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IDIOPATHIC CALCIUM OXALATE NEPHROLITHIASIS: A CELLULAR DISEASE

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Abstract

Physico-chemical, metabolic and hormonal theories regarding the pathogenesis of calcium oxalate nephrolithiasis do not sufficiently explain many features of this disease. The recent findings of an abnormally faster oxalate self-exchange and higher phosphorylation of band 3 in erythrocytes of idiopathic calcium oxalate stone formers suggest the hypothesis that nephrolithiasis may be a cellular disease, characterized by a defect in the function of the anion-exchange. The cellular anomaly seems genetically controlled. Band 3 anion exchanger function seems to be biochemically regulated through modulation of band 3 phosphorylation, which depends on cyclic AMP- and phospholipid-sensitive Ca^{2+} independent protein kinases. In this light, a reduced glycosaminoglycan concentration in the erythrocyte membranes of stone formers might play a role, as these molecules exert a strong inhibitory effects on band 3 phosphorylation and anion transport *in vitro* and *in vivo*. An *in vivo* trial was performed in which stone formers were administered glycosaminoglycans orally. A reduction in oxalate excretion, and oxalate renal clearance, and a simultaneous correction of the abnormal RBC oxalate flux and band 3 phosphorylation were observed. These data suggest the existence of a link between the erythrocyte abnormality and oxalate transport by the kidney and gut.

Key Words: Nephrolithiasis, oxalate, glycosaminoglycans, band 3 protein, erythrocyte, band 3 phosphorylation, oxalate erythrocyte self-exchange, protein kinase, renal tubular acidification defect, urate erythrocyte self-exchange.

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Introduction

No less than 76% of all kidney stones are made up of calcium oxalate (CaOx), and in 80% of the cases we are dealing with idiopathic forms of nephrolithiasis (Nordin *et al.*, 1979). The physico-chemical theory of lithogenesis, explains stone formation by the precipitation, growth and crystalline aggregation of several lithogenic salts in the urine, and has contributed greatly to the understanding of the complex problem of the etiology and pathogenesis of calcium urolithiasis (Fleisch, 1978). Calcium oxalate and calcium phosphate are potentially the most insoluble lithogenic salts under the ionic conditions normally present in urine; indeed, their supersaturation level even in the urine of non-stone forming subjects is very close to the point of spontaneous precipitation.

The risk of forming CaOx stones is determined by the degree of calcium salt supersaturation, and the activity level of factors stabilizing (inhibitors) and destabilizing (promoters) their nucleation, crystal-growth and crystal-aggregation (Robertson *et al.*, 1978). Idiopathic calcium nephrolithiasis (ICN) is currently interpreted as the consequence of an imbalance between these factors (Robertson *et al.*, 1978; Baggio *et al.*, 1982).

The principal determinants of urine calcium salt saturation are urine pH, calcium and oxalate excretion, and diuresis. Most studies addressed the role of calcium ion in promoting ICN. In the last decade, however, the pathogenetic role of oxalate has been reevaluated in the light of physico-chemical considerations and clinical findings (see for a review Smith, 1988). Indeed minimal changes in urinary oxalate concentration produced increase in CaOx supersaturation than equal variations in calcium concentration (Finlayson, 1974). Moreover, severity of disease and entity of crystalluria were well correlated with oxaluria, rather than calciuria (Robertson and Peacock, 1980).

While urine supersaturation clearly is necessary for stone formation, it does not seem the sole prerequisite, as urine supersaturation by the main lithogenic salts is frequently present even in non-stone forming subjects

(Robertson *et al.*, 1968). These results led to the concept that there exist substances in urine that can inhibit crystal nucleation, growth and aggregation; citrate, pyrophosphate, magnesium, glycoproteins and glycosaminoglycans (GAGs) seem endowed with such activity; in reference to GAGs, an inhibitory effect on CaOx crystal growth *in vitro* was proven (Kok *et al.*, 1988; Gjaldbaek 1982), and an excretion deficit in stone formers was documented (Robertson *et al.*, 1978; Baggio *et al.*, 1982; Caudarella *et al.*, 1983; Martelli *et al.*, 1985; Baggio *et al.*, 1987a; Nikkilä, 1989; Michelacci *et al.*, 1989), although not unanimously (Samuell, 1981; Hesse *et al.*, 1986; Fellström *et al.*, 1986; Hwang *et al.*, 1988). Moreover in nephrolithiasis, not only a quantitative, but also a qualitative defect exists, because the degree of GAG sulfation is higher than normal (Foye *et al.*, 1976; Fellström *et al.*, 1986).

