D levels in our patients; nevertheless, the finding of low PTH in our study is consistent with that of Krapf's. Correction of metabolic acidosis normalized the calcium balance, serum phosphorus, and PTH resulting in an increase in bone mass. The elevated osteoid surface and volume, the reduction in mineralizing surface, the falls in mineral apposition rate and adjusted apposition rate, and the prolonged mineralization lag time suggest toward a presence of mineralization defect in dRTA. Nevertheless, the degree of increased osteoid thickness, together with its negative correlation with the adjusted apposition rate, a cardinal feature of osteomalacia, was only borderline significant [32]. The explanation may be the high variability of the osteoid thickness among our patients, which in turn suggests the presence of heterogeneity of bone disease in dRTA. Previously, both osteomalacia and osteoporosis have been reported in association with metabolic acidosis [18,33]. Our findings also indicate that some patients have a histologic feature of osteomalacia associated with an increased osteoid thickness while others fall in a group of low bone turnover osteopenia accompanied by a normal to decreased osteoid thickness. The existence of diversity of bone disease in dRTA is further supported by the wide range of values reported on the mineralizing surface and mineralization lag time. The most likely causative factors for the defective mineralization in our patients were phosphate depletion and, perhaps, abnormal vitamin D metabolism. After successful correction of metabolic acidosis, the parameters associated with mineralization and bone formation improved considerably. In addition, alkaline therapy might also improve osteoblast function suggested by the increase in bone formation rate per osteoblast. While the negative calcium and phosphate balance and, perhaps, impaired osteoblast function contributed to the low bone mass, cell-mediated bone resorption did not seem to play a major role. Previous in vitro studies demonstrated enhanced osteoclastic bone resorption during metabolic acidosis [9,34]. Later on, Frick and Bushinsky [35] discovered that RANKL RNA expression was upregulated in mouse calvariae incubated in acidic media, suggesting that metabolic acidosis stimulates osteoclast differentiation. In addition to the proliferative effect on bone cells, PTH is also a potent stimulation for RANKL expression and osteoclast differentiation [36,37]. Thus, the absence of enhanced bone resorption in our patients could be explained by the presence of low PTH resulting in the reduction in osteoblast and osteoclast populations and the suppression of osteoclast differentiation. Substantial evidence suggested that growth hormone or insulin-like growth factor axis was suppressed during metabolic acidosis [38,39]. Therefore, in addition to the low PTH, the impaired growth hormone or insulin-like growth factor system, whose effect directly promotes cellular proliferation and differentiation, might also be responsible for the overall reduction in bone cell populations. After alkaline therapy, the parameters associated with bone resorption were unchanged. One would have expected the reduction in osteoclast number and/or activity after correction of acidosis; however, such findings might have been prevented by the rise of the PTH.

Additional analysis on the bone matrix proteins demonstrated a significant increase in osteocalcin expression within the bone matrix after correction of metabolic acidosis. Osteocalcin, produced and secreted almost exclusively by cells of osteoblast origin, is well-known as a marker of osteoblast function and its serum level correlates with bone formation [11,40]. The increased osteocalcin expression, which might have occurred secondarily to the enhanced osteoblast function after correction of metabolic acidosis, corresponded to the improvement in bone formation and mineralization. In vitro studies using osteoblast culture model demonstrated the inhibition of osteoblast function by metabolic acidosis; for example, metabolic acidosis impaired osteoblastic collagen synthesis [8] and reversibly inhibited the expression of osteoblastic genes including type I collagen, matrix Gla protein, and osteopontin [41]. Our finding provides in vivo evidence on the inhibitory effect of metabolic acidosis on osteoblastic gene expression and its improvement after alkaline therapy. In support of our result, others have found an increase in serum osteocalcin after correction of metabolic acidosis corresponding to the improvement in bone mineral balance [42,43].

Osteonectin is another NCP that involves in the process of bone formation. Osteonectin-deficient mice have decreased bone formation and profound osteopenia [13]. Culture of bone marrow stromal cells and osteoblasts obtained from these animals revealed compromised osteoblast formation, maturation, and survival [44]. Previous in vitro study using osteoblast culture model in acidic medium found no alteration in the expression of osteonectin mRNA compared to control culture at neutral pH [41]. We also found no significant difference in the expression of osteonectin within the bone matrix before and after alkaline therapy, suggesting that the effect of metabolic acidosis on bone matrix protein expression might be selective.

Osteopontin and bone sialoprotein belong to the same family protein, which contains an RGD (Arg-Gly-Asp) cell attachment sequence, therefore play roles in the regulation of adhesion and the attachment and spreading of osteoclasts to the bone surface [45]. Osteopontin mRNA was found in both osteoblasts and osteoclasts [14] and especially highly expressed in the resorption lacunae and in the osteoclasts at immediate resorption surfaces [15]. In a study by Ihara et al. [36], PTH-induced increase in TRAP-positive cells was absent in osteopontin-deficient bones, emphasizing the role of osteopontin in osteoclast differentiation and bone resorption. In our study, six of seven patients had decreased osteopontin expression within the bone matrix after alkaline therapy. The expression of bone sialoprotein was not significantly altered. The discrepancy between our result and that of Frick and Bushinsky [41], who found decreased osteopontin mRNA in osteoblasts cultured in acidic media, could be because osteopontin production is almost undetectable in osteoblasts actively expressing osteocalcin [46];

therefore, the reduction in its expression might be secondary to the decreased production by osteoclasts rather than osteoblasts. Since RANKL is upregulated during metabolic acidosis; thus, the reduction in RANKL-stimulated osteoclast differentiation might be the reason for decreased osteopontin expression after alkaline therapy. PTH has been known to stimulate RANKL and osteopontin expression [37,47]; therefore, the increase in PTH levels after acidosis correction could overshadow the alterations in osteopontin expression and histomorphometric parameters related to bone resorption.

We found no significant alteration in the distribution of NCPs before and after alkaline therapy, suggesting minor roles of the distribution of the proteins in abnormal bone remodeling in patients with dRTA. The variability in the expression of NCPs among different patients might be due to sex and age differences in the composition of bone matrix [48]. This observation, however, will require further study in a larger number of patients.

Our study confirmed previous reports of preserved antigenicity within the mineralized compartment of the bone embedded in plastic [23,49]. The staining within the cellular components could be improved with embedding and polymerization performed at lower temperature (−15 to −20°C) [50]. The data on NCPs was limited by the semiquantitative nature of immunohistochemistry; however, the method we applied required measurements of at least 40 trabecular bone areas in multiple bone sections cut at different levels, which has been shown previously to be sufficient to obtain representative data with a small confidence intervals [23].

In summary, our data demonstrated abnormal bone remodeling in patients with dRTA characterized by low turnover bone disease with defective mineralization. Alteration of NCPs expression suggested the effect of metabolic acidosis on bone cells in vivo. Alkaline therapy improved bone formation through the restoration of bone mineral balance and, perhaps, enhanced osteoblast function. Further studies are required to elucidate the effect of chronic metabolic acidosis on bone resorption in vivo.

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