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ORIGINAL PAPER

Induction of apoptosis with cisplatin enhances calcium oxalate crystal adherence to inner medullary collecting duct cells

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Abstract Attachment of stone crystals to tubular epithelium may initiate kidney stone formation. We previously reported that apical nucleolin related protein (NRP) expression during mitosis enhance attachment of Ca oxalate monohydrate crystals (COM). Some forms of injury may also increase affinity for crystals. We examined changes in subcellular localization of NRP during the course of cisplatin-induced apoptosis in cultured inner medullary collecting duct cells. Caspase-3 activation and chromatin condensation followed by nuclear fragmentation occurred after 20 h exposure to cisplatin, indicating the development of apoptosis. Cells were fixed without permeabilization and stained for surface NRP. Cells with condensed chromatin showed little or no cytoplasmic or apical NRP. Those at an early stage of nuclear fragmentation had cytoplasmic but not apical NRP and cells with advanced nuclear fragmentation were positively stained for apical NRP. Membrane proteins isolated by apical biotinylation and precipitated with avidin were analyzed by Western blot. Apical NRP was markedly increased after cisplatin compared to control, while expression of the apical marker, GP-135, and other putative attachment protein were unchanged. Hyaluronic acid was decreased. Cultures with apoptotic cells demonstrated increased adherence of COM that was inhibited by the polyanion (poly)aspartic acid. We conclude that pre-existing apoptotic injury may promote calcium oxalate crystals attachment to renal tubular epithelium via apical NRP expression.

Keywords Calcium oxalate · Apoptosis · Nucleolin · Nephrolithiasis · Inner medullary collecting duct · Caspase

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Introduction

We as well as others have proposed that attachment of Ca oxalate crystals to renal epithelium is required for stones to develop [1–3]. These investigators have demonstrated specific attachment of stone crystals to cultured renal tubule cells. The attachment of crystals can be mediated by several membrane-associated molecules: phosphatidyl serine, hyaluronan, annexin II, NRP, and possibly, osteopontin [4–8].

Nucleolin related protein (NRP), in particular, is a surface-associated protein of cultured inner medullary collecting duct (IMCD) cells that selectively adsorbs to COM and binds to membrane skeletal elements in a Ca-dependent fashion [9]. NRP is highly homologous to rat nucleolin and demonstrates the same arrangement of peptide domains. Both forms are recognized by anti-nucleolin antibody preparations. Nucleolin itself is a major nucleolar protein, highly expressed in exponentially growing cells, and can be translocated between the cell cytoplasm and nucleus [10]. There are reports of cell surface expression in addition to ours [11–19]. NRP has a highly acidic region (asp + glu = 62% of residues) that has been shown to be capable of mediating COM attachment [7].

Undisturbed, well-formed cell monolayers and, presumably, cells in vivo, are quite resistant to crystal adherence [20, 21]. We have ascribed the resistance to

attachment to the confinement of molecules with crystal-binding properties to domains other than the apical membrane. When tight junctions between cells are disrupted in the course of cellular proliferation or migration or when cells are exposed to a number of different agents, apical membranes seem to express constituents with affinity for COM [7, 20].

NRP is found on both apical and basolateral cell membranes of cultured IMCD cells but expression declines with increasing organization of the monolayer. However, cells induced to divide to cover defects in well-organized monolayers show increased NRP expression and a greater affinity for COM [7]. Based on these and other studies, it has been proposed that certain types of injury or other perturbations of cells result in enhanced adherence of COM through increased surface expression of molecules that can mediate attachment. Along with increased cell division, already demonstrated to enhance crystal attachment, injury to cells also may result in apoptosis. The purpose of the current study was to determine whether apoptosis induced by cisplatin is associated with enhanced surface expression of NRP and whether such expression leads to enhanced COM attachment.

Methods

Cell culture and cisplatin treatment

Inner medullary collecting duct (IMCD) cells were cultured as described previously [4]. Exposure to cisplatin was for 20 h at the indicated concentrations.