The causes that may lead to an imbalance between saturation and urinary inhibition might reside in systemic, metabolic, or renal anomalies. The latter are certainly very important in the idiopathic forms of nephrolithiasis; in fact, primary tubular loss of calcium (Pak, 1979), reduced urinary citrate excretion due in part to partial defect in renal acidification (Minisola *et al.*, 1989), and altered excretion of renal tubular epithelium constituents, such as renal enzymes (Baggio *et al.*, 1983a), glycosaminoglycans, and Thamm-Horsfall glycoprotein (Gambaro *et al.*, 1984) have been often reported. Several studies addressed whether the tubular anomalies were primitive and therefore pathogenetically important, or only a consequence of stone formation, but no firm conclusion was reached (Jaeger *et al.*, 1986); nonetheless, there is evidence that at least some anomalies are primitive in nature.

From this brief digression, it appears evident that ICN has been viewed above all as a physico-chemical problem, or a metabolic and hormonal disorder. While studies based on these approaches have been of great importance, several aspects are still unexplained, leading to the recent concept that cellular anomalies might be determinant in ICN pathogenesis (Baggio *et al.*, 1984a, 1986a).

In this review, we will discuss findings in support of this theory, and their possible clinical and pharmacological applications.

The Theory of Cellular Anomalies as the Pathogenetic Factor

In developing a cellular hypothesis of this disease, we considered that the co-existence of familial occurrence, tubular disease associated with ICN, and anomalies in the systemic handling of oxalate might suggest the presence of a generalized cellular defect. Other obser-

vations buttressed this idea, in particular, the change in the epidemiology and clinical pattern of urinary stone disease (Robertson and Peacock, 1982), and the obvious impact of environment, as seen in hypertension, in which a possible primary cellular alteration is highly suspected; moreover, further clinical and physico-chemical observations of the effect of even small increase in urinary oxalate concentration on the lithogenetic process.

Alterations in Cellular Transport of Oxalate

The study of oxalate self-exchange through the erythrocyte membrane under conditions of equilibrium supported the working hypothesis. Anomalous transmembrane flux in red blood cells (RBC) was present in 70-80% of the subjects studied (Baggio *et al.*, 1986a). Other workers corroborated this finding (Jenkins *et al.*, 1988; Narula *et al.*, 1988). As this disorder is not present in secondary forms of calcium nephrolithiasis, it seems characteristic of ICN, and moreover, genetically controlled, with a mendelian-type segregation. The simplest explanation is that the increased erythrocyte flux has the features of an autosomal, monogenic dominant trait with complete penetration, and variable expression. However, a polygenic inheritance with frequent and linked genes, and two different thresholds (one for increased flux, and the other for nephrolithiasis) cannot be ruled out.

In the families we studied, this anomaly was also present in non stone-forming subjects. Moreover nephrolithiasis, which was detected only in subjects with the anomaly, appeared more frequently in males, and only after the second decade of life. These observations further stressed the impact of environmental (diet, etc.) and sex-linked factors.

We then tried to clarify the relationships between altered cellular oxalate flux and ICN; that is, whether we were dealing with a pathogenetic or a casual association, and if the anomaly could constitute a risk marker for ICN. Follow-up studies in five families revealed five new cases after five years of observation, and these occurred only in subjects with the cellular anomaly (unpublished). This finding suggested that this cellular trait constitutes a risk marker of the disease.

