



รายงานวิจัยฉบับสมบูรณ์

โครงการ ผลกระทบของภาวะความเป็นกรดกับ
การเจริญเติบโตของเซลล์กระดูก

โดย พ.ญ. สีนี ดิษฐบรรจง

สิงหาคม 2548

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คณะแพทยศาสตร์รพ.รามธิบดี

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณ ศ.นพ.รัชตะ รัชตะนาวิน คณบดีคณะแพทยศาสตร์รพ.รามาธิบดี ที่ช่วยชี้แนะ ให้การสนับสนุน และให้โอกาสในการทำวิจัย สำนักงานกองทุนสนับสนุนการวิจัย สำหรับเงินทุน นางปิยะนุช ระเด่นอาหมัด ผู้ช่วยวิจัยที่ขยันและความละเอียดรอบคอบ สำนักงานวิจัย และเจ้าหน้าที่ในหน่วยงานต่างๆ ของคณะแพทยศาสตร์รพ.รามาธิบดี ที่อำนวยความสะดวกในการใช้เครื่องมือในการทำวิจัย

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Abstract

Project Code: RSA4580037

Project Title: Effect of metabolic acidosis on osteoblast differentiation

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Distal renal tubular acidosis (dRTA) is characterized by impairment of distal tubular acid secretion resulting in persistent metabolic acidosis. Recent examination of bone histology of dRTA patients showed markedly decreased bone formation with impaired bone matrix mineralization that is not entirely explained by an alteration in the mineral balance. Data from in vitro studies suggests a direct inhibitory effect of metabolic acidosis on osteoblast function. We investigated the effects of chronic metabolic acidosis on osteoblast differentiation from mesenchymal precursor cells (MSCs). Human MSCs were isolated from bone marrow aspiration specimen. Osteoblast differentiation was induced in the presence of dexamethasone in culture. Concentrated HCl was added to lower the medium pH. The expression of various osteoblastic genes was determined by quantitative real-time RT-PCR. Our isolated MSCs displayed characteristic spindle-shaped morphology. Flow cytometry revealed characteristic MSC surface marker profile. The mRNA expression of early osteoblast transcription factor, cbfa-1, and the major bone matrix protein, type I collagen, reached its peak at day 5-10 in control culture. Chronic metabolic acidosis was found to enhance the expression of cbfa-1 and type I collagen in a dose dependent manner. In contrast, the mRNA expression of osterix (a transcription factor downstream of cbfa-1) and alkaline phosphatase (an enzyme normally upregulated in intermediately mature osteoblasts) as well as alkaline phosphatase enzyme activity was decreased. The study on the expression of mature osteoblast marker, osteocalcin is currently ongoing. Impaired bone matrix mineralization was demonstrated by a reduction in both calcium deposition and number of bone nodules visualized by von Kossa staining. In conclusion, chronic metabolic acidosis enhanced early osteoblast differentiation while attenuating bone matrix mineralization.

Keywords: acidosis, osteoblast, mesenchymal, renal tubular acidosis

บทคัดย่อ

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ภาวะความเป็นกรดในเลือดเกิดจากการที่ไตขับกรดออกจากร่างกายไม่ได้ ผู้ป่วยที่มีภาวะนี้มักมีความผิดปกติของระดับแคลเซียม ฟอสเฟต วิตามินดี และ ฮอร์โมนพาราไธรอยด์ในเลือด ส่งผลให้มีการลดลงของการสร้างกระดูกทำให้มีมวลกระดูกลดลง การศึกษาในห้องทดลองยังพบว่าภาวะความเป็นกรดสามารถยับยั้งการทำงานของเซลล์กระดูกได้โดยตรง เนื่องจากเซลล์กระดูกเจริญเติบโตมาจาก mesenchymal stem cells (MSCs) คณะผู้วิจัยจึงทำการศึกษาผลกระทบของภาวะความเป็นกรดต่อการเจริญเติบโตของเซลล์กระดูก โดยใช้ MSCs นี้ได้มาจากไขกระดูกของคนปกติ นำมาเพาะเลี้ยงในห้องทดลอง (cell culture) แล้วกระตุ้นให้เซลล์เหล่านี้เจริญเติบโตไปเป็นเซลล์กระดูกโดยการผสม dexamethasone, β -glycerophosphate และ L-ascorbate phosphate เข้าไปในสารเลี้ยงเซลล์ และอาศัยการเติมกรด hydrochloric ปริมาณเล็กน้อย เพื่อให้ได้ระดับ pH ประมาณ 7.1-7.4 จากนั้นศึกษาการเปลี่ยนแปลงของระดับ mRNA โดยใช้วิธี quantitative real-time RT-PCR และ โปรตีนที่สกัดจาก MSCs เป็นระยะๆ โดยเริ่มตั้งแต่ระยะแรกที่เริ่มกระตุ้นด้วย dexamethasone จนกระทั่งระยะหลังที่เซลล์เปลี่ยนแปลงไปเป็นเซลล์กระดูกที่เจริญเติบโตเต็มที่ นอกจากนี้ยังได้ศึกษา mineralization ของเซลล์กระดูกโดยใช้วิธีการย้อมแบบ Von Kossa ผลการทดลองพบว่า MSCs ที่เพาะเลี้ยงได้มีรูปร่างเรียวยาว (spindle shape) และจากการทำ flow cytometry สามารถยืนยันได้ว่าเซลล์ที่ได้เป็น MSCs จริง ภาวะความเป็นกรดมีผลกระตุ้นให้มีการเพิ่มขึ้นของ cbfa-1 (ซึ่งเป็น transcription factor ที่จำเพาะและจำเป็นต่อการเจริญเติบโตของ osteoblast) และ type I collagen mRNA (90% ของ bone matrix protein ที่สร้างจาก osteoblast) ใน young osteoblasts เมื่อเปรียบเทียบกับเซลล์ที่เพาะเลี้ยงใน pH 7.4 ในขณะที่ osterix (downstream transcription factor ของ cbfa-1) และ alkaline phosphatase ซึ่งปรากฏในเซลล์กระดูกที่เจริญเติบโตในระดับกลาง มีปริมาณลดลง ขณะนี้คณะผู้วิจัยกำลังทำการศึกษาปริมาณของ osteocalcin mRNA ซึ่งพบในเซลล์กระดูกที่เจริญเติบโตเต็มที่อยู่ เมื่อย้อมเซลล์กระดูกเหล่านี้ด้วยวิธี Von Kossa พบว่าเซลล์ที่เลี้ยงในสภาวะที่เป็นกรดมีปริมาณ bone matrix mineralization ลดลง สรุปได้ว่าภาวะความเป็นกรดสามารถกระตุ้นการเจริญเติบโตของเซลล์กระดูกในระยะเริ่มต้น แต่ยับยั้งการเกิด bone matrix mineralization