Immunofluorescence

For these studies, cells were grown on 24-mm^2 coverslips and fixed with freshly prepared 3.7% paraformaldehyde in phosphate buffered saline (PBS). Then the cells were washed and rendered permeable with 0.1% Triton X-100, blocked with 2% bovine serum albumin (BSA) in PBS, incubated with a polyclonal antiserum against cleaved caspase-3 at a dilution of 1:400 and rhodamine-labeled secondary antibody. Cultures stained for NRP were not rendered permeable but were incubated with anti-human NRP monoclonal antibody (10 μg/ml) followed by rhodamine-conjugated goat anti-mouse antibody (Chemicon International, Temecula, CA, USA). Cells stained for activated caspase, or surface NRP were also stained with the nuclear dye DAPI, 2 μg/ml for 10 min. The coverslips were mounted on microscope slides and examined by epifluorescence microscopy using individual filters for DAPI (blue) and activated caspase (red) or DAPI and NRP (red).

Confocal microscopy

Cells that were examined using confocal microscopy were fixed with 3.7% paraformaldehyde and incubated with primary antibody as described above. After washing with PBS, the cells incubated with antibody are exposed to secondary antibody in PBS that contained 2% BSA for 1 h, washed again, and then incubated with goat anti-rabbit antiserum conjugated with fluorescein diluted 1:250 in BSA/PBS for 1 h. The cells were washed again and stained with wheat germ agglutinin (WGA) conjugated with rhodamine (Sigma, St. Louis, MO, USA) at a concentration of 10 μg/ml for 30 min. Control experiments omitting the primary antibody did not show any staining. Immunofluorescence was observed by confocal microscopy using the Leica TCS SP2 system (Leica Microsystems, Heidelberg, Germany).

Surface biotinylation and precipitation of membrane proteins

Cultured IMCD cells were grown on plastic until confluent. Sulfo-NHS-SS-biotin (Pierce Biotechnology, Rockford, IL, USA) was used to biotinylate apical proteins by exposure at a concentration of 0.5 mg/ml in PBS containing 1 mM of both $CaCl_2$ and 1 mM $MgCl_2$) for 30 min. Unbound biotin was washed out with PBS that contained 0.1 M glycine. Then the cells were lysed as described previously, and the lysates were incubated with NeutrAvidin + (Pierce Biotechnology) for 1 h at 4°C and washed five times with lysis buffer [9]. Precipitated proteins were released from the beads by boiling in sample buffer in the presence of 50 mM dithiothreitol. Western blotting of lysates and NeutrAvidin + precipitates was performed using a monoclonal anti-nucleolin antibody (clone 4E2; Medical and Biologic Laboratories, Naka-ku, Nagoya, Japan), as well as polyclonal antisera against osteopontin [22], activated caspase 3 (Cell Signaling Technology, Beverly, MA, USA), annexin II (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti–GP-135 (gift of J. H. Schwartz, Boston University Medical School).

Hyaluronic acid assay

Inner medullary collecting duct (IMCD) cells (passage 24) were grown in six plastic flasks and were divided randomly into two groups of three flasks. Three flasks served as controls and three were incubated with cisplatin for 20 h at a concentration of 25 μM. At the end of the incubation period, all media were collected from control and cisplatin-treated cells, and then the cells were lysed as described previously [9]. The supernatant fraction of the lysate was recovered post centrifugation (23,000g × 10 min × 4°C)

and both it and samples of the medium were concentrated by ultrafiltration using a 10 kDa cutoff filter (Amicon Ultra 4, Millipore). Hyaluronic acid in cell lysate supernatants and media were measured using a commercial kit (Corgenix, Inc., Broomfield, CO, USA) employing hyaluronic acid binding protein. The reactions were performed in a microtitration plate and the optical density at 450 nm was read in a plate reader. The values were normalized for protein content with a coomassie blue-based protein assay using BSA as a standard (Bio-Rad Laboratories, Hercules, CA, USA).

CaOx monohydrate crystal attachment

Crystals were formed by slow mixing of solutions of 10 mM $CaCl_2$ and 10 mM Na oxalate containing [^{14}C] oxalate, followed by continuous mixing of the crystal suspension for 1 week at 6°C. Then crystals were separated by centrifugation, washed with water and then methanol, and, finally, dried in air at 95°C for 1 h [23].