The use of erythrocyte anion channel-inhibiting substances showed that the anomalous oxalate transport was related to an alteration in band 3 cell protein function (Baggio *et al.*, 1984b). Proteins immunologically correlated with band 3 red cell protein were also detected in the gut and kidney. Moreover, it was demonstrated that band 3 renal protein in the basolateral membrane of intercalated alpha cells of the collecting duct is encoded by the same gene that encodes band 3 erythrocyte protein (Demuth *et al.*, 1986; Brosius *et al.*, 1989; Kudricki and

Shull, 1989). These cells are deputized to proton secretion (Steinmetz, 1986), and band 3 protein has a very important role due to its anion transporting function, particularly chloride and bicarbonate (Steinmetz, 1986).

On the basis of these biological considerations, we investigated the presence of functional alterations in the gut and kidney in subjects with the red cell anomaly. We found that a more rapid intestinal oxalate absorption (Baggio *et al.*, 1986a) and abnormalities in distal acidification (Gambaro *et al.*, 1988) were associated with increased erythrocytic flux. The former naturally may translate into urinary oxalate excretory peaks, and hence into peaks of urinary supersaturation for calcium oxalate; the latter have been repeatedly observed in ICN, and are generally considered a consequence of the nephropathy (Jaeger *et al.*, 1986), despite their possible pathogenetic role in calcium-phosphate lithiasis, and definite causal role in overt type 1 renal tubular acidosis. However, low distal tubular proton secretion, as seen in this type of acidification defect, opposes our theory of a hyperfunctioning anion carrier because, if the same RBC defect is also present in alpha intercalated cells, proton secretion would be favored, giving rise to more acidic urine. However, studies in this sense in ICN patients without acidification defects, showed that bicarbonate infusion caused a significantly higher urine to blood PCO_2 gradient in subjects with higher RBC oxalate self-exchange, compared to those with normal oxalate flux. We reasoned that if the anion exchanger were hyperactive, it would have been more sensitive to peritubular bicarbonate concentration, one of the main factors controlling the activity of the basolateral anion exchanger of alpha intercalated cells (Breyer *et al.*, 1986). These two apparently contradictory results are still puzzling us. Although further study is mandatory, some very preliminary data suggest a redundant proton secretion, at least by some CaOx stone formers. In these patients, the production of a more acidic urine after an acid load could facilitate CaOx stone formation. In other words, therefore, not only an incomplete form of distal renal tubular acidosis might exist in some stone formers, but also a higher than normal proton excretion in another subset of patients.

Alterations in the Red Blood Cell Anion Carrier

The biochemical basis of the anomalous erythrocyte oxalate flux might reside in an altered phosphorylation state of the membrane proteins, and in particular band 3, which is the known anion carrier in red blood cells (Cabantchik *et al.*, 1978). Compared to controls, in fact, phosphate incorporation is greater in the erythrocytic membranes of stone formers (Baggio *et al.*, 1986b, 1987b).

The hypothesis of a causal link between the two cellular abnormalities is suggestive, and supported by evidence that drugs, such as stilbenes, which bind covalently with the protein and inhibit its phosphorylation, in a parallel manner also reduce cell transport of oxalate (Baggio *et al.*, 1984b), as well as the observation that both anomalies seem dependent upon the intracellular energy level, as ATP depletion induces a reduction in both oxalate flux and band 3 phosphorylation (Baggio *et al.*, 1986b). However, it is still debated whether anion transport modulation involves phosphorylation of band 3, and other membrane proteins (Bursaux *et al.*, 1984; Clari *et al.*, 1990; Yannoukakos *et al.*, 1991). Preliminary results from our laboratory evidenced a close relationship between the level of band 3 phosphorylation and its function as an anion exchanger. In fact, anion transport was unmodified by agents mediating cyclic AMP-, or phospholipid-sensitive Ca^{2+} -dependent protein kinases that are responsible for phosphorylation of some red cell membrane proteins, other than band 3 (Raval and Allan, 1985). This is the case in adenylylase activation by forskolin, and in endogenous diacylglycerol increase following RBC incubation with phospholipase C and Ca^{2+} , as well as the activation of protein kinase C by phorbol myristate acetate or short incubation with oleil-2-acetyl-rac-glycerol (OAG). On the other hand, agents able to modify the phosphorylation state of the erythrocyte anion carrier, such as low molecular weight heparin or longer incubation with OAG, also induce changes in oxalate transmembrane self-exchange. These findings also suggest that cyclic AMP- and phospholipid-sensitive Ca^{2+} -independent protein kinases are critical modulators of band 3 function. Thus, our current views on the possible mechanisms involved in the origin of the erythrocytic anomalies associated with ICN suggest a primary structural defect in band 3 protein might be proposed, even though it is extremely unlikely that a primitive defect involves simultaneously two proteins, band 3 and 2 which are both abnormally phosphorylated in stone formers (Baggio *et al.*, 1986b). However, we are addressing this aspect by evaluating the possible existence of a linkage between the increased RBC oxalate flux and chromosome 17, where band 3 gene is located (Lux *et al.*, 1989). Our first attempt failed because the large family we studied was not sufficiently informative in reference to the probe employed.

