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Osteogenic changes in kidneys of hyperoxaluric rats

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ABSTRACT

Many calcium oxalate (CaOx) kidney stones develop attached to renal papillary sub-epithelial deposits of calcium phosphate (CaP), called Randall's plaque (RP). Pathogenesis of the plaques is not fully understood. We hypothesize that abnormal urinary environment in stone forming kidneys leads to epithelial cells losing their identity and becoming osteogenic. To test our hypothesis male rats were made hyperoxaluric by administration of hydroxy-L-proline (HLP). After 28 days, rat kidneys were extracted. We performed genome wide analyses of differentially expressed genes and determined changes consistent with dedifferentiation of epithelial cells into osteogenic phenotype. Selected molecules were further analyzed using quantitative-PCR and immunohistochemistry. Genes for runt related transcription factors (RUNX1 and 2), zinc finger protein Osterix, bone morphogenetic proteins (BMP2 and 7), bone morphogenetic protein receptor (BMPR2), collagen, osteocalcin, osteonectin, osteopontin (OPN), matrix-gla-protein (MGP), osteoprotegrin (OPG), cadherins, fibronectin (FN) and vimentin (VIM) were upregulated while those for alkaline phosphatase (ALP) and cytokeratins 10 and 18 were downregulated. In conclusion, epithelial cells of hyperoxaluric kidneys acquire a number of osteoblastic features but without CaP deposition, perhaps a result of downregulation of ALP and upregulation of OPN and MGP. Plaque formation may additionally require localized increases in calcium and phosphate and decrease in mineralization inhibitory potential.

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1. Introduction

Kidney stone formation is a common chronic disease and its lifetime prevalence is increasing in United States as well as the rest of the world [1,2]. The increase in stone prevalence is associated with simultaneous escalation in cost of taking care of patients, which has already reached over ten billion dollars/year. But this figure does not include the hidden costs of nephrolithiasis, the impact on renal functions and quality of life [3]. New epidemiological studies also provide the evidence that stone formation is a risk factor for developing hypertension, chronic kidney disease and end stage renal disease [4–7]. The number of stone episodes and surgical interventions is highly correlated with reduction and loss of renal function [3]. Obesity, hypertension, diabetes, chronic kidney disease and metabolic syndrome, all of which are on the rise, are also risk factors for stone formation in the adult population [5,8]. In the case of pediatric urolithiasis, analysis of PHIS database showed that stone patients had significantly higher odds of hypertension and obesity than the controls [9].

Calcium oxalate (CaOx) kidney stones develop attached to Randall's plaques (RP), subepithelial deposits of calcium phosphate (CaP) on renal papillary surface, or Randall's plugs, crystal aggregates blocking the terminal collecting ducts [10–14]. The plaques themselves originate deep inside the renal interstitium associated with the basement membranes of loops of Henle, collecting ducts or blood vessels [15,16]. Mechanisms involved in the initial formation of the RPs are still unclear. Pathogenesis is generally considered a passive, unregulated physicochemical process [17]. In our opinion [18], as well as some others [19] plaque and stone formation are actively regulated processes similar to vascular calcification in the kidneys in which vascular smooth cells (VSMC) acquire osteogenic phenotype [20–22]. Exposure of VSMC to elevated levels of calcium and phosphate triggers osteogenic transformation of VSMC [23–26]. Transformation involves increased expression of osteoblast specific genes and decrease in smooth muscle cell markers [27,28]. Bone morphogenetic proteins, BMP 2 and BMP 4, and Wnt signaling pathways are activated through up-regulation of transcription factor, runt-related transcription factor 2 (RUNX2). Cells produce matrix proteins. Reactive oxygen species are likely involved in the VSMC transformation to osteogenic phenotype by regulating RUNX-2 transcription factor [29,30]. It is our hypothesis that abnormal urinary environment such as hyperoxaluria, hypercalciuria and hypocitraturia and associated oxidative stress, produce specific changes in the renal

Abbreviations: CaOx, calcium oxalate; CaP, calcium phosphate; RP, Randall's plaque; RUNX, runt-related transcription factor; BMP, bone morphogenetic protein; BMPR, bone morphogenetic protein receptor; ALP, alkaline phosphatase; OPN, osteopontin; MGP, matrix-gla-protein

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Table 1
The list of forward and reverse primers used for quantitative Real Time PCR designed using Primer-BLAST (National Center for Biotechnology Information, NCBI, and National Institute of Health, NIH).