To determine CaOx crystal attachment to cultured IMCD, cells were seeded at uniform density and grown to confluence on glass cover slips. The cover slips were washed twice with artificial urine (AU) [24] and incubated for 20 min in AU containing 0.48 mg/ml of well-dispersed ^{14}C-COM crystals (0.013 μCi/ml) at 37°C. After incubation, the cover slips were affixed to glass microscope slides with petroleum jelly and placed individually in slowly stirred AU at 37°C for 1 min. The cover slips were then transferred to scintillation vials containing 0.5 ml of 6 N HCl to dissolve the cells; then scintillation fluid was added and radioactivity counted. In each individual experiment, 10 cover slips of cisplatin-treated cells were used, and 10 cover slips of cells exposed to vehicle (dimethylsulfoxide) from the same set of cultures served as controls.

StatMost (DataMost, Salt Lake City, UT, USA) was used to compare the difference between different conditioned media in the same cell line. Both one-way ANOVA and an unpaired *t* test were used to confirm the conclusions.

Results

Apoptosis in cultured IMCD cells

Exposure of cultured IMCD cells to cisplatin induced apoptosis. This is demonstrated by the development of nuclear fragmentation and staining for activated caspase in the cells. Figure 1a shows a representative field of IMCD cells showing cells in varying stages of apoptosis, as revealed by staining of nuclei using DAPI and activated caspase.

Surface expression of NRP in apoptotic IMCD cells

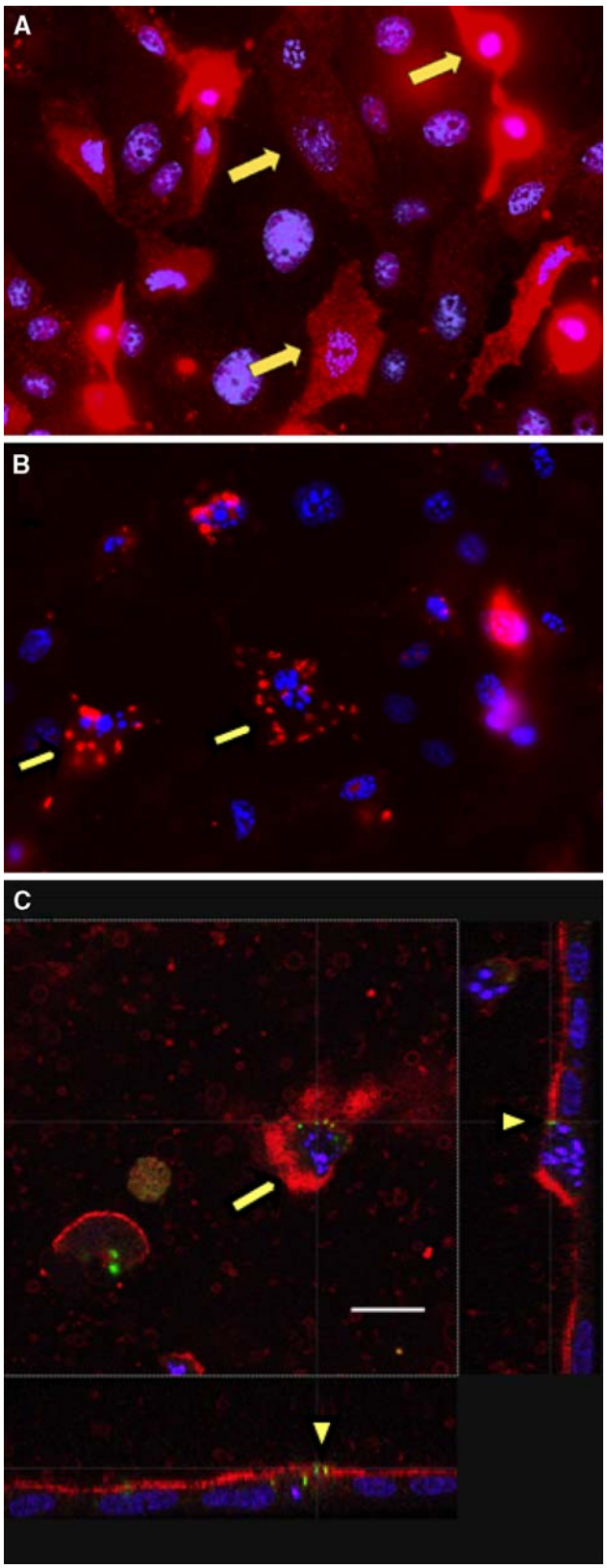
Apoptosis induced by cisplatin in IMCD cells was associated with surface expression of NRP. This is shown in Fig. 1b, which is the overlay of the image of the DAPI staining in a field of cisplatin-treated IMCD cells and the immunofluorescent signal for NRP in the cells prior to permeabilization. Figure 1c shows a confocal image of an apoptotic cell demonstrating colocalization of the surface marker WGA and the immunofluorescent signal for NRP.