The hypothesis of an imbalance between AMP- and Ca^{2+} -insensitive protein kinases, and phosphatases seems more interesting, and in this way it might be more easily explained the link between RBC band 3 anomalies, and the oxalate carriers in the proximal tubule and in gut that have only structural and functional homologies with band 3 protein, and are encoded by different genes (Alper *et al.*, 1987). Despite their differ-

ences, these anion/oxalate carriers might depend upon the same regulatory mechanisms, thus explaining parallel derangements in their function. The putative imbalance in kinase activities might involve both the enzymes themselves, or modulating effectors of their activities. This idea is supported by our finding of abnormally low GAG level in erythrocytes from stone formers (Baggio *et al.*, 1990), as these molecules exert inhibitory effects on band 3 phosphorylation and anion transport *in vitro* and *in vivo* (see below) (Baggio *et al.*, 1988, 1990, 1991a, 1991b).

Thus, the possibility exists that transport of other ions also dependent upon the same mechanism would be abnormal. Indeed, we recently observed a higher urate self-exchange in ICN patients (Gambaro *et al.*, 1990), even though inconstantly associated with the faster oxalate transport, so raising the possibility that exchange is specific for a certain ion, at least at some step of the transport and/or its regulatory mechanism. Alternatively, a different hierarchy of affinity for carrier between anions could explain these results.

Clinical and Pharmacological Considerations

That some cell alterations may be considered a possible primary metabolic defect in ICN opens new perspectives for defining the risk of nephrolithiasis, and preventing recurrences pharmacologically. Concerning the former, risk markers for nephrolithiasis are not yet available at present. In fact, the anomalies associated with this disease (hypercalciuria, hyperoxaluria, low urinary citrate levels, defect in crystal agglomeration inhibiting activity in urine, etc.) have been proposed as risk markers only on the basis of case-control investigations between stone-formers and non-stone-formers, without being tested in prospective studies to verify their predictivity of the disease. On the other hand, based on our family follow-up study (see above), increased red blood cell oxalate flux might be a means to detect subjects at risk of ICN. This would make it possible to establish dietary or other types of regimes in these subjects, in order to prevent the disease from acting on other non-genetic risk factors. However, as the impact of environment on ICN and its possible relationship with the cellular anomaly is not yet sufficiently understood, further study is needed before this type of application can be suggested.

In reference to pharmacologic prophylaxis, drugs able to modulate the observed cellular abnormalities could modify urinary physico-chemical conditions, and hence the natural history of the disease. Several molecules have been studied in this sense, and findings were favorable regarding cellular activity, and effect on urinary physico-chemical balance in many cases. Nonethe-

less, how they would influence the natural history of nephrolithiasis is not known, as long-term prospective studies are not yet available.

Drugs capable of favorably modifying the natural history of the disease, such as hydrochlorothiazide (Maschio *et al.*, 1982), are undoubtedly able to correct oxalate self-exchange in red blood cells (Baggio *et al.*, 1986a), and change band 3 protein phosphorylation kinetics (Baggio *et al.*, 1987b). Other drugs, such as amiloride (Baggio *et al.*, 1987c; Clari *et al.*, 1986) and the calcium antagonists (Baggio *et al.*, 1986c; Gambaro *et al.*, 1987) were able to normalize erythrocyte oxalate flux, and the latter also induced a change in the urinary excretion of calcium.