คำหลัก: acidosis, osteoblast, mesenchymal, renal tubular acidosis

Introduction

Distal renal tubular acidosis (dRTA) is a clinical syndrome characterized by impaired renal excretion of ammonium and titrable acid resulting in persistent metabolic acidosis. The presence of chronic metabolic acidosis results in various metabolic consequences, including hypokalemia, hypercalciuria, hypophosphatemia and abnormal bone metabolism¹⁻³. Recent bone histologic studies of dRTA patients from our laboratory revealed findings of osteopenia and suppressed bone formation when compared to healthy controls⁴ (Table 1). Correction of metabolic acidosis with alkaline therapy improved the abnormality⁵ (Table 2). 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.

Table 1 Bone histomorphometry of dRTA patients compared to normal controls

Histomorphometric Parameters	dRTA	Normal
Osteoblast Surface (Ob.S/BS %)	0.78 ± 1.03	2.6 ± 1.1
Osteoclast Surface (Oc.S/BS %)	0.05 ± 0.03	0.13 ± 0.23
Bone Formation Rate (BFR/BS $\mu\text{m}^3/\mu\text{m}^2/\text{day}$)	0.02 ± 0.02*	0.07 ± 0.045

* $P < 0.05$ compared to normal controls. Adapted from Domronkitchaiporn et al⁴.

Table 2 Bone histomorphometry of dRTA patients before and after alkaline therapy

Histomorphometric Parameters	Pre alkaline	Post alkaline
Osteoblast Surface (Ob.S/BS %)	1.19 ± 0.88	1.79 ± 0.21
Osteoclast Surface (Oc.S/BS %)	0.04 ± 0.04	0.05 ± 0.06
Bone Formation Rate (BFR/BS $\mu\text{m}^3/\mu\text{m}^2/\text{day}$)	0.02 ± 0.02	0.06 ± 0.03*

* $P < 0.05$ compared to pre-alkaline. Adapted from Domronkitchaiporn et al⁵.

Previously, Bushinsky et al 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^{6, 7}. Later on, the effect of metabolic acidosis on bone cells was examined. Using an in vitro model of bone organ culture derived from neonatal mouse calvariae, Krieger et al observed that short-term incubation of calvariae in acidic medium suppressed type I collagen synthesis and lowered alkaline phosphatase enzyme activity⁸. In a later study, the same group examined the effect of acute metabolic acidosis on

primary bone cells. Incubation of calvariae-derived osteoblasts in acidic medium for 30 minutes resulted in marked decrease in *erg-1* and type I collagen gene expression⁹ (figure 1). Furthermore, chronic metabolic acidosis diminished the expression of matrix Gla protein (figure 2) and osteopontin mRNA (figure 3) of primary osteoblasts derived from the same source and impaired bone matrix mineralization¹⁰. This evidence supports the existence of both cellular and non-cellular effect of metabolic acidosis on bone.

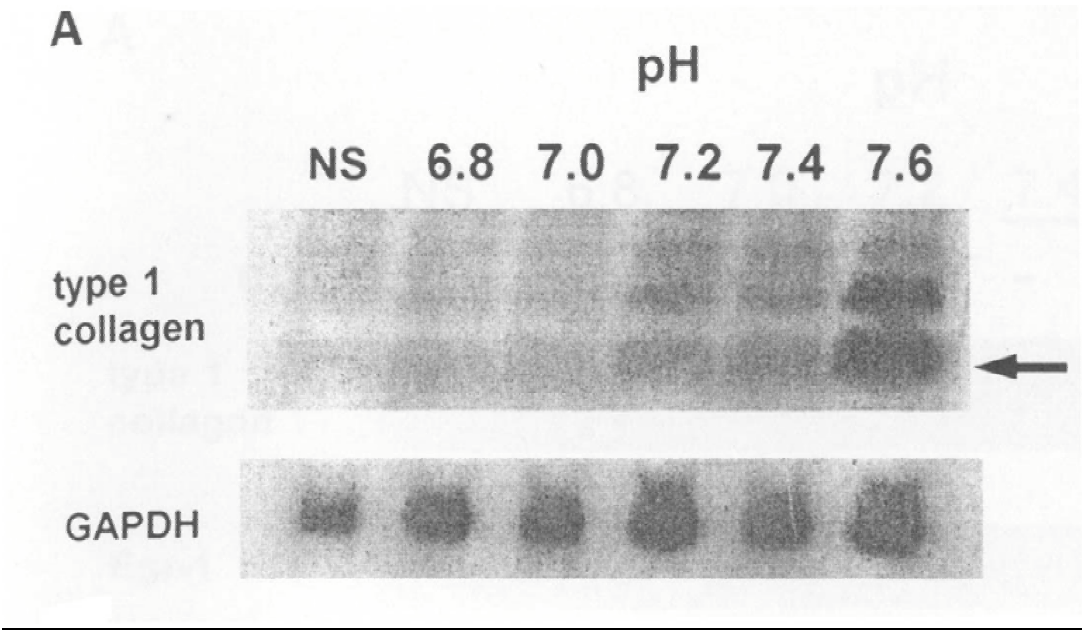


Figure 1 Northern blot analysis of type I collagen mRNA expression in calvariae-derived osteoblasts incubated in acidic medium for 30 minutes. Adapted from Frick et al⁹.