To verify that the increased NRP is expressed in the apical membrane, surface biotinylation was performed and the precipitated proteins stained for GP-135, an apical membrane marker, as well as NRP. Figure 2a shows a Western blot demonstrating increased apical expression of NRP, with less being present among the biotinylated proteins at a lower cisplatin concentration and a greater amount with a higher concentration.

Precipitated biotinylated apical proteins were probed for expression of other putative attachment molecules. Surface expression osteopontin and of annexin II were not altered in the cultures exposed to cisplatin (Fig. 2b). Hyaluronic acid, another molecule that has been shown to mediate attachment of crystals to cells, was measure in medium and cell lysates from cisplatin-treated cells. Compared with controls, treatment with cisplatin was associated with a decreased content of hyaluronic acid. In lysates, the control value was 123 ± 31 ng/mg protein; in the supernatant, the value was $1{,}539 \pm 93$ ng/mg protein. In the cisplatin treated cells the values were lower; 9 ± 2 and 468 ± 74 ng/mg protein, respectively (both significantly different from controls at the $P < 0.05$ level).

Enhanced COM crystal attachment in apoptotic IMCD cells

Cultured IMCD in whom apoptosis had been induced by cisplatin demonstrated enhanced COM attachment. Figure 3 reports the results of exposure of cells to two different concentrations of the agent, engendering different concentration dependent degrees of apoptosis as revealed by the amounts of activated caspase present in cell lysates. A similar concentration dependent enhancement of COM attachment was observed. To examine the possibility that exposure of phosphatidyl serine occurring during the course of apoptosis could be mediating enhanced crystal attachment, the effect of Annexin V on cisplatin-mediated crystal attachment was studied. This substance has been shown to block the enhanced attachment mediated by enriching cells with phosphatidylserine-containing liposomes [4]. As shown in Fig. 4, Annexin V was ineffective at blocking the enhanced crystal attachment associated with cisplatin-induced apoptosis. This suggests that the



◄ **Fig. 1** Cisplatin induces apoptosis in cultured IMCD cells. IMCD cells were exposed to 25 μM cisplatin for 20 h. **a** Cells were permeabilized with Triton X-100, fixed with paraformaldehyde and reacted with an antiserum against activated caspase, using a secondary antibody conjugated with TRIC. The nuclei were also stained with DAPI, as described in the text. The cells were viewed and photographed with filters producing a *blue* color for the DAPI and *red* for the activated caspase fluorescent signals. *Arrows* indicate cells in various stages of apoptosis; early nuclear condensation in the center, more advanced nuclear condensation at the bottom, and fully developed apoptotic bodies with nuclear collapse at the top. **b** IMCD cells were exposed to cisplatin, except that they were not permeabilized prior to fixation and they were reacted with a monoclonal antibody against human nucleolin, and then rhodamine-conjugated secondary antibody. As shown by *arrows*, cells with fragmented nuclei indicating apoptosis, also demonstrate a signal for nucleolin, *arrows*. *Bar* 16 μm. **c** Confocal microscopy of surface expressed nucleolin. Cultures exposed to cisplatin were washed without permeabilization, fixed, reacted with anti-nucleolin antibody, and then a fluoresceinated secondary antibody. Cells were stained with Texas Red-conjugated wheat germ agglutinin and observed by confocal microscopy. Apoptotic cells demonstrate fragmented nuclei (*arrow*). The cell surface is indicated by the *red signal*, indicating the cell surface stained by wheat germ agglutinin. Surface localized nucleolin is indicated by the *green* signal in the Y–X and Y–Z sections (*arrowheads*). *Bar* 10 μm

presumably enhanced surface phosphatidylserine occurring with apoptosis does not, in this setting, mediate the increased COM attachment observed.

