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ORIGINAL PAPER

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Renal Tubular cell injury and fibronectin

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Abstract We recently reported that fibronectin (FN: 230 kDa) was oversecreted from the renal tubular cells as a result of the stimulation of COM crystals, and inhibited the adhesion of COM crystals to renal tubular cells. In the study presented here, we investigated whether FN can prevent injury to renal tubular cells caused by oxalate and COM crystals and tried to identify the relation with inhibition of crystal adhesion. The protective effect of FN against renal tubular cell injury by exposure to oxalate and COM crystals was examined by measuring the lactate dehydrogenase (LDH) activity and using a non-radioactive proliferation assay. Moreover, crystal-cell interaction was morphologically assessed by means of scanning electron microscopy (SEM). LDH was reduced significantly by the addition of FN in a dose-dependent manner. Cell viability increased significantly with the addition of FN, also in a dose-dependent manner. Moreover, the morphological SEM study showed that few crystals were attached to the surface of cells when FN was added compared to the number of adhering crystals when FN was not added. FN was found to have an inhibitory effect on renal tubular cell injury caused by exposure to oxalate and COM crystals. We speculate that the inhibitory effect of FN on the adhesion of COM crystals is related to the inhibitory effect of exposure to COM crystals on renal tubular cell injury.

Keywords Fibronectin · MDCK cell · Cell injury · Oxalate · Calcium oxalate monohydrate crystal

Introduction

The prevalence of urolithiasis in Japan was 100.8 per 100,000 people in 1995 [1], and this rate has been increasing over the last 30 years. More than 60% of these patients suffer from urolithiasis caused by calcium oxalate stones. However, the mechanism of stone formation is not yet clearly understood. It is thought that adhesion of COM crystals on the surface of renal tubular cells and the subsequent cellular response may constitute critical pathogenic steps in the development of renal stones. The mechanisms involved in crystal adhesion and injury to renal tubular cells have been studied in Madin-Darby canine kidney (MDCK) cells [2–4]. It has been shown that high oxalate levels and/or COM crystals can produce cell injury and altered cell physiology, which can have an impact on crystal adhesion [5–8]. Some researchers found that urinary glycosaminoglycans (GAGs) prevent crystal adhesion to renal tubular cells [9, 10]. GAGs can also restore the anti-adherence properties of an injured urothelium, thereby preventing crystal adhesion. Fibronectin (FN: 230 kD) [11] is a multifunctional a2-glycoprotein distributed throughout the extracellular matrix and body fluids. Many investigators have demonstrated that FN, because of its cell adhesive action, is related to biological processes such as morphogenesis, wound healing and metastasis [12–16]. We recently reported that FN, oversecreted from the renal tubular cells as a result of the stimulation of COM crystals, inhibited the adhesion of COM crystals to renal tubular cells as well as aggregation of the COM crystals in vitro [17]. In the study presented here, we investigated whether FN can protect renal tubular cells against the injury caused by oxalate and COM crystals, and tried to identify the relation with inhibition of crystal adhesion.

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Materials and methods

Cell culture

MDCK cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown at 37°C in Dulbecco's modified eagle medium containing 5% fetal calf serum and 1% gentamicin in a 5% carbon dioxide and 95% air atmosphere. We cultured 3 ml of cells in suspension (4×10^5) was cultured in a 35-mm culture dish.

Oxalate and COM crystal preparation

Oxalate as potassium oxalate was added using a stock solution of 10-mM potassium oxalate in normal sterile saline passed through filters with a pore size of 0.2 mm. COM was prepared by adding equal volumes of 1 mM CaCl2 and 10 mM $K_2C_2O_4$ together at room temperature. After standing for 10 min., COM crystals were treated with ultrasound for 15 min to obtain uniform crystal conditions [18]. Crystal purity was determined by high-resolution X-ray powder diffraction. Crystal morphology was examined by light microscopy and was found to be primarily spherulitic, about 1 mm in diameter [19].