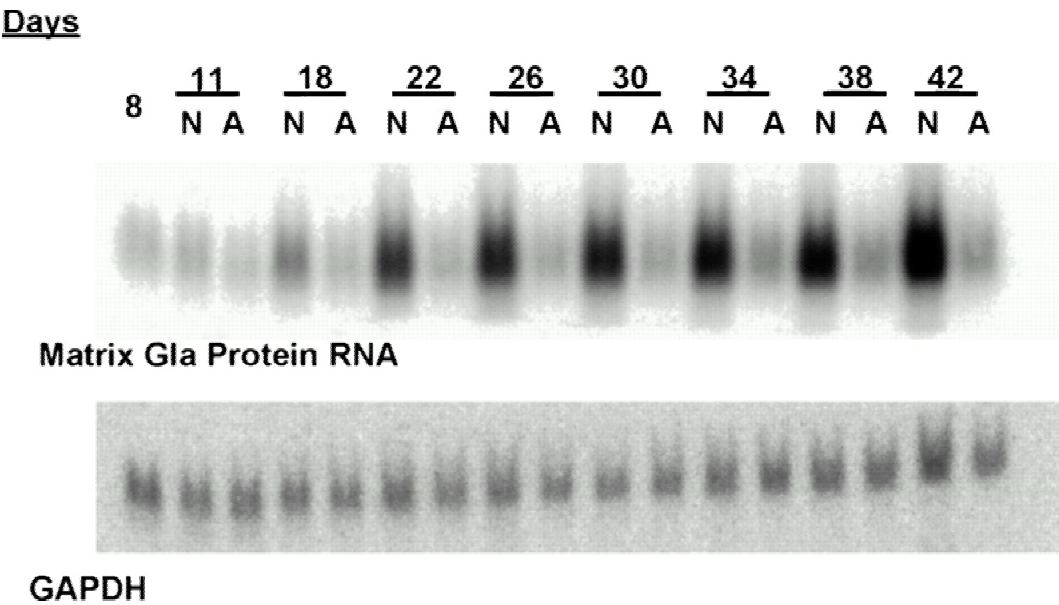


Figure 2 Northern blot analysis of matrix Gla protein mRNA expression of calvariae-derived osteoblasts incubated in acidic medium for 11-42 days. N = normal pH, A = acidosis. Adapted from Frick et al¹⁰.

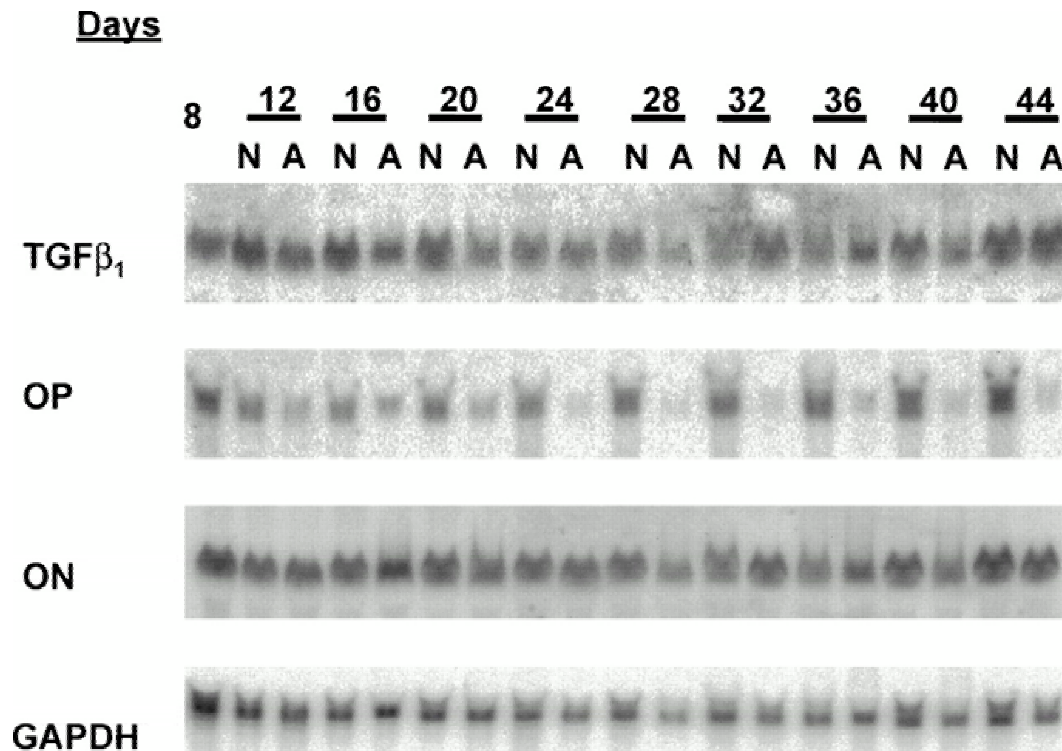


Figure 3 Northern blot analysis showed mRNA expression of various bone matrix proteins of calvariae-derived osteoblasts incubated in acidic medium for 12-44 days. N = normal pH, A = acidosis, OP = osteopontin, ON = osteonectin. Adapted from Frick et al ¹⁰.

Osteoblasts originate from mesenchymal precursor cells (MSCs) in the bone marrow. These cells contribute to replacement of osteoblasts in bone turnover and fracture healing throughout life. Under appropriate conditions, multipotent MSCs can also differentiate into chondrocytes, adipocytes and fibroblasts. A wide variety of systemic and local factors appears to regulate osteoprogenitor proliferation and differentiation, a sequence that is characterized by a series of cellular and molecular events distinguished by differential expression of osteoblast-associated genes, including those for specific transcription factors and matrix proteins ¹¹ (figure 3). In vitro, induction of osteogenic differentiation from MSCs can be achieved in the presence of dexamethasone (Dex), β-glycerophosphate (β-GP) and ascorbate phosphate (Asp) ¹². Mature osteoblasts synthesize and secrete bone matrix including type I collagen and various NCPs ready for subsequent mineralization. Of these NCPs, osteocalcin and osteonectin have been known to involve in the process of bone formation and mineralization ¹³⁻¹⁵, while osteopontin and bone sialoprotein play roles in bone resorption ¹⁶⁻¹⁹. Metabolic acidosis, through its effect on osteoblasts 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, 20}. The diagnosis was mostly based on clinical and roentgenographic findings. Bone pain was relieved and osteomalacia improved after alkaline therapy ^{3, 21}. 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 the first part of our study, we examined the NCP composition in non-decalcified bone sections of dRTA patients before and after alkaline therapy. Additional information on mineralization parameters will be analyzed by bone histomorphometry. In the second part of our study, we studied the sequential changes in the expression of osteoblastic genes in human MSCs cultured in normal and acidic environment during in vitro osteogenic induction.