We have proposed that increased surface expression of NRP causes enhancement of COM attachment by providing increased numbers of membrane-associated anionic sites [7]. To test this, we exploited the effect of the soluble anion poly(aspartic) acid, which we have previously shown abrogates the effect of the surface expressed acidic sequence of NRP. As shown in Fig. 5, the presence of a soluble polyanion homopolymer completely abolishes the effect of cisplatin on crystal attachment.

Discussion

The current studies are the first report of which the authors are aware demonstrating that apoptosis per se, not associated with exposure to oxalate, is associated with enhancement of COM attachment. This enhancement is correlated with the degree of apoptosis in the cultures as measured by activated caspase 3 expression and appears to be mediated by surface expression of NRP. NRP was identified among a group of proteins associated with the cell membrane that have pronounced affinities for COM crystals and has previously been reported to enhance COM attachment during recovery from "wounding" cultures and when overexpressed in the apical membrane [7, 9].

Surface expression of phosphatidylserine is also a feature of apoptosis, and its enrichment in the apical cell membrane in cultured IMCD cells has been demonstrated to enhance crystal attachment [4, 25]. Enhanced crystal attachment associated with increased phosphatidylserine

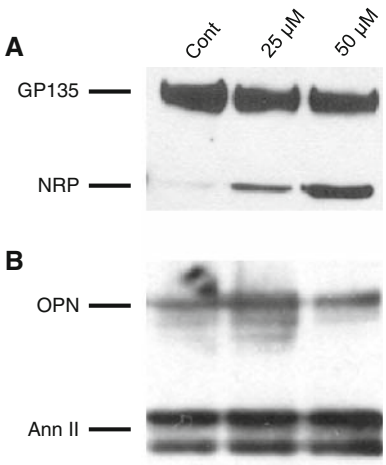


Fig. 2 Western blot of surface nucleolin and other putative attachment molecules. IMCD cells were exposed to the indicated concentrations of cisplatin for 20 h. The cells were surface biotinylated, lysed, and the biotinylated proteins precipitated with streptavidin beads. The proteins were solubilized in sample buffer, subjected to PAGE, and transferred to membranes. **a** Western blotting was performed using the anti-nucleolin antibody and an antiserum against GP-135, a cell surface marker. There is a small amount of surface nucleolin in the control cells; however, exposure to cisplatin increased the surface-localized nucleolin markedly and in what appears to be a concentration-dependent manner. **b** The blots were stripped and re-probed with antisera against osteopontin and annexin II. Both control cultures and cisplatin treated cultures demonstrate similar signal levels for these proteins

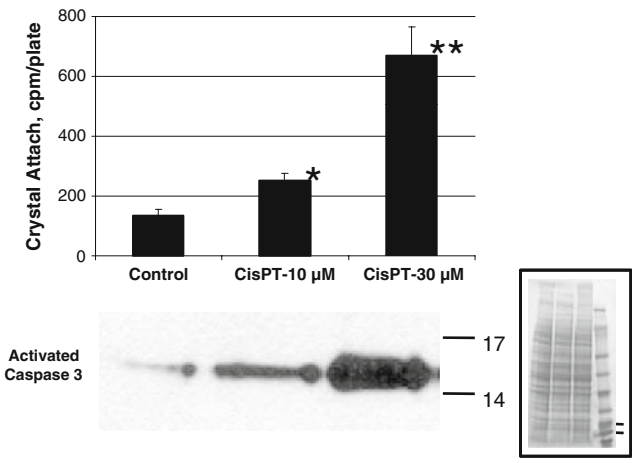


Fig. 3 Crystal attachment is enhanced in cells made apoptotic with cisplatin (CisPT). IMCD cells were grown on glass coverslips and exposed to the indicated concentrations of cisplatin. They were exposed to ^{14}C-COM crystals in AU, washed, and counted for adherent radioactivity. The *top portion* of the figure indicates crystal attachment as adherent radioactivity; the *bottom portion* indicates apoptosis as caspase by Western blotting of protein lysates of identically handled coverslips. As shown, crystal adherence increased in parallel with activated caspase content with cisplatin exposure, in concentration-dependent manner. The *inset* at the right shows protein staining of relevant lanes of the gel indicating approximately equivalent levels of loading. The 17 and 14 kDa markers are indicated on the ladder in the right-most lane. *Significantly different from control; **significantly different from 10 μM cisplatin