Glycosaminoglycans are also able to influence red cell oxalate flux (Baggio *et al.*, 1988; 1991a), and probably exert this effect through their powerful modulating action on membrane kinases (Hathaway *et al.*, 1980; Lecomte and Boivin, 1981; Meggio *et al.*, 1982; Boivin *et al.*, 1985), which are deputized to the phosphorylation of the anion carrier.

Glycosaminoglycans are considered important inhibitors of lithogenesis; their urinary excretion is frequently reduced in ICN patients; a decreased erythrocyte GAG content has also been reported in ICN patients (Baggio *et al.*, 1990), and their synthesis in the fibroblasts of stone-forming subjects seems reduced (Caudarella *et al.*, 1987). Since it is possible that an anomaly which is present in one cellular line might also be present in other cell compartments, the pathogenetic role of glycosaminoglycans in ICN might be more complex than previously thought.

In vitro, these drugs and in particular the low molecular weight heparins, heparan sulfate and dermatan sulfate, albeit with different efficacy, are able to inhibit band 3 protein phosphorylation, as well as red blood cell oxalate flux at concentration of 2.5 $\mu\text{g/ml}$ (Baggio *et al.*, 1988), which is much lower than that needed to inhibit protein kinase C (200 $\mu\text{g/l}$) (Wise *et al.*, 1982). Our findings are consistent with data from Hathaway *et al.* (1980), Lecomte and Boivin (1981), and Clari and Ferrari (1983) demonstrating that casein kinase is inhibited by heparin at concentrations of a few $\mu\text{g/l}$ or ng/l , which are much lower than that needed for protein kinase C. This was one more reason to exclude the protein kinase C-dependent pathway from the pathogenesis of the cellular abnormality observed in nephrolithiasis.

To explore the possible clinical application of these drugs in the prevention of stone relapses, and also to obtain deeper insights into the pathogenesis of ICN, as well as establish a definitive pathogenetic link between the RBC oxalate anomaly and nephrolithiasis, we searched for a link between the erythrocyte abnormality and oxalate transport by the kidney and gut.

Our rationale was that if we were able to correct the RBC oxalate anomaly *in vivo*, oxalate transporters in the kidney and in gut might respond similarly, leading to changes in urinary excretion and clearance of oxalate, since these carriers have molecular and functional similarities with erythrocyte band 3 (Alper *et al.*, 1987). GAGs seemed a good candidate to verify this hypothesis, as they are able to correct both oxalate self-exchange and band 3 phosphorylation in erythrocytes.

For clinical use, oral administration is, of course, preferable, but this route for GAGs is currently still considered unreliable; it is generally thought that they are catabolized at the intestinal epithelial level to monosaccharides devoid of any pharmacological activity. This is not true; Larsen *et al.* (1986) demonstrated the presence of mainly oligo-, di- and mono-saccharides, and to a minor extent octa- and tetra-saccharides in the blood following a single oral dose of ³⁵S-heparin or ³H-heparin. At the same time an anti-factor Xa appeared, probably due to the larger heparin fragments. These workers also reported that oral administration of heparin in the presence of cortisone induced a systemic anti-angiogenic effect (Folkman *et al.*, 1983). Norman *et al.* (1985) found that the oral administration of a synthetic GAG, sodium pentosan polysulphate, was followed by a slight but significant increase in the negative zeta potential in urine, a measure of the polyanionic inhibitory activity of crystallization. Intestinal absorption of intact or large fragments of heparin and dermatan sulphate was recently demonstrated (Jaques *et al.*, 1991, Dawes *et al.*, 1989).

We employed a mixture of GAGs extracted from animals approved for human use in Italy, which is composed of two GAGs that are highly active *in vitro* on RBC abnormalities (70% a heparin-like fraction, and 30% dermatan sulphate). Pharmacokinetic studies disclosed that almost 50% of this drug is absorbed after oral administration, and 25% is excreted unmodified by the kidney (unpublished). In view of these findings, we may estimate that at least 15 mg/day of the drug are absorbed unaltered through the intestinal epithelium (daily dose 60 mg time 25%). If the distribution of this heparinoid is in the vascular space alone (approximately 5 l), its plasma concentration would be about 3 mg/l which, based on our *in vitro* data is certainly sufficient to inhibit RBC oxalate flux up to 55%, and RBC band 3 phosphorylation up to 30%. Indeed, following oral administration, this drug was effective on RBC anomalies (Baggio *et al.*, 1991a) and at the same time exerted a hypooxaluric action (Baggio *et al.*, 1991b).