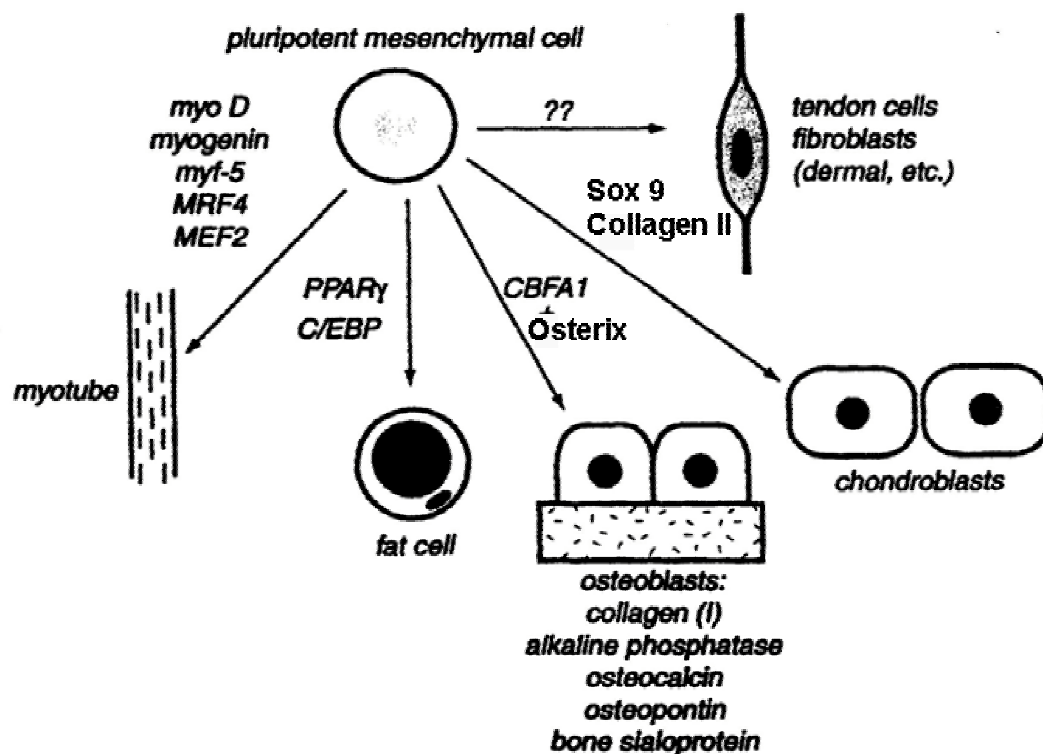


Figure 4 Multipotent MSCs can differentiate into osteoblasts, chondrocytes, adipocytes and myocytes under appropriate condition through the expression of lineage specific transcription factors.

Objectives

Part 1

1. To determine the presence of impaired bone matrix mineralization in dRTA patients
2. To determine whether alkaline therapy improves osteoblast function.
3. To examine the expression of various NCPs in bone sections of dRTA patients before and after alkaline therapy by immunohistochemistry

Part 2

1. To isolate and characterize bone marrow-derived MSCs by using flow cytometry and its ability to differentiate into osteoblasts and adipocytes in vitro
2. To determine the effect of chronic metabolic acidosis on MSCs cell proliferation
3. To determine the expression of various osteoblastic genes during in vitro osteogenic induction in normal and acidic condition by quantitative real-time RT-PCR
4. To examine the effect of chronic metabolic acidosis on bone matrix mineralization by von Kossa staining and calcium assay

Summary of results

Part 1

1. Bone matrix mineralization was impaired in majority of dRTA patients while fewer patients had histologic feature of adynamic bone disease
2. Alkaline therapy improved osteoblast function suggested by the increase in bone formation rate/number of osteoblasts.
3. The expression of osteocalcin increased significantly after alkaline therapy.
4. Osteopontin expression decreased in all but one patient.
5. There was no alteration in osteonectin and bone sialoprotein expression before and after alkaline therapy.

Part 2

1. Isolated bone marrow-derived MSCs showed characteristic MSC surface marker profile and were able to differentiate into osteoblasts and adipocytes in vitro.
2. Chronic metabolic acidosis dose-dependently attenuated cellular proliferation
3. Chronic metabolic acidosis enhanced the expression of early osteoblastic genes including cbfa-1 (a specific osteoblast transcription factor) and type I collagen (a major bone matrix protein).
4. Chronic metabolic acidosis suppressed the expression of alkaline phosphatase mRNA as well as its enzyme activity.
5. The expression of osterix, another essential osteoblast transcription factor downstream of cbfa-1, was diminished by metabolic acidosis.
6. Metabolic acidosis impaired bone matrix mineralization.
7. We are still confirming the expression of mature osteoblastic gene, osteocalcin.

Conclusion

Part 1

Abnormal bone remodeling in patients with dRTA is 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.

Part 2

Chronic metabolic acidosis directly affects osteoblast differentiation from MSCs. Osteoblast differentiation is enhanced in the early stages, whereas bone matrix mineralization is impaired. The roles of heightened cbfa-1 mRNA on the expression of other osteoblastic genes will require further study.

Materials and Methods

Part 1

Patients

Subgroup of dRTA patients who completed one 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 reported²⁰. 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, one 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 one year with 60 mEq/day of potassium citrate in two-divided dose 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-hour 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, 22}. Serum iPTH was determined by an immunoradiometric assay (ELSA-PTH; CIS BioInternational, GIF-sur-Yvette Cedex, France). The normal serum iPTH was 10 to 60 pg/mL.

Bone mineral density

Bone mineral density (BMD; g/cm²) 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 one year of alkaline therapy using protocol reported previously⁴. In brief, bone specimens of 5 mm in diameter and 20-30 mm in length were fixed for 24 hours 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 μ m 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- μ m 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 standardized nomenclature²³. 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 to 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, MD²⁴.

