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Association of Randall's Plaques with Collagen Fibers and Membrane Vesicles

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Abstract

Background—Idiopathic calcium oxalate (CaOx) kidney stones develop by deposition of CaOx crystals on Randall's plaques (RP). Mechanisms involved in RP formation are still unclear.

Objective—It is our hypotheses that RP formation is similar to vascular calcification involving components of extracellular matrix including membrane bound vesicles (MV) and collagen fibers. In order to verify our hypothesis we critically examined renal papillary tissue from stone patients.

Methods—4 mm cold-cup biopsies of renal papillae were performed on fifteen idiopathic stone patients undergoing PCNL. Tissue was immediately fixed and processed for analyses by various light and electron microscopic techniques.

Results and Limitations—Spherulitic CaP crystals, the hallmark of RP's, were seen in all samples examined. They were seen in interstitium as well as laminated basement membrane of tubular epithelia. Large crystalline deposits comprised of dark elongated strands mixed with spherulites. Strands showed banded patterns similar to collagen. Crystal deposits were surrounded by collagen fibers and membrane bound vesicles. Energy dispersive x-ray microanalyses (EDX) and electron diffraction identified the crystals as hydroxyapatite.

The number of kidneys examined is **small and urinary data was not available for all the patients.**

Conclusions—Results presented here show that crystals in the Randall's plaques are associated with both the collagen as well as MV. Collagen fibers appeared calcified and vesicles contained crystals. We conclude that crystal deposition in renal papillae may have started with membrane vesicle induced nucleation and grew by addition of crystals on the periphery within a collagen framework.

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Keywords

Randall's Plaque; Hydroxyapatite; Stone Formation; Ectopic Calcification; Biomineralization

Introduction

Investigations of the human renal papillary autopsies and biopsies have provided evidence that idiopathic stones are formed attached to renal papillary surfaces at sites of interstitial plaques of pre-formed calcium phosphate (CaP), the so-called Randall's plaques¹. It has been proposed that Randall's plaques (RP) themselves start by the deposition of apatitic CaP crystals in the basement membrane of the Loops of Henle^{2,3}. Mechanisms involved in the formation and growth of RPs are poorly understood. In the absence of animal models of RP formation we are looking for mineralogical signatures⁴ in the plaque itself using a variety of ultrastructural techniques, as has previously been done in cases of pathological mineralization in other organ systems such as bone and blood vessels⁵. Our study was performed specifically to examine plaque growth in the renal interstitium. We hypothesized that RPs grow by addition of more crystals which are formed by heterogeneous nucleation. To determine the substrate which promotes crystallization of CaP and growth of RP, we examined renal papillary tissue from idiopathic CaOx stone patients obtained at the time of stone removal.

Materials and Methods

After IRB approval patients undergoing percutaneous nephrolithotomy (PCNL) for management of large intrarenal calculi were consented to participate in the study. Percutaneous renal access was obtained by the treating urologist at the time of PCNL. At the completion of stone removal, a papilla was selected for biopsy and intraoperative image recorded using a Karl Storz Tricam Endoscopic camera (Culver City, CA). The papilla was accessed utilizing a 24-F rigid Karl Storz Nephroscope or 15-F flexible Karl Storz cystonephroscope and 4 mm cold-cup biopsy obtained and tissue sample stored in 10% formalin fixation. All stones were analyzed (ARUP Labs, Salt Lake City UT). Two 24-hour urine metabolic evaluations were performed one-month following PCNL by Litholink Inc. (Chicago IL).

Twelve idiopathic calcium oxalate stone formers, as defined by stone composition of <50% calcium oxalate, no brushite component and >50% basic calcium phosphate or uric acid component, were included in this study. Following fixation kidney tissue was processed for light and electron microscopic analyses using standard techniques described in other publications^{6,7}. All samples were examined by light and transmission electron microscopy (TEM). Six specimens were examined by SEM. Because of small specimen size, some were first examined by scanning electron microscopy (SEM) and then TEM and **histology**. Paraffin embedded sections were examined after hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) staining. Specimens, which showed distinct calcium deposits were additionally stained for osteopontin (OPN) as previously described⁸ as well as collagen using standard Masson's trichrome protocol.

Crystal deposits were analyzed using energy dispersive x-ray microanalyses (EDX) in association with SEM⁹. Five of the 15 specimens had large interstitial deposits which allowed us to perform selected area electron diffraction. Electron diffraction micrographs were analyzed by using the three most prominent diffraction lines of aluminum to translate the measured diameter of the diffraction rings in millimeters to the ASTM interplanar "d" spacing's in Angstroms. This camera constant was then used to translate the measured

diameters of the electron diffraction lines of the experimental samples to Angstroms for comparison to the extensive library of diffraction standards assembled at Crystal Identification Center and Molecular Structure Laboratory, Medical College of Wisconsin.