Gene	Primer	Sequence
Fibronectin (*Fn1*)	Fn1_F	5′-GTGGCTGCCTTCAACTTCTC-3′
	Fn1_R	5′-GTGGGTTGCAAACCTTCAAT-3′
Runt-related transcription factor-2 (*Runx2*)	Runx2_F	5′-TCCCATCTGCTAGAAGTGTT-3′
	Runx2_R	5′-TTAGCCAGCTCACTTTCTTC-3′
Osterix/SP7	Sp7_F	5′-AAGCCATACACTGACCTTTC-3′
	Sp7_R	5′-GTGGGTAGTCATTGGCATAG-3′
Bone morphogenetic protein-2 (*Bmp2*)	Bmp2_F	5′-ACCAGACTATTGGACACCAG-3′
	Bmp2_R	5′-AATCCTCACATGTCTCTTGG-3′
Bone morphogenetic protein-7 (*Bmp7*)	Bmp7_F	5′-ATGGCCAACGTGGCAGAGAA-3′
	Bmp7_R	5′-CAGCCCAGGTCTCGGAAGCT-3′
Bone morphogenetic protein receptor, type II (*Bmpr2*)	Bmpr2_F	5′-ATAGGCGTGTGCCAAAAATC-3′
	Bmpr2_R	5′-GCTAGGGATTCGAGCTTGTG-3′
Cytokeratin 8 or keratin 8 (*Krt8*)	Krt8_F	5′-AGCCAGAGTACCAGCCCTAA-3′
	Krt8_R	5′-ACAATTGAGTTGGGCATTGGC-3′
Cytokeratin 18 or keratin 18 (*Krt18*)	Krt18_F	5′-ATATCCGTGTCCCGCTCTGT-3′
	Krt18_R	5′-TCGTTCAGGTCTTGCATGGT-3′
Vimentin (*Vim*)	Vim_F	5′-TTCTCAGCACCACGATGACC-3′
	Vim_R	5′-TGCTGAGCTCGTTTCTATCCC-3′

epithelial cells. The cells lose their epithelial character and become osteogenic. We decided to test our hypothesis in a rat model of hyperoxaluria. Since many genes and pathways are involved we performed genome wide analysis of differentially expressed genes in the kidneys and determined changes consistent with the dedifferentiation of epithelial cells into bone producing cells.

We investigated the expression of genes considered to be involved in epithelial transformation and bone morphogenesis including runt related transcription factors (RUNX) [31,32], zinc finger protein Osterix [33], bone morphogenetic proteins (BMP1-7) [34], bone morphogenetic protein receptor (BMPR) [35], collagens [36], alkaline phosphatase (ALP) [37], osteocalcin [31], osteonectin [38], osteopontin (OPN) [39] matrix-gla-protein (MGP) [21], osteoprotegrin (OPG) [40], cytokeratins [41], cadherins [42], fibronectin (FN) [43] and vimentin (VIM) [44]. Epithelial cells express high levels of cytokeratin 8, 10, 18, as well as E-cadherin, while mesenchymal cells express N-cadherin, fibronectin and vimentin [45]. BMPs play significant role in osteoblast differentiation and interact with BMPRs [46,47]. Collagens are major constituent of extracellular matrix [48], control matrix remodeling and are involved in calcification [49] including growth of Randall's plaques [50]. ALP is a membrane associated enzyme and plays critical role in both physiological and pathological calcification [51,52]. RUNX2 also known as core-binding factor alpha-1 is a key transcription factor associated with osteoblast differentiation [53,54]. Osterix is a zinc-finger containing transcription factor essential for osteoblast differentiation [46]. This report shows the changes that occur in the expressions of these molecules of interest in renal tissues in response to sustained hyperoxaluria and crystal formation. We discuss how these changes point to a number of molecular processes that may be involved in the deposition of CaP crystals in the renal interstitium and formation of plaques.