Immunohistology

Immunohistochemistry was performed on the bone sections as described previously by Derkx et al²⁵. 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% H₂O₂ 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 hour. 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 hours 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, rinse 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 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 ²⁵. Briefly, a CCD color video camera (Sony, Japan) mounted on a microscope (Zeiss, Germany) with a 10x 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 μ m sections cut at steps of 50-100 μ m in the same bone biopsy. This has been shown previously to be sufficient to obtain representative data with a small confidence interval ²⁵. 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 (in order 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.

Part 2

Isolation and culture of bone marrow derived MSCs

Bone marrow samples were obtained using a bone marrow biopsy needle inserted through posterior iliac crest of a healthy bone marrow donor after an informed consent. Bone marrow mononuclear cells (BMMCs) were separated by density gradient centrifugation with 1.073 g/ml Percoll solution (Sigma, MO, USA). Briefly, 10 ml of heparinized bone marrow cells were mixed in an equal volume of Dulbecco's Modified Eagle's Medium (DMEM) (BioWhittaker, MD, USA) and centrifuged at 900g for 10 min at room temperature. The washed cells were resuspended in DMEM at a density of 4×10^7 cells/ml, and 5 ml aliquot was layered over 1.073 g/ml Percoll solution and centrifuged at 1,000g for 30 min at room temperature. The interface mononuclear cells were collected and washed twice with DMEM. Total cell count and cell viability were evaluated by 0.2% Trypan blue exclusion. A total of 2×10^6 cells/ml of BMMCs were cultured in DMEM complete medium supplemented with 10% fetal bovine serum (FBS) (GibcoBRL, NY, USA) and 1% penicillin-streptomycin (GibcoBRL) at 37°C in 5% CO₂ incubator. On day 3 of cultivation, non-adherent cells

were discarded and this process was repeated every 4 days. Upon 90% confluency, MSCs were trypsinized by 0.05% trypsin (Gibco BRL) and passaged for the next expansion. This study has been approved by ethical committee on Research Involving Human Subjects at Ramathibodi hospital, Mahidol University.

Flow cytometry analysis of cultured bone marrow derived MSCs

Bone marrow derived adherent cells (at the end of 4th passage) were trypsinized and adjusted to $5-10 \times 10^6$ cells/ml. 100 μ l of adjusted cells were incubated with 10 μ l of following monoclonal antibodies, CD-14PE, CD-34FITC, CD45-FITC, HLA-DR PE and CD-105FITC (Becton-Dickinson Pharmingen, Heidelberg, Germany) at 4°C in the dark. After 20 min of incubation, 2 ml of PBS/2% FBS solution was added to each monoclonal antibody-treated cells. The mixtures were then centrifuged at 2,500 rpm for 10 min followed by removal of supernatant. These steps were repeated again following fixation of the cells with 0.5% paraformaldehyde. Flow cytometry analysis was performed using Cellquest software program.

Osteogenic and adipogenic differentiation

Studies were performed in subconfluent culture of human MSCs between passages 4-8. These cells have previously been shown to remain undifferentiated through multiple passages and their osteogenic potential is preserved up until passage 10-15²⁶. A normal karyotype and telomerase activity were found to be maintained even at passage 12²⁷. MSCs were grown in multi-well tissue culture plates (Nunc, Denmark) until 50-60% confluency when DEX 100 nM (Sigma, MO, USA), β -GP 10 mM (Sigma) and Asp 0.1 mM (Sigma) were added to induce osteogenic differentiation (day 0). This medium will be referred to as osteogenic differentiation medium (OM). In the dose response experiments, concentrated hydrochloric acid (HCl) was added (at day 0) at the concentration of 1 μ l/ml of culture medium to achieve an approximate pH of 7.25 (HCL 1), 2 μ l/ml for pH 7.15 (HCL 2) and 3 μ l/ml for pH 7.0 (HCL 3). MSCs grown in the culture medium without HCl (HCL 0) served as control (pH 7.4). Time course experiments were performed with the cells grown in HCL 2 culture medium. MSCs cultivated in the absence of OM represent negative controls. For adipogenic differentiation, DEX 1 μ M, 3-isobutyl-1 methylxanthine 100 μ g/ml (Sigma) and insulin 10 μ g/ml (Sigma) were added to confluent culture of MSCs. Medium was changed twice weekly.

Cell proliferation assay

Cell proliferation was evaluated by MTT assay at various time intervals. The yellow tetrazolium MTT (Sigma, MO, USA) was reduced by metabolically active cells resulting in intracellular purple formazan, which was solubilized and quantified by spectrophotometric means. The experiments were performed in 12-well tissue culture plates. MSCs were grown in the presence of OM without HCl (HCL 0) and with HCl (HCL 1, HCL 2 and HCL 3). After the cells were rinsed twice with DMEM without phenol red, 1 ml of 0.5 mg/ml MTT solution was added to each well. After 3 hours of incubation at 37°C in CO2 incubator, the purple precipitate formed within the cells. MTT was removed and intracellular purple formazan was solubilized with 1 ml of 0.04 M HCl in absolute isopropanol. Absorbance was recorded at 570 nm with background subtraction at 650 nm.

Studies of osteoblastic gene expression by quantitative real-time RT-PCR.

Total RNA was isolated from culture cells at various time intervals using Trizol (Life Technologies, NY, USA) method as described by manufacturer. cDNAs were reverse transcribed from 1 µg of total RNA using Reverse Transcription System (Promega, WI, USA) with random hexamer as primer as described by manufacturer. cDNAs obtained from the reaction were diluted 1:5 in DNase free water. Quantitative real-time RT-PCR was performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems, CA, USA). 3-6 µl of cDNAs was analyzed in 25 µl reaction of Taqman® Universal PCR mastermix (Applied Biosystems). Multiplexed PCR reaction was performed with both target and reference genes (18S rRNA) in the same reaction. Each sample was analyzed in triplicate. The probe for 18S rRNA was fluorescently labeled with VIC™ and TAMRA (Applied Biosystems), whereas the probes for gene of interest were labeled with 6-carboxyfluorescein (FAM) and TAMRA (Applied Biosystems). Primer concentrations were 300 nM except for type I collagen and 18S rRNA where the concentrations were 25 nM. Probe concentrations were 100-150 nM for the target genes and 50 nM for 18S rRNA. The nucleotide sequences of primers and probes for 18S rRNA, cbfa-1, type I collagen (Coll I), osterix (Osx), alkaline phosphatase (ALP) and osteocalcin (OC) are shown in Table 3. Relative expression levels of the gene of interest, normalized by the amount of 18S rRNA, were calculated by Sequence Detection Software version 1.2 (Applied Biosystems) using Relative Quantification Study approach. The average values of ΔC_t s from each sample were used for statistical analysis.