Results

Results of stone and urine analyses are presented in Table 1

Intraoperative images of the papillae clearly showed RPs (Figure 1A) as well as attached stones (Figure 1B). Once renal papillary tissues were fixed for microscopic analyses, plaques appeared as small bulges or protrusions on the papillary surface. A closer examination using SEM showed these bulges were not completely covered with the surface epithelium (Figures 2 A, B). Part of the epithelial covering appeared to have sloughed off exposing the underlying structures. Loss of surface epithelium revealed a granular surface which was followed by a fibrous layer (Figure 2 C). Crystal deposits or plaque were present underneath the fibrous layer (Figure 2 D). The outer surface of the plaque was composed of 1-5 μm diameter spherical units, referred to as spherulites. These spherulites had a rough external surface giving an appearance of a pin cushion and were mixed with thin long fibers running between them (Figure 3 A). Some fibers appeared deeply embedded in the crystals. Fusion of the units and loss of their identity inside the plaque was apparent when plaques were fractured to examine the interior, which revealed mostly continuous concentric layers (Figure 3 B). However, higher magnification of fractured surfaces occasionally revealed the presence of spherulites therein. EDX microanalyses of the spherulites showed the presence of calcium and phosphorus only, indicating that the deposits were made of CaP (Figure 4 A). Calcium and phosphorus were also seen on EDX analysis of some fibers (Figure 4B) indicating that they also contained CaP deposits.

TEM analysis of the plaque showed basically two types of calcifications. All specimens contained spherical deposits in the interstitium as well as in the laminated basement membrane of the renal tubules (Figure 5 A). Some specimens had only few such deposits that required sustained efforts to visualize them while others had a large number of spherical crystals both around the tubules as well as in the interstitium. The spherical units ranged in size from less than 0.5 μm to 2 μm across and showed a number of distinct internal concentric laminations (Figure 5 B). Some sections clearly showed that concentric laminations inside the spherulites contained radially arranged crystallites which could also be seen in grazing sections. The size of the spherical units and number of concentric layers seen in their cross section may depend upon the plane of sectioning¹⁰. As a result, a 0.5 μm spherical unit may actually be bigger. Spherulites present in the basement membrane generally appeared embedded in an amorphous matrix (Figure 5 A,B), sometime associated with membrane bound vesicles (Figure 5 A) while those in the interstitium were surrounded by both collagen fibers and cellular degradation products, including membrane bound vesicles. Small vesicles approximately 200 nm across were seen with dark contents (Figure 6 A, B), which could be nucleating crystals. Grazing sections of the spherical units showed crystals sticking out of their surface (Figure 6C).

Large calcifications were located in the interstitium and surrounded by collagen fibers with characteristic banding patterns, as well as membranous cellular degradation products and an amorphous matrix (Figures 7 A-D). The deposits were comprised of dark elongated strands mixed with spherulites (Figure 7 B) and were apparently produced by their aggregation. Strands present on the periphery often showed banding patterns, highly correlated with the banding pattern of the nearby collagen fibers (Figure 7 D). In some sections the periphery of the deposits appeared nodular (Figure 7A).

Higher magnifications showed the center of the deposits to be more crystalline with radiating needle shaped crystals while the periphery appeared less crystalline.

Electron diffraction analyses (Figure 8 A, B, C) revealed that all of them had some hydroxyapatite diffraction rings. There were less intense rings at the periphery (Figure 8 B, C) compared to those taken at the center (Figure 8 A) indicating that peripheral hydroxyapatite was less crystalline than the central one. In addition, crystals when present were smaller on the periphery in comparison with those present in center of the deposits.

Discussion

Ever since Randall proposed his hypothesis that renal papillary subepithelial deposits act as the initiating lesion of idiopathic renal calculi ¹, a number of morphological studies of renal papillae obtained from kidneys of stone formers as well as non stone formers have been performed. Some of the earliest electron microscopic studies were performed by Cooke and associates ^{11, 12} and Haggitt and Pitcock ¹³. Haggitt and Pitcock examined kidneys from 100 randomly selected autopsies and performed electron microscopy on selected specimens. They found alizarin positive laminated spherules in the interstitium adjacent to the collecting ducts. TEM showed laminated spherical bodies in close association with collagen fibers in the interstitium as well as basement membrane of the collecting ducts. Cooke studied 62 normal kidneys and found calcification in 43. He reported that calcification was invariably located in the basement membrane of the loops of Henle from where it extended into the medullary interstitium. Later electron microscopic examination of nephrectomy specimens from three normocalcaemic, normotensive patients localized the mineral deposits in association with collagen mainly around the loops of Henle. Some collecting ducts and blood vessels were also involved.