Effect of FN on injury to MDCK cell caused by exposure to oxalate

To determine whether FN can protect renal tubular cell against injury caused by oxalate, we conducted two experiments to assess cell injury. In the first experiment, we measured the cytosolic enzyme lactate dehydrogenase (LDH) activity released from MDCK cells in the culture supernatant in order to determine and quantify cell injury. After the MDCK cells had grown to confluency, the medium was aspirated and replaced with 0.05 M tris hydrochloric acid buffer with five concentrations of FN, 0, 0.5, 1.0 and 5.0 μg/ml, for 1 h at 37°C. After removal of the culture medium, the cells were rinsed with Hanks' balanced salt solution and exposed to 0.5 mM oxalate. After the medium had been centrifuged at 1,500 rpm for 10 min, LDH activity of the supernatant was measured with a commercially available kit (Boeringer Mannheim, Castle Hill, Australia).

Cell viability was evaluated by MTT assay for the second experiment. After MDCK cells (2×10^5/well, 96-well plate) had grown to confluency, the medium was aspirated and replaced with 0.05 M tris hydrochloric acid buffer with five concentrations of fibronectin, 0, 0.5, 1.0 and 5.0 μg/ml, for 1 h at 37°C. After the culture medium had been removed, the cells were rinsed with Hanks' balanced salt solution and exposed to 0, 0.25, 0.5, and 1.0 mM oxalate for 1 h at 37°C. MTT assay [20] was performed.

Effect of FN on injury to MDCK cell caused by exposure to COM crystal

To determine whether FN can prevent injury to the renal tubular cells caused by COM crystals, we conducted two experiments for evaluation of the cell injury. For the first experiment, we measured LDH activity for the identification and quantification of cell injury. After the MDCK cells (5×10^5/well, 6-well plate) had grown to confluency, the medium was aspirated and replaced with 0.05 M tris hydrochloric acid buffer with five concentrations of FN, 0, 0.5, 1.0 and 5.0 μg/ml, for 1 h at 37°C. After removal of the culture medium, cells were rinsed with Hanks' balanced salt solution and exposed to 500 mg/ml of COM crystals. After the medium had been centrifuged at 1,500 rpm for 10 min, LDH activity of the supernatant was measured with a commercially available kit.

Cell viability was evaluated by MTT assay for the second experiment. After MDCK cells (2×10^5/well, 96-well plate) had grown to confluency, the medium was aspirated and replaced with 0.05 M tris hydrochloric acid buffer with five concentrations of FN, 0, 0.5, 1.0 and 5.0 μg/ml, for 1 h at 37°C. After the culture medium had been removed, the cells were rinsed with Hanks' balanced salt solution and exposed to 500 mg/ml COM crystals for 0.5 h, 3 h and 24 h. MTT assay [20] was performed.

Morphological examination by scanning electron microscopy (SEM)

After the MDCK cells (5×10^5/well, 6-well plate) had grown to confluency, the medium was aspirated and replaced with 0.05 M tris hydrochloric acid buffer with two concentrations of FN, 0 and 5.0 μg/ml, for 1 h at 37°C. After removal of the culture medium, the cells were rinsed with Hanks' balanced salt solution and exposed to 500 mg/ml of COM crystals. For SEM, the cells or crystals were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h on ice, while the samples were postfixed on OsO_4 in 0.1 M cacodylate buffer for 2 h on ice. The samples were then dehydrated through a graded ethanol series at 10-min intervals and finally critical point dried. After being mounted on stubs, the samples were spray-coated with a conductive layer and examined under a HITACHI S-8000 microscope.

Statistical analysis

Values are expressed as the mean plus or minus standard error. Statistical analysis was performed with the Mann-Whitney U test. Differences were considered significant at $p < 0.05$.

Results

Inhibitory effect of FN on injury to MDCK cell caused by exposure to oxalate

Zero concentration of FN was used as control. As the concentration of FN increased, the quantity of LDH tended to decrease. While there were no significant differences at the FN concentration of 0.5 μg/ml, there were significant differences at the FN concentrations of 1.0 μg/ml and 5.0 μg/ml. The release of LDH from cells not treated with oxalate was below 5 IU/l (Fig. 1).

As for the MTT assay, as the oxalate concentration increased, cell viability tended to decrease and cell injury tended to increase. Furthermore, as seen in the LDH study, with an increase in the FN concentration, cell viability tended to increase and there were significant differences at the FN concentration of 1.0 mg/ml. The cell viability of cells not treated with oxalate was over 80% (Fig. 2).