Table 3 Primer and Probe Sequences

Gene	Forward and reverse primers (5'-3')	Probe (5'-3')
18 S rRNA	CGGCTACCACATCCAAGGAA GCTGGAATTACCGCGGCT	TGCTGGCACCAGACTTGCCCTC
Cbfa-1	GCCTTCAAGGTGGTAGCCC CGTTACCCGCCATGACAGTA	CCACAGTCCCATCTGGTACCTCTCCG
Coll I	CAGCCGCTTCACCTACAGC TTTTGTATTCAATCACTGTCTTGCC	CCGGTGTGACTCGTGCAGCCATC
Osx	CCCCACCTCTTGCAACCA CCTTCTAGCTGCCCCACTATTTCC	CCAGCATGTCTTGCCCCAAGATGTCTA
ALP	GACCCTTGACCCCCACAAT GCTCGTACTGCATGTCCCCT	TGGACTACCTACCTATTGGGTCTCTTCGA GCCA
OC	GAAGCCCAGCGGTGCA CACTACCTCGCTGCCCTCC	TGGACACAAAGGCTGCACCTTTGCT

Alkaline phosphatase enzyme activity assay

Alkaline phosphatase enzyme activity was measured based on its ability to convert a substrate, *p*-nitrophenyl phosphate, to a yellow colored product, *p*-nitrophenol. The absorbance of *p*-

nitrophenol can be determined at 405 nm in a microtiter plate reader. The experiments were performed in 12-well tissue culture plates. After cell layers were rinsed twice with calcium and magnesium free PBS, 0.3 ml of 2 mg/ml *p*-nitrophenyl phosphate solution (Sigma) in 0.75 M AMP (Sigma) with 2 mM magnesium chloride, pH10.3, was added to each well. Cells were incubated in 37°C water bath for 30 min and the reaction was stopped by adding 0.3 ml of 50 mM NaOH to each well. Protein concentration was determined using Bradford Reagent (Sigma) as described by manufacturer. Absorbance of *p*-nitrophenol was normalized by protein concentration. Standard curve was constructed using *p*-nitrophenol standard solution (Sigma). For each sample and standard, assay was performed in duplicate.

Measurement of calcium content

The deposition of calcium in mineralization nodules was determined based on Calcium-o-Cresolphthalein Complexone method. Calcium yielded purple-colored product when formed complex with a chromogenic substrate, o-cresolphthalein Complexone, in an alkaline medium. The intensity of color, measured at 575 nm, is directly proportional to calcium concentration in the sample. The experiments were performed in 12-well tissue culture plates. All the glasswares were cleaned and soaked overnight in 3% HCl, rinsed with distilled water and dried before use. After cell layers were rinsed twice with calcium and magnesium free PBS, decalcification was performed by adding 200-500 µl of 0.6 N HCl into each well. Cells were left overnight at 4°C and 50 µl of samples were transferred to the test tubes containing 1 ml of colored solution (0.1% o-cresolphthalein complexone (Sigma) and 1% 8-hydroxyquinoline (Sigma)) the next morning. To provide an alkaline environment, 1 ml of AMP, pH 10.7, was added to each tube and the mixtures were incubated at room temperature for 5 min before measurement. Absorbance of calcium was normalized by protein concentration. Serially diluted stock CaCO₃ was used as standards. For each sample and standard, assay was performed in duplicate.

Von Kossa staining

The experiments were performed in 25 mm² tissue culture dishes. MSCs were allowed to grow in the presence of OM with or without HCl until mineralization appeared. After washing the cells twice in calcium and magnesium free PBS, cell layers were fixed with 10% formalin for 30 min, washed thoroughly with distilled water and incubated with 2% silver nitrate in front of 60 watt lamp for 15 min. Aluminum foil was placed around the dish and the lamp in order to reflect the light.

Statistical Analysis

Results are presented as mean ± standard deviation (SD) on the first part of the study of dRTA patients and as mean ± standard error of measurement (SEM) on the second part of the study on MSCs. Student *t* test was used to compare group means of two samples. The difference was considered significant at *P* value below 0.05.

Results

Part 1

Patient characteristics are shown in Table 4. 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 5. Full details on blood and 24-hour urine chemistries before and after potassium citrate therapy have been reported previously⁵. 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.

Table 4 Patient characteristics at presentation

Patient No	Age years	Height cm	Weight kg	Sex	Blood/Urine pH	U r i n e Am m o n i u m 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

^aduration of symptoms, for example, muscle weakness, renal stone or fracture

Table 5 Blood (per liter) and urine (per day) chemistries of dRTA patients

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

^aSignificant difference when compared to the corresponding baseline value (P < 0.05)

^bSignificant difference when compared to the corresponding baseline value (P < 0.01)

^cData obtained from the 28 normal farmers who were permanent residents of Khon Kaen province

Table 6 Bone mineral density (g/cm²) 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