In recent years, Stoller and associates as well as Evan and associates have meticulously investigated Randall's plaques. Stoller et al. performed high resolution radiography ¹⁴ of cadaveric kidneys and found that 57% of the kidneys had subepithelial Randall's plaques which extended deep within the papillae and were intimately associated with collecting ducts and vasa recta. von Kossa positive spherical CaP deposits were identified scattered in the interstitium as well as around the collecting ducts and blood vessels. They proposed a pathway for idiopathic stone formation involving the vascular system ¹⁵. Evan and associates performed exhaustive morphological studies of renal papillae from stone patients with a variety of causes ¹⁶ and concluded that all idiopathic calcium stones develop attached to the subepithelial Randall's plaques ¹⁷. They also confirmed the earlier observations of Cooke that Randall's plaques begin in the basement membrane of the loops of Henle. Osteopontin was identified in the crystal matrix interface while heavy chain of inter-alpha-inhibitor was localized in the crystal matrix itself. There was no evidence of cell injury, inflammation, interstitial fibrosis or intratubular crystal deposition in the renal biopsies from idiopathic stone formers. They hypothesized that deposits migrate from the basement membrane of the loops of Henle into the surrounding interstitium and become associated with type 1 collagen, fusing into a syncytium in which islands of mineral appear to float in an organic sea ^{16, 18}.

Our studies show CaP crystals as spherical units with radially arranged crystals, the hallmark of apatite crystals particularly in kidney stones ⁹. They range in size from half a micron to a few microns and are found loosely scattered around the tubules and in their basement membranes and then extending into the interstitium. Large interstitial deposits show both spherical units as well as long strands with distinct banding pattern suggestive of collagen fibers. Cellular degradation products including membrane bound vesicles were associated with the calcified deposits. Apparently CaP crystals start as small spherical units

which grow from less than a micron to a few microns in diameter and in the process aggregate to form larger deposits which grow by further addition of crystals on the periphery. During maturation the aggregated spherical units fuse with each other. As a result they lose their identity in the interior of the deposits while spherical units can still be seen on the periphery of the growing plaque. During the outward growth they come in contact with the collagen fibers and membranous degradation products which also calcify. Thus the extension of plaque into the interstitium and beyond is through outward growth by the addition of crystals on the periphery through aggregation and calcification of the collagen and membranous vesicles.

That calcification proceeds through heterogeneous nucleation of CaP crystals by membranes and collagen fibers is common in both physiological as well as ectopic conditions. Physiological calcification occurs primarily in extracellular matrices of bone, cartilage and teeth. Ectopic calcification takes place in soft tissues in response to injury and mineral imbalance¹⁹, the latter is most likely the cause of initial deposition around the basement membrane of the loops of Henle²⁰. It was originally thought that while physiological calcification was a highly regulated process, ectopic calcification was a passive process involving spontaneous precipitation of basic CaP in response to tissue injury and cell death. Recent investigations have however provided the evidence that ectopic calcification is also a regulated process and crystal deposition at various sites in the body is a result of an imbalance between forces that inhibit precipitation and those that promote it²¹. This is similar to what happens during stone formation²².

Both physiological and ectopic calcification start through heterogeneous nucleation of crystals by membranes lipids of the so-called matrix vesicles or similar entities, and propagate in a scaffolding of collagen fibers^{5, 23, 24}. We have provided evidence that cell membrane lipids may also be involved in kidney stone formation. We have demonstrated the presence of lipids and membranes in the matrices of CaOx kidney stones²⁵. Matrices of CaOx crystals induced *in vitro* in human urine also showed the presence of membranes and lipids.²⁶ Membrane vesicles isolated from the rat renal tubular brush border promoted the formation of CaOx crystals in a buffered metastable solution²⁶ and CaP in artificial urine²⁷.

Animal model studies have also shown an involvement of cell membranes in crystallization. Crystals of CaOx experimentally induced in rat kidneys were always seen in association with membrane fragments^{6, 28}. The female weanling rats given AIN-76 diet became hypercalciuric and produced intratubular concretions of CaP.^{28,29} Calcification started in association with calcium rich vesicles budding from the brush border of the tubular epithelium.

Conclusions

Results of our studies presented here provide additional information about Randall's plaques. In particular we demonstrate the structural similarities between the RPs and ectopic calcification. Stone formation as a form of ectopic calcification has previously been proposed^{19, 30}. Apparently Randall's plaques start as small, discrete spherical entities, perhaps as a result of the mineral imbalance, but grow by addition of more crystals through mineralization of membranous vesicles and collagen fibers. Confirmation of our findings requires further studies of biopsies from a larger number of well characterized patients with different types of stones.