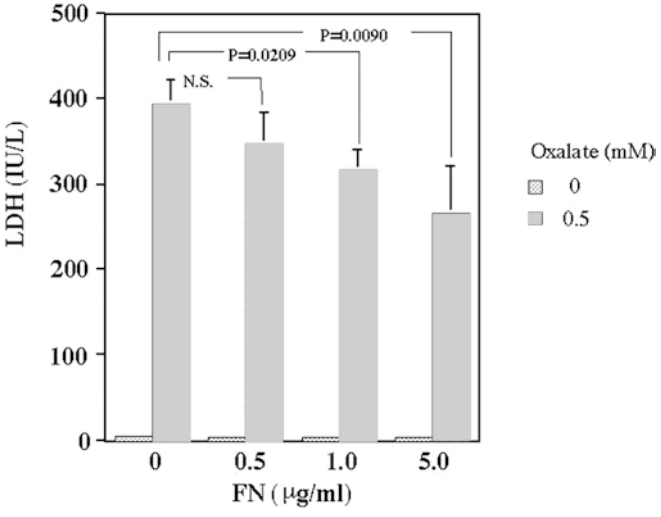

Fig. 1 Inhibitory effect of FN on injury to MDCK cell caused by exposure to oxalate: the investigation by LDH. There were significant differences at the FN concentrations of 1.0 mg/ml and 5.0 mg/ml. The release of LDH from cells not treated with oxalate was below 5 IU/l

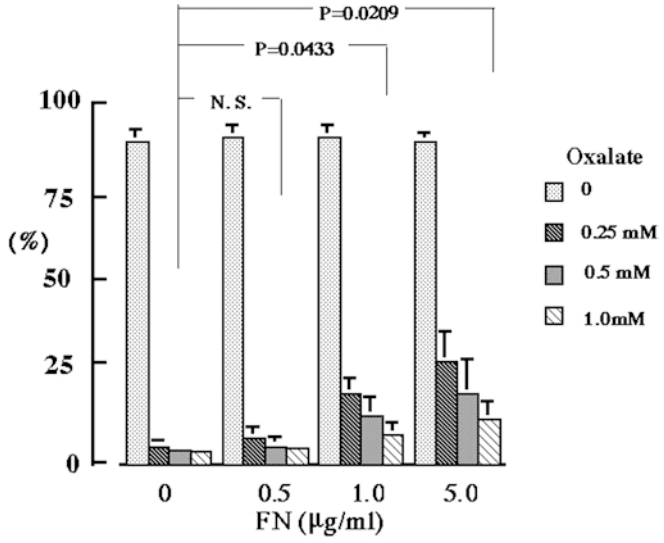

Fig. 2 Inhibitory effect of FN on injury to MDCK cell caused by exposure to oxalate: the investigation by MTT assay. The cell viability tended to increase with an increase in FN concentration, and there were significant differences at the FN concentration of 1.0 mg/ml. The viability of cells not treated with oxalate was over 80%

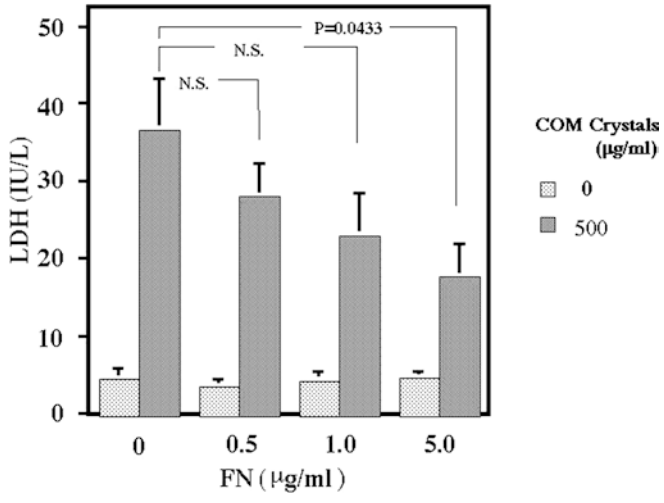

Fig. 3 Inhibitory effect of FN on injury to MDCK cell caused by exposure to COM crystals: the investigation by LDH. An increase in the concentration of FN tended to be accompanied by a decrease in the quantity of LDH. There were no significant differences at the FN concentration of 0.5 mg/ml and 1.0 mg/ml, but there were at the FN concentration of 5.0 mg/ml. The release of LDH from cells not treated with oxalate was below 5 IU/l