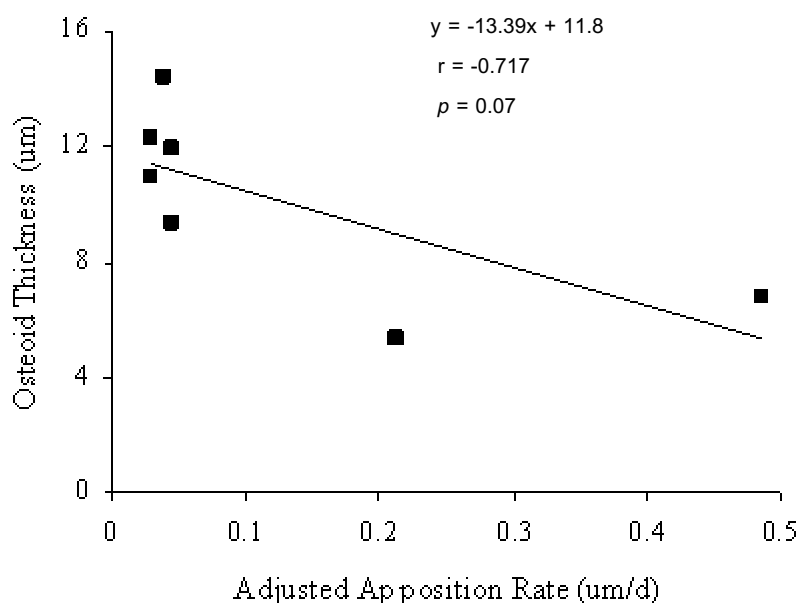
Bone mineral densities at baseline and the end of the study period are shown in Table 6. The basal BMD values of dRTA patients were lower than those of normal controls in all studied areas. After one year of alkaline therapy, there were significant elevations in the BMDs of total femur and trochanter of femur ($P < 0.05$). Bone histomorphometric data before and after the treatment is presented in Table 7. 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/osteoid surface and adjusted apposition rate were accompanied by the prolongation of mineralization lag time ($P < 0.05$). Bone formation rate per bone surface was suppressed at baseline ($P < 0.05$). While 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 5). No positive staining for the aluminum was found in any of the bone specimens.

Table 7 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) $\mu m/d$	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) $\mu m/d$	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) $\mu m^3/\mu m^2/y$	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) $\mu m^2/cell/d$	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)

^aSignificant difference when compared to the corresponding normal value ($P < 0.05$)^bSignificant difference when compared to the corresponding baseline value ($P < 0.05$)^cAj.AR was calculated from : Aj.AR = [(MS/OS) x MAR]/100^dMlt was calculated from : Mlt = O.th/Aj.AR^eBFR/BS was calculated from : BFR/BS = [(MS/BS) x 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^fBFR/N.Ob was calculated from BFR/N.Ob = MAR x [osteoid perimeter (μm)/number of osteoblasts]

Figure 5 Relationship between osteoid thickness (μm) and adjusted apposition rate ($\mu\text{m}/\text{day}$) of dRTA patients before treatment with alkaline.



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 between the proteins was observed. As shown in figure 6, osteocalcin (Fig 6A 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) (Fig 6C and D). A significant increase in the area of osteocalcin staining from $16.65 \pm 9.25\%$ in bone biopsy at initial diagnosis to $22.26 \pm 8.16\%$ after alkaline treatment ($P < 0.04$) was observed (Fig 7A). Six of seven patients showed decreased expression of osteopontin with an average of $28.91 \pm 10.4\%$ at initial biopsy to $22.3 \pm 3.76\%$ post alkaline ($P = 0.16$) (Fig 7B). Osteonectin and bone sialoprotein diffusely stained the bone matrix and cement lines (Fig 6E and F). The area of staining of osteonectin ($19.46 \pm 10.92\%$ and $21.62 \pm 9.52\%$, $P = 0.5$) and bone sialoprotein ($11.94 \pm 4.37\%$ and $12 \pm 3.76\%$, $P = 0.98$) were not significantly different post alkaline therapy (Fig 7C and D). After a careful examination of the sections obtained from initial biopsy and one year after alkaline treatment, there was no alteration in the distribution of any of the proteins.