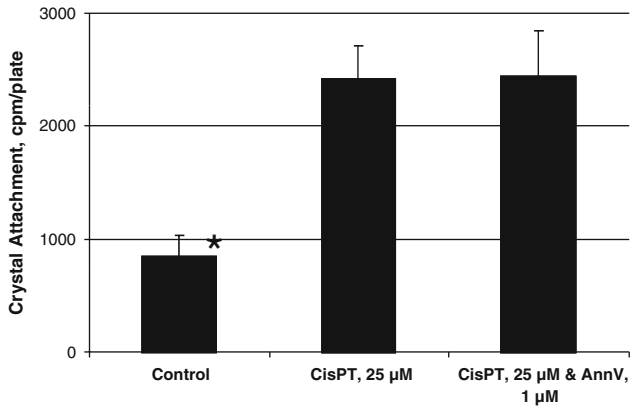


Fig. 4 Annexin V (AnnV) fails to antagonize cisplatin associated COM crystal adherence. Crystal attachment was performed as described in the previous figure except, in this case, with and without 1 μM annexin V. *Significantly different from both CisPT and CisPT and AnnV, which were not different from each other

exposure was blocked by Annexin V [4]. However, exposure of phosphatidyl serine occurring in the course of cisplatin-induced apoptosis does not appear to be responsible for the enhanced crystal attachment, as it was not blocked by Annexin V. Perhaps the surface exposure of phosphatidylserine is not significant at the time point at which crystal attachment was studied, or, more, likely, perhaps the phosphatidylserine sites are occluded by the apparently more superficially attached NRP [9]. In any case, we have no direct evidence bearing on this issue.

Exposure of cultured cells to oxalate and induction of hyperoxaluria in animals induces apoptosis [26, 27]. However, such exposure, except insofar as oxalate dissolution from crystals occurred during the 20-min exposure to crystals, was not a part of the experimental design of these studies. Exposure of cells to crystals themselves apparently can also induce apoptosis [28]. This effect cannot be responsible for the results shown in Fig. 3, as the cells in each group were exposed to the same amount of crystals for the same period of time. In addition, in the current studies, apoptosis was clearly in evidence prior to the exposure to crystals.

Others and we have proposed that the attachment of COM to cells is specific and that it may be mediated by one or more attachment molecules. Molecules other than NRP and phosphatidylserine that have been implicated in the process of attachment include glycosaminoglycans, such as hyaluronan [4, 5]. Other investigators have presented evidence for the mediation of attachment by sialic acid-containing glycosidic side chains of proteins otherwise unidentified [29]. Other proteins with Ca-binding properties, such annexin II and, possibly, osteopontin, could also mediate attachment [6–8].

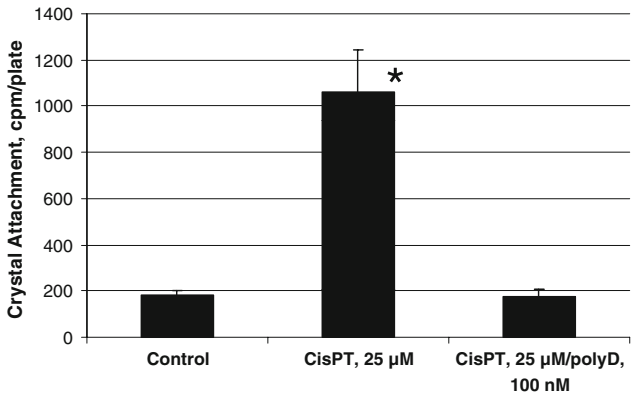


Fig. 5 Polyanion exposure antagonizes cisplatin associated COM crystal adherence. Crystal attachment was performed in IMCD cells exposed to cisplatin for 20 h with and without 100 nM poly(aspartic) acid (polyD). The polyanion completely prevented the enhanced COM attachment. *Significantly different from both Cont and CisPT/polyD, which were not different from each other