Inhibitory effect of FN on injury to MDCK cell caused by exposure to COM crystals

Figure 3 shows the inhibitory effect of FN on injury to MDCK cells caused by exposure to COM crystals in the LDH study. Similar to the case of oxalate, an increase in the concentration of FN tended to be accompanied by a decrease in the quantity of LDH. There were no significant differences at the FN concentration of 0.5 μg/ml and 1.0 μg/ml, but there were at the FN concentration of 5.0 μg/ml. The release of LDH from cells not treated with oxalate was below 5 IU/l.

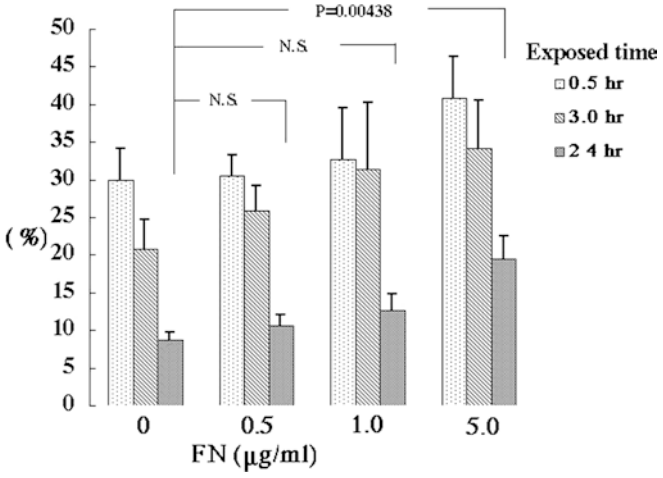

Fig. 4 Inhibitory effect of FN on injury to MDCK cell caused by exposure to COM crystals: the investigation by MTT assay. The cell viability tended to increase in proportion to the concentration of FN. There were no clear differences between the effects of exposure times of 0.5 h and 3 h. However, cell viability decreased to approximately 50% after the 24 h exposure

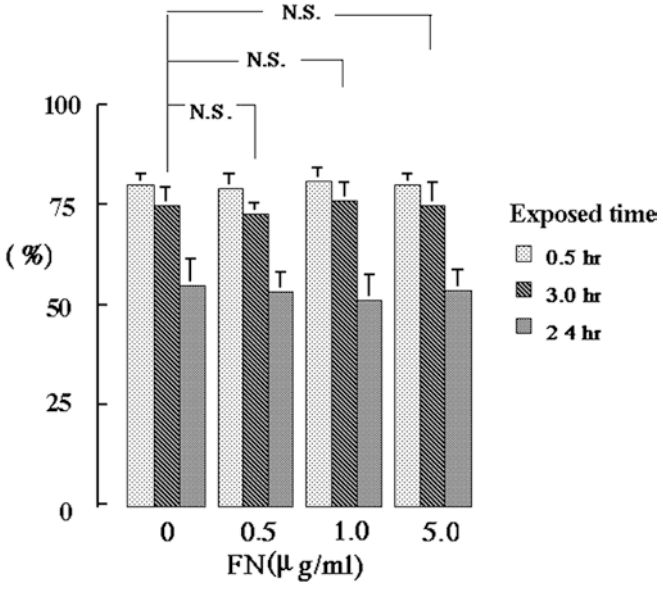

Fig. 5 The effect of FN on injury to MDCK cell: the investigation by MTT assay. The cell viability of cell not treated with COM crystals tended to decrease as the exposure times increased. But there were no significant differences between exposure times of 0.5 h, 3 h and 24 h

The inhibitory effect of FN on injury to MDCK cells caused by exposure to COM crystals in the MTT assay is shown in Fig. 4. Cell injury was studied with the MTT assay using the COM crystals by changing the duration of exposure. As the FN concentration increased, cell viability tended to increase. There were no clear differences between the effects of exposure times of 0.5 h and

3 h. However, cell viability decreased to approximately 50% after the 24-h exposure. The cell viability of cells not treated with COM crystals tended to decrease as the exposure times increased. But there were no significant differences between exposure times of 0.5 h, 3 h and 24 h (Fig. 5).