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Key of Definitions

RP	Randall's Plaques
MV	Membrane Bound Vesicles
CaOx	Calcium Oxalate
EDX	Energy Dispersive X-ray Microanalyses
CaP	Calcium Phosphate
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy
H&E	Hematoxylin and Eosin
PAS	Periodic Acid Schiff
OPN	Psteopontin

Figure 1.

Intraoperative images of renal papillae. **A.** Randall's plaques appear cream colored spots on renal papillary surfaces. Stone analyses showed 80% CaOx dihydrate and rest CaP. **B.** Stone attached to the papillary surface. Analysis showed 10% CaOx monohydrate, 60% CaOx dihydrate and rest CaP)

Figure 2.

Scanning electron microscopy of the Randall's plaque. **A.** Randall's plaque appears as a protrusion on the papilla. Papillary surface epithelium (E) is sloughing exposing the sub-epithelial surface (SE). Dotted Bar = 100μm **B.** Higher magnification of an area in A showing surface epithelium (E), sub- epithelial surface (SE), as well as area of exposed fibrous layer (F) underneath. Dotted Bar = 33.3 μm **C.** Higher magnification of the fibrous layer. Dotted Bar = 20μm **D.** Spherulites of various diameters under the fibrous layer. Fibers as well as amorphous substances are also present. Dotted Bar = 15μm

Figure 3.

A. Higher magnification of the spherulites (SP) showing needle shaped crystallites on the surface as well as fibers (arrows) embedded therein. Dotted Bar = 4.29μm **B.** Surface of an internal layer of fused spehrulites. Dotted Bar = 30μm

Figure 4. Energy dispersive x-ray microanalysis of **A.** spherulites, and **B.** the fibers connecting them.

Figure 5. Transmission electron microscopic features of the Randall's plaque

A. A necrotic tubule is surrounded by laminated spherulites (arrows). They range in diameter from smaller than half a micron to approx 5 micron in diameter. Bar = 5 μ m **B.** Higher magnification of the spherulites with distinct concentric laminations and radial striations, present in an amorphous matrix under the renal tubular epithelium. Bar = 1 μ m

Figure 6.

Higher magnification showing vesicular structures in and around basement membrane of the renal papillary collecting ducts. **A.** Small (arrowheads) and large vesicles (arrows) are dispersed in the basement membrane. Bar = 500nm **B.** A membrane (arrow) bound vesicle. Dotted Bar = 200nm **C.** Basement membrane of a collecting duct showing spherulites in grazing section. Surface appears uneven with projecting crystallites (arrows, compare with SEM appearance of the surface in Figure 3A). Collagen fiber (C) with banding pattern is also visible. Bar = 500nm

Figure 7.

Transmission electron microscopic appearance of calcified deposits in the renal interstitium.

A. Edge of the deposit demonstrating fusing spherulites (arrows, compare with SEM appearance in Figure 3B). Some vesicular entities are also visible. Bar = 500nm **B.** Another area of large calcified deposit showing longitudinally running strands (ST). The deposit is surrounded by a matrix with collagen fibers (C). Bar = 5µm **C.** Fusion of spherical units (SP) of various dimensions with each other as well as calcified collagen produced a large calcified deposit surrounded by collagen fibers (C). Laminations can still be seen inside the peripheral spherulites (SP). Arrows point to the basement membrane of a collecting duct. Bar = 2µm **D.** Higher magnification of an area on the periphery of a large calcified deposit showing longitudinally running strands (ST). Notice the banding pattern (arrows) in the calcified strands. Compare it with the banding pattern in the nearby collagen fiber (C). Bar = 1µm

Figure 8. Selected area electron diffraction analyses at various sites on the calcified deposits showed different levels of crystallinity with **A** being mostly crystalline to **C** being near amorphous.

Table 1

24-hour urinary stone risk profiles and stone analyses for some of the patients investigated.

Stone Analyses	Calcium mg/day	Oxalate mg/day	Citrate mg/day	pH	SS CaOx	SS CaP	Cr 24/kg
100% COM	204	43	556	6.83	5.53	1.67	20
70% COD, 30% CaP	224	22	334	5.83	9.82	2.02	14.9
80% COD, 20% CaP	248	46	1136	6.63	6.4	1.81	15.9
100% COM	212	28	650	5.59	4.92	NA	NA
90% COM, 10% COD	332	29	557	6.14	8.0	3.12	24.6
100% COM	77	49	304	5.96	5.23	0.45	18.3
10% COM, 60% COD, 30% CaP	293	24.7	301	5.5	1.4	NA	NA