2. Materials and methods

2.1. Animal procedures

The experiments described herein were performed in Sprague-Dawley rats purchased from Harlan Labs, Inc. The studies were approved by the University of Florida's IACUC and were conducted in accordance with the recommendations of the NIH Guide for the Care and Use of Laboratory Animals. All procedures are detailed in our earlier publications [55–57]. In brief, two groups of 6 rats each, average weight of 150 g, were placed in metabolic cages with free access to food and water. Rats in group 1 were fed a normal rat chow diet and given sterile water. Rats in group 2 were fed the same chow as group 1 rats, but supplemented with 5% (w/w) hydroxy-L-proline (HLP). At the end of day 28, all rats were euthanized and their kidneys removed. From each rat, one kidney was used for RNA isolation, while the second was placed in 10% phosphate buffered formalin for histological analyses.

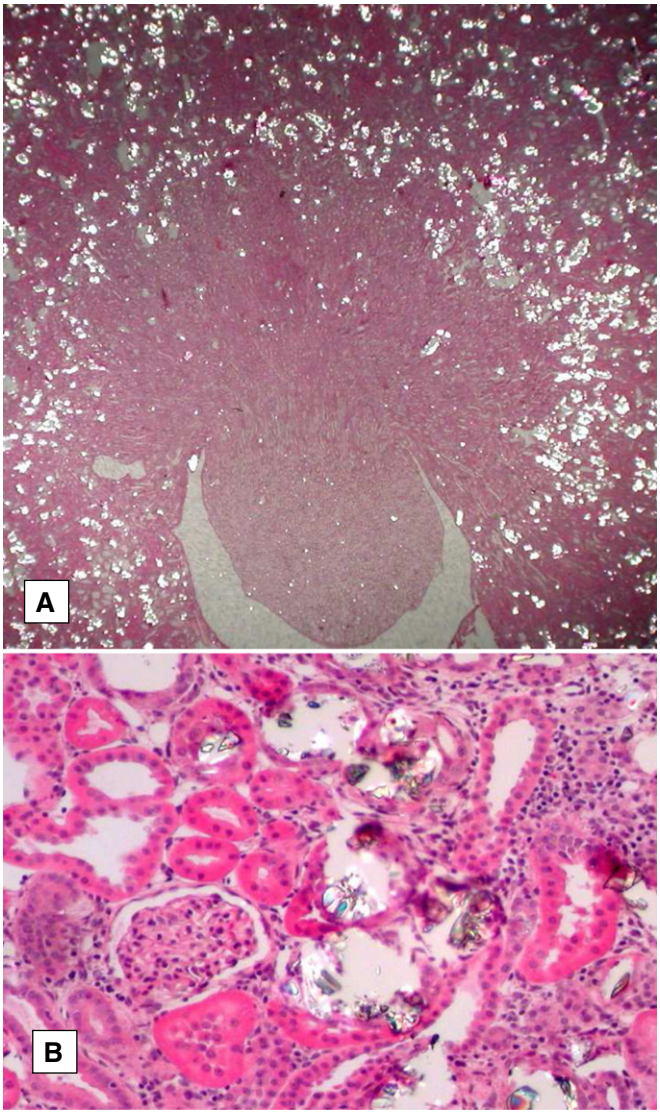

Fig. 1. H&A stained section of a kidney from hyperoxaluric rat on the 28th day of feeding on hydroxyl-L-proline (HLP). A. Low mag image showing both cortical and medullary segments of the kidney. Calcium oxalate (CaOx) crystal deposits appear as bright spots and most of them are located in the cortical renal tubules. Original X2.5. B. High magnification image showing a glomerulus and tubules with and without CaOx crystals. Glomerulus and tubules without the crystals appear normal. Tubules with crystals are dilated with many fold increase in their luminal diameter and compressed lining epithelia. Renal interstitium shows signs of inflammation. Original X45.

2.2. RNA extraction and differential expression of genes by microarray analysis

Each rat kidney excised for RNA isolation was surgically separated into medulla and cortex, then snap frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated concurrently from each of the specimens using the RNeasy Mini-Kit (QIAGEN, Valencia, CA) as per the manufacturer's instructions. Microarray hybridizations were carried out with each of the RNA specimens using the Illumina™RatRef-12