Morphological examination by scanning electron microscopy

In comparison with the control MDCK cells, the cells exposed to COM crystals showed deformation, an expanded nucleus and crystals tangled up with microvilli and cilia (Fig. 6 A, B). Figure 7 shows scanning electron microscopy (SEM) of cells exposed to COM crystals with and without FN. Few crystals attached to the surface of cells when FN was added compared with adherence without this addition.

Discussion

In recent years, some researchers have emphasized that the interaction between crystals and renal tubular cells, including the adhesion or endocytosis of crystals to cells, are important factors in urolithiasis formation [9, 21, 22]. Moreover, some reports have suggested the involvement of renal tubular cell injury in the crystal–cell interaction process [4,19]. We demonstrated in a previous study that FN was oversecreted from the renal tubular cells as a result of stimulation by COM crystals, and inhibited the adhesion of COM crystals to renal tubular cells as well as aggregation of the COM crystals in vitro [17]. We therefore hypothesized that FN would have a protective function against renal tubular cell injury caused by oxalate and COM crystals.

First, we observed the effect of FN on injury to renal tubular epithelial cells by either oxalate or COM crystals. Wiessner et al. reported that both individual cell injury and generalized cell monolayer injury result in the presentation of different cell surfaces and that both types of injury result in an increased affinity for crystal adhesion. They also stated that both mechanisms could be important, either independently or collectively, for the retention of microcrystals adhering to renal collecting duct cells in urolithiasis [19]. As the physiological concentration of FN in normal urine is well known [23], we performed assays at 0.1, 0.5, 1.0 and 5.0 μg/ml. In this experiment, changes in the release of LDH and of cell viability determined by MTT assay [20] were measured to evaluate cell injury. LDH is a stable cytosolic enzyme present in all cells and is released upon damage of the plasma membrane. The increase in the amount of enzyme activity correlates with the number of lysed cells in the damaged plasma membrane. We found that LDH induced by either oxalate or COM crystals increased in a concentration-dependent manner. LDH decreased significantly as a result of the addition of FN, and these effects were clearly reduced by FN in a dose-dependent manner. Moreover, the cell viability as determined by MTT assay increased significantly following the addition

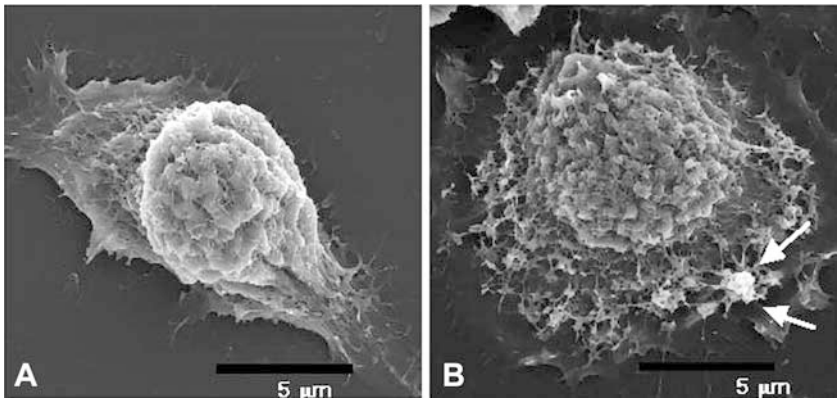

Fig. 6A, B Morphological examination by scanning electron microscopy of MDCK cells exposed to COM crystals. In comparison with the control MDCK cells, the cells exposed to COM crystals showed deformation, an expanded nucleus and crystals tangled up with microvilli and cilia. **A** COM crystals (−); **B** COM crystals (+)

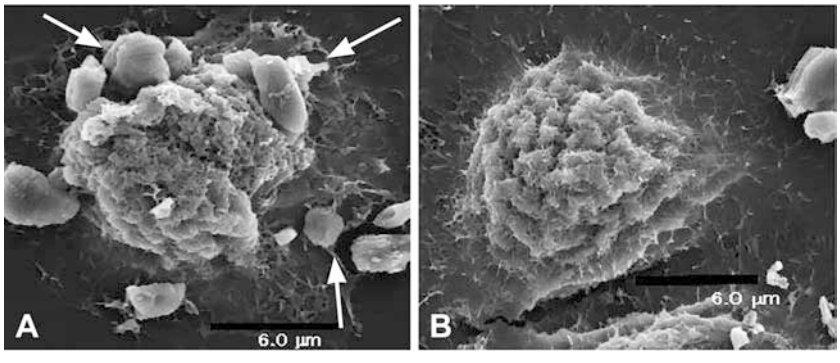

Fig. 7A, B Morphological examination by scanning electron microscopy of MDCK cells exposed to COM crystals with and without FN. Few crystals attached to the surface of cells when FN was added compared with adherence without this addition. **A** FN (−); **B** FN (+)

of FN, also in a dose-dependent manner. Therefore, we concluded that FN exerts an inhibitory activity on cell injury caused by either oxalate or COM crystals.

Lieske et al. reported that all effective anions, such as nephrocalcin and uropontin, inhibited the adhesion of crystals to cells by coating the crystalline surface [24]. This suggests that molecules present in tubular fluid can defend the kidney cell surface against adhesion of COM crystals. We speculated that the inhibitory activity of FN on the adhesion of COM crystals to renal tubular cells was related to the inhibitory activity by FN against cell injury.

Next, the cells injured by COM crystals and attached crystals were morphologically examined for SEM. As also reported previously by Hackett et al. [25], we found that the crystals were tangled up with microvilli and cilia, that the nucleus had expanded, and that adjacent cells had been structurally altered. Our results also showed that in comparison with the control MDCK cells, the cells exposed to COM crystals showed deformation, expansion of the nucleus and crystals tangled up with microvilli and cilia. In addition, the extent of injury to cells after the addition of FN was minor as compared with that of cells without the addition. Moreover, few crystals were attached to the surface of cells when FN was added compared to the number of adhering crystals when FN was not added. This constituted morphological confirmation that FN plays a protective role in cell injury caused by COM crystals and inhibits the adhesion of COM crystals to renal tubular cells.

Interactions between COM crystals and renal tubular cells have been examined by a number of investigators. Wiessner et al. reported that the mechanism of crystal adhesion to renal tubular cells is based on crystal interaction with basolateral or basement membrane components. These components may become exposed as a result of the loss of cell polarity seen in several disease states in association with tissue injury, ischemia in kidney tubules, microvillus inclusion disease, and polycystic kidney disease [19]. We consider that the COM crystal adhesion can easily result from the injury to renal tubular epithelial cells caused by COM crystals. In our experiment, the addition of FN had an inhibitory effect on renal tubular cell injury caused by exposure to COM crystals. Moreover, in a previous study we demonstrated that FN was oversecreted from the renal tubular cells as a result of stimulation by COM crystals. On the basis of these results, we hypothesize that the cell injury caused by COM crystals will be protected by the function of wound healing which FN have, and FN inhibits crystal adhesion by coating the surface of the renal tubular epithelial cells.

Conclusions

FN was shown to have an inhibitory effect on renal tubular cell injury resulting from exposure to oxalate and COM crystals. We speculate that the inhibitory effect of FN on the adhesion of COM crystals is related to its inhibitory effect on renal tubular cell injury caused by exposure to COM crystals. We therefore believe that FN may act as an inhibitor of calcium oxalate urolithiasis.

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References

1. Yoshida O, Terai A, Ohkawa T, Okada Y (1999) National trend of the incidence of urolithiasis in Japan from 1965 to 1995. Kidney Int 56: 1899
2. Lieske JC, Norris R, Toback FG (1997) Adhesion of hydroxyapatite crystals to anionic sites on the surface of renal epithelial cells. Am J Physiol 270: F224
3. Verkoelen CF, Romijin JC, De Bruijin WC, Cao LC, Schroder FH (1995) Association of calcium oxalate monohydrate crystals with MDCK cells. Kidney Int 48: 129
4. Verkoelen CF, Van Der Boom BGAB, Houtsmuller AB, Schroder FH, Romijin JC (1998) Increased calcium oxalate monohydrate crystal binding to injured renal tubular epithelial cells in culture. Am J Physiol 274: F958
5. Lieske JC, Swift H, Martin T, Patterson B, Toback FG (1994) Renal epithelial cells rapidly bind and internalize calcium oxalate monohydrate crystals. Proc Natl Acad Sci U S A 91: 6987
6. Scheid C, Koul H, Hill WA, Luber-Narod J, Kennington L, Honeyman T, Jonassen J, Menon M (1996) Oxalate toxicity in LLC-PK1 cells: role of free radicals. Kidney Int 49: 413
7. Scheid C, Koul H, Hill WA, Luber-Narod J, Jonassen J, Honeyman T, Kennington L, Kohli R, Hodapp J, Ayvazian P, Menon M (1996) Oxalate toxicity in LLC-PK1 cells: a line of renal epithelial cells. J Urol 155: 1112
8. Khan SR, Byer KJ, Thamilselvan S, Hackett RL, McCormack WT, Benson NA, Vanghn KL, Erdos GW (1999) Crystal-cell interaction and apoptosis in oxalate-associated injury of renal epithelial cells. J Am Soc Nephrol 10: S457
9. Gill WB, Jones KW, Ruggiero KJ (1982) Protective effects of heparin and other sulfated glycosaminoglycans on crystal adhesion to injured urothelium. J Urol 127: 152
10. Verkoelen CF, Romijin JC, Cao LC, Boeve ER, De Bruiji WC, Schro FH (1996) Crystal-cell interaction inhibition by polysaccharides. J Urol 155: 749
11. Ebisuno S, Kohjimoto Y, Tamura M, Ohkawa T (1995) Adhesion of calcium oxalate crystals to Madin-Darby canine kidney cells and some effects of glycosaminoglycans or cell injuries. Eur Urol 28: 68
12. Hynes RO (1985) Molecular biology of fibronectin. Annu Rev Cell Biol 1: 67
13. Dufour S, Duband JL, Kornblihtt AR, Thiery JP (1988) The role of fibronectin in embryonic cell migrations. Trends Genet 4: 198
14. Dufour S, Duband JL, Humphries MJ, Obara M, Yamada KM, Thiery JP (1988) Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple adhesion site on fibronectin molecules. EMBO J 7: 2661
15. Barnes JL, Torres ES, Michell RJ, Peters JH (1995): Expression of alternatively spliced fibronectin variants during remodeling in proliferative glomerulonephritis. Am J Pathol 1447: 1361
16. Jarnagin WR, Rockey DC, Koteliansky VE, Wang SS, Bissell DM (1994) Expression of variant fibronectins in wound healing: cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis. J Cell Biol 127: 2037
17. Tsujihata M, Miyake O, Yoshimura K, Kakimoto K, Takahara S, Okuyama A (2000) Fibronectin as a potent inhibitor of calcium oxalate urolithiasis. J Urol 164: 1718
18. Ebisuno S, Kohjimoto Y, Tamura M, Inagaki T, Ohkawa T (1997) Histological observations of the adhesion and endocytosis of calcium oxalate crystals in MDCK cells and in rat and human kidney. Urol Int 58: 227

19. Wiessner JH, Hasegawa AT, Hung LY, Mandel GS, Mandel NS (2001) Mechanisms of calcium oxalate crystal attachment to injured renal collecting duct cells. Kidney Int 59: 637
20. Romijin JC, Verkoelen CF, Schroder FH (1988) Application of The MTT assay to human prostate cancer cell lines in vitro: establishment of hormone-stimulated growth and drug-induced cytostatic and cytotoxic effects. The Prostate 12: 99
21. Bigelow MW, Wiessner JH, Kleinman JG, Mandel GS (1996) Calcium oxalate-crystal membrane interactions: dependence on membrane lipid composition. J Urol 155: 1094
22. Lieske JC, Leonard R, Toback FG (1995) Adhesion of calcium oxalate monohydrate crystals to renal epithelial cells is inhibited by specific anions. Am J Physiol 268: F604
23. Nakatsuka S, Tanaka H (1987) Clinical evaluation of urinary fibronectin. Kawasaki Igaku Kaishi 13: 339
24. Lieske JC, Deganello S, Toback FG (1999) Cell-crystal interactions kidney stone formation. Nephron 81 [Suppl]: 8
25. Hackett RL, Shevock PN, Khan SR (1994) Madin-Darby canine kidney cells are injured by exposure to oxalate and to calcium oxalate crystals. Urol Res 22: 197