In the apoptotic IMCD cells produced by cisplatin exposure, there was no evidence for increased surface expression of putative attachment molecules, with the exception of NRP. In fact, hyaluronan expression was decreased in the apoptotic cells. Although we cannot rule out the possibility that molecules not yet described was mediating the increased COM attachment, it seems likely that NRP is playing a significant role in this process. This is supported by the observation that the acidic homopolymer polyD completely abolished the increased COM attachment observed in the cisplatin-treated cells, as reported previously for COM attachment mediated by the acidic region of this molecule [7].

There are two competing hypotheses for crystal retention in stone disease. Evan and coworkers have suggested that in patients with typical Ca oxalate stone disease, the stones form on a nucleus of Ca phosphate crystals that originally form in the basement membranes of thin limbs of the loop of Henle [30]. Deposits of these crystals form what has classically been called Randall's plaques. These investigators propose that the plaques erode the papillary surface epithelium to trigger Ca oxalate crystallization by heterogeneous nucleation of COM, COD, or both, eventually producing the clinical stone.

The alternate hypothesis is that stone crystals of any variety arise by nucleation within renal tubules, possibly in the longest loops of Henle, and then become attached to the apical surface of tubules cells near the papillary tip. It is also possible that both mechanisms are operative under different circumstances, as proposed by Evan [30].

One category of such circumstances includes situations in which cells are damaged in some way. Either directly in the process of cell injury or during its repair, putative attachment molecules not normally present on the apical surface of cells are expressed or normally expressed molecules are increased in amount [5, 7]. These may provide sites for crystal attachment when circumstances are present that permit crystal nucleation in the renal tubules.

There are potential clinical implications of the current observations. As reviewed by Khan [31], oxalate exposure induces apoptosis. Thus, both the mild hyperoxaluria often observed in idiopathic stone formers, the more marked hyperoxaluria observed in various genetic forms of hyperoxaluria, or the even greater degrees of hyperoxaluria seen in enteric hyperoxaluria may induce apoptosis in renal tubule epithelial cells, enhancing the attachment of CaOx crystals when they form. However, a relation between apoptosis per se and crystal deposition or stone localization has never been directly observed.

A correlation between a decreased degree of apoptosis and crystal deposition has been observed in animals treated with green tea, and hepatocyte growth factor has been reported to reduce both apoptosis and both crystal deposition in animals and crystal attachment in cell culture [32, 33]. In addition, apoptosis and crystal deposition was diminished in ethylene glycol treated animals given atorvastatin [34]. However, others have not reported such a correlation [27, 35, 36]. In renal cell carcinomas, for instance, in which CaOx deposition is frequently observed, there was no correlation between the numbers of apoptotic cells and CaOx deposition [37]. The lack of direct correlation, especially, in animal experiments or in clinical situations may be a consequence of the increased complexity of these systems compared to the cell culture, where the many other determinants of crystal formation and attachment are more easily controlled. It should be emphasized here that the current study is the first showing induction of apoptosis by independent means not involving oxalate or calcium oxalate crystal exposure enhances COM attachment.

Polycystic kidney disease is another condition in which an increased susceptibility to stone formation has been observed [38]. Here, most other factors conducive to stone formation are usually absent. In particular, patients with this condition cannot, in general, produce highly concentrated urine [39]. This would tend to protect against stone formation. The occurrence of urine chemistry abnormalities except for hyperoxaluria does not correlate with stone development in this condition; however, larger renal volume appears to [40]. While this suggests that structural alterations may be playing a role in promotion of stone formation, the number and size of cysts were not associated with the development of nephrolithiasis, suggesting that the relation between structural abnormalities and stone formation is not simple. However, both increased mitotic figures and apoptotic cells have been observed in polycystic kidneys [41]. Thus, it seems reasonable to

hypothesize that the increased surface expression of attachment molecules may induce crystal attachment and contribute to stone formation in this condition or in others with or without other factors known to contribute to stone formation.

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Conflict of interest statement None of the authors has any financial interests in companies that may have a financial interest in the information contained in this manuscript.

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