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Evaluation of apoptosis and cell proliferation in experimentally induced renal cysts

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Abstract Cell proliferation and apoptosis in renal cysts induced by streptozotocin, alloxan and ferric-nitrilotriacetate were investigated in rats. In the kidneys of all treated animals dilated tubules at the cortico-medullary region, large cysts, glomerular cysts and tubular dilation in the medullary area were found. Both cell proliferation and apoptosis were increased in the epithelium of the non-dilated tubules, in the mesangial and interstitial cells. Cells lining the dilated tubules or cysts demonstrated apoptosis but their proliferating activity was low. By calculating the proliferation–apoptosis ratio we found that alloxan did not change the balance between the two mechanisms. Meanwhile streptozotocin resulted in an increased apoptosis and ferric-nitrilotriacetate in an increased cell proliferation. p53 expression might be responsible for the uncontrolled proliferation in rats treated with ferric-nitrilotriacetate as this oncoprotein was diffusely present in tubular cell nuclei. The observed apoptosis seemed to be independent of bcl-2 oncoprotein expression. We assume that the initial factor in such cystogenesis should be a cellular injury due to direct toxic or to the diabetogenic effect of the drugs. The latter is more likely as all the animals were hyperglycemic and insulin treatment following administration of streptozotocin prevented the morphologic changes.

Key words Rat · Renal cysts · Cyst induction · Cell proliferation-Apoptosis

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Introduction

Polycystic kidney disease is characterized by progressive dilation of tubules and glomerular capsules leading to renal failure. The pathogenesis of the disease is still uncertain although the gene and its protein product responsible for dominant and recessive polycystic kidney disease have been reported [8]. The difficulties in understanding the pathogenesis of cystic disease arise from the fact that obtaining human cystic kidneys in different stages of cyst formation is often problematic. Therefore growing attention is being given to animal models. There are a number of chemicals known to induce renal cysts, such as alloxan [19], diphenylamine and diphenylthiazole [1], ferric-nitrilotriacetate (Fe-NTA) [10] and streptozotocin (STZ) [9]. In experimental models the tubular dilation is thought to be related to a metabolic change in tubular epithelial cells or to the drug-induced defect in elasticity of tubular basement membrane, or perhaps due to tubular obstruction [1]. In human polycystic kidney disease cell proliferation, altered basement membrane production, impaired cell polarity are theorized as being involved in cystogenesis [6]. The finding that bcl-2-deficient mice with fulminating lymphoid apoptosis presented polycystic kidney disease [20] raised interest in the possible connection between programmed cell death and polycystic kidney disease [14, 22]. Apoptosis, which is a prominent feature in metanephric development, is regulated by growth factors and oncogenes [11]. In polycystic kidney disease apoptosis was found to be significantly increased [14].

In the present study we used animal models of renal cystic conditions induced by STZ, alloxan, and Fe-NTA. STZ is derived from *Streptomyces achromogenes* and has diabetogenic, antimicrobial, and antitumoral activity. It is used as an experimental inducer of permanent diabetes by its direct toxicity to pancreatic β cells. As STZ is chemically related to dimethylnitrosamine it can therefore induce renal cancer in rats [15]. Alloxan also has a diabetogenic effect by causing selective necrosis of the β

cells of the pancreas. Kidneys are also affected, showing changes varying from swelling to necrosis, which may lead to uremia and death [20]. Fe-NTA causes severe acute and subacute renal tubular necrosis through Fe-induced free radical chemistry and also induces renal tumors in animals [10]. The induced changes represented a relatively early stage of cyst formation. We wished to clarify whether apoptosis and cell proliferation could be considered as initiating factors in cystogenesis. As both conditions are influenced by oncogenes we wished to determine whether the observed apoptosis and cell proliferation were associated with abnormal expression of bcl-2 and p53 oncoproteins.

Materials and methods

Animals and treatments

Approval for this study was obtained from the Ethical Committee for Animal Experimentation of the University Medical School of Debrecen (65/1