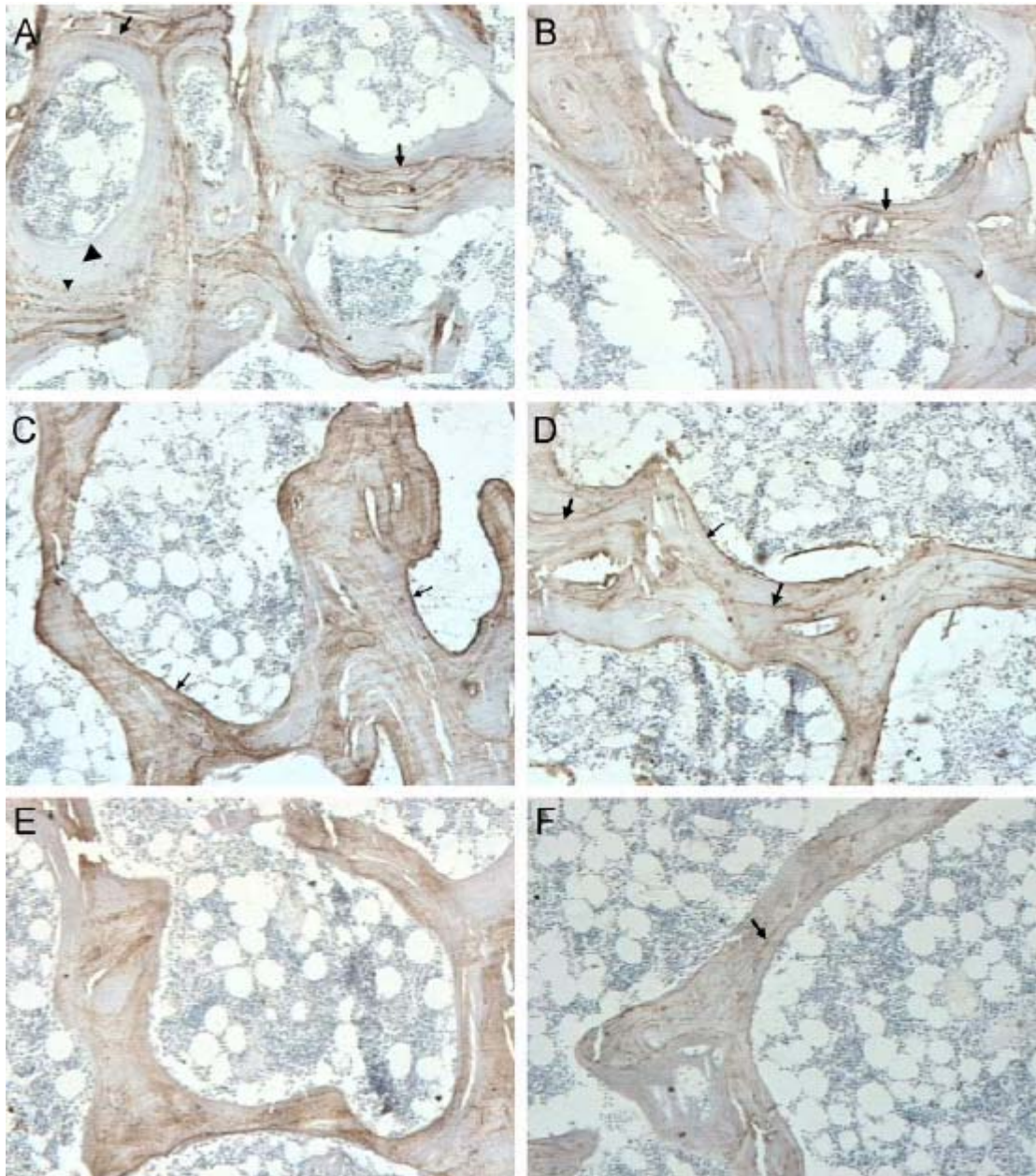


Figure 6 Immunohistochemistry of NCPs in bone sections of patients with dRTA pre and post alkaline treatment. Each protein has specific pattern of distribution but an overlap in localization between the proteins was observed. (A) Osteocalcin pre-alkaline; (B) Osteocalcin one year post-alkaline treatment. 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, x100.

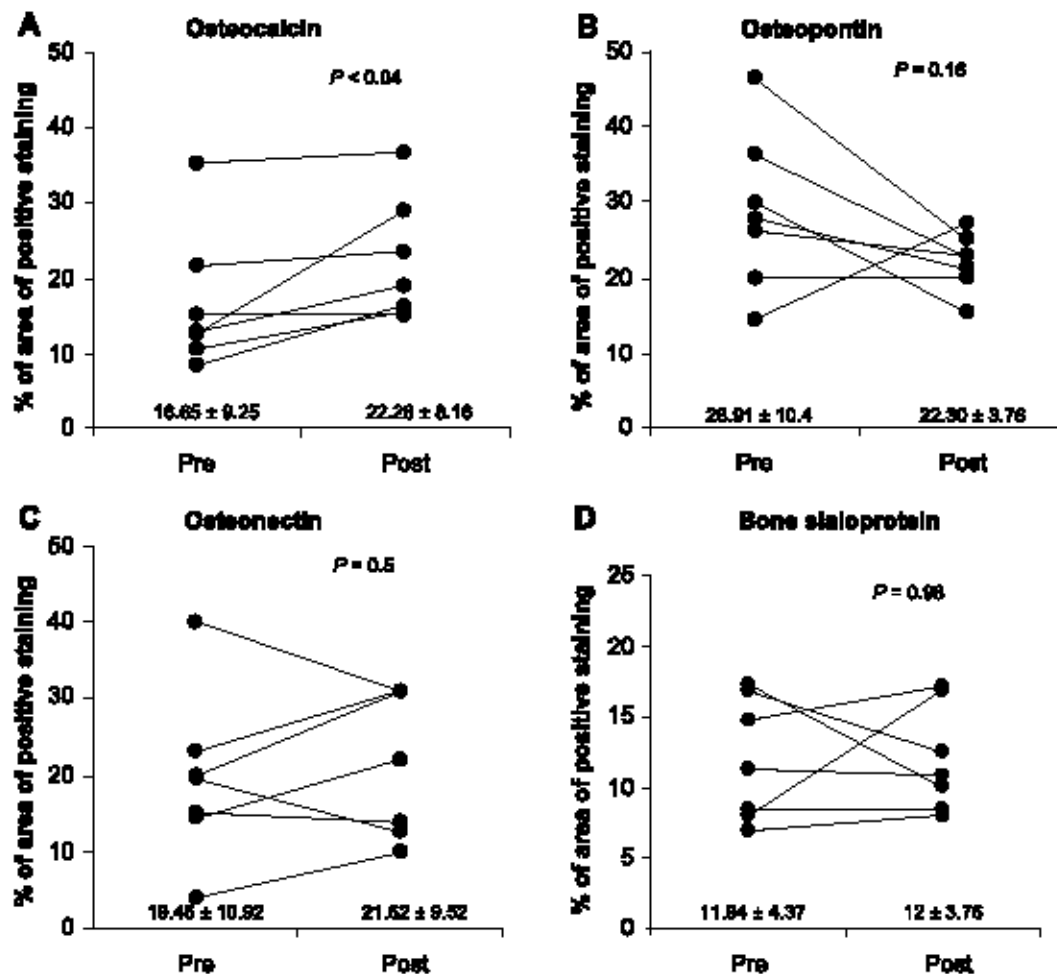


Figure 7 Quantitation of NCPs expression using digital image analysis pre and post-alkaline treatment. Data was expressed as percentage of area of positive staining/mineralized bone matrix area (A) Osteocalcin; (B) Osteopontin; (C) Osteonectin; (D) Bone sialoprotein. Osteocalcin expression significantly increased after alkaline treatment. Six of seven patients had decreased osteopontin expression.

Part 2

First, we isolated and characterized MSCs derived from bone marrow aspiration specimen. After BMMCs isolated from Percoll gradient were plated, the adherent MSCs gave rise to colonies and exhibited characteristic spindle-shaped morphology (Figure 8A). Flow cytometry analysis of the cells in the 4th passage revealed characteristic MSC surface marker profile (CD14^{neg}CD45^{neg}CD34^{neg}HLA-DR^{neg}CD105^{pos})²⁷ (Figure 9A–D). To determine the ability of MSCs to differentiate into osteoblasts and adipocytes, MSCs were cultivated in the presence of OM and adipogenic inducing medium respectively. After 20 days in culture, cell colonies displayed bone-like nodular aggregates of matrix mineralization (Figure 8B) noticeable on von Kossa staining (Figure 13). MSCs cultivated in the absence of OM never mineralized (data not shown). Lipid-rich vacuoles were observed after 7 days of adipogenic induction (Figure 8D). Cell proliferation of cultured human