Although this drug, following intra-venous administration, was able to reduce renal oxalate clearance, its hypooxaluric effect under steady-state conditions, such as those of the 15 day trial, must also be necessarily due

to intestinal inhibition of dietary oxalate absorption (Baggio *et al.*, 1991b). As glycosaminoglycans do not bind oxalate (Baggio *et al.*, 1983b) we believe they act at the intestinal level with a mechanism similar to that most probably acting on the proximal tubular epithelium, that is by modifying the phosphorylation of band 3-related transporters and, therefore, cellular oxalate flow. It cannot be ruled out that the hypooxaluric effect may be explained by a reduction in endogenous oxalate synthesis, or oxalate precipitation in the body of oxalate as a calcium salt, but these hypotheses seem unlikely.

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Discussion with Reviewers

W.G. Robertson: If thiazides correct the oxalate transport excess in the red cells from stone formers, why do they not reduce urinary oxalate excretion, since this would be anticipated from the authors' model?

Authors: Our data show no relationship between 24 hour urinary oxalate and the flux rate constant of the oxalate self-exchange in RBC. It seems that the difference between ICN patients with increased or normal oxalate flux lies in a different time course of oxaluria after oral challenge with oxalate. In fact, 8 hours after challenge, oxalate recovery from the urine is the same regardless of the presence or not of the RBC abnormality; however, subjects with increased RBC oxalate self-exchange displayed a faster excretion of oxalate in the first 2-4 hours (Baggio *et al.*, 1986a). It is true that thiazides were never shown to reduce urinary oxalate excretion, but we wonder if they cannot modify the time course of oxaluria after meals.

B. Hesse: To what extent did urinary oxalate excretion decrease during treatment with GAGs, and was the decrease significantly correlated with reduced erythrocyte oxalate self-exchange and band 3 protein phosphorylation?

Authors: 15 day oral treatment with GAGs promoted a reduction in urinary oxalate of 25%, in erythrocyte oxalate self-exchange of 35%, and in band 3 phosphorylation of 24% (Baggio *et al.*, 1991b). The three variables were not statistically correlated.

Reviewer V: Have GAGs decreased stone formation in patients with ICN?

Authors: To our knowledge there is only one open clinical study in which the effect of GAGs on stone relapses was evaluated. Danielson *et al.* (1988) reported that in 96 frequent relapsing stone forming patients (some of whom had enteric disorders), pentosan polysulphate, 400 mg/daily, decreased the disease activity during a mean treatment time of 22 months. Pentosan polysulphate, a synthetic GAG, was orally administered at a very high dosage capable of increasing the inhibition of calcium oxalate crystal growth in urine. No effect was observed on oxalate excretion. We have no experience with this drug on RBC oxalate self-exchange, and unfortunately it is not possible to match this drug with the animal extractive GAG used in our study due to their different chemical structure, and to the different bioavailability (pentosan polysulphate has a very low oral bioavailability, 0.5-1%).

M. Akbarieh: The authors suggested that red blood cell anomaly is a risk marker of ICN. How can the authors explain this hypothesis, and how it might be tested?

Authors: When we published our family studies on the genetics of the RBC anomaly in 1986, we observed that not all the subjects presenting the anomaly had passed stones (Baggio *et al.*, 1986a). After a 5 year follow-up, 5 of those subjects became stone-formers. It is remarkable that only those with the RBC anomaly formed urinary stones; we believe that this is a clear demonstration that abnormal oxalate self-exchange in erythrocytes is a marker of the risk to become a stone-former. Of course, to strengthen this conviction, more families must be studied with a longer follow-up, and more insights into the physiopathological meaning of the cellular anomaly in terms of nephrolithiasis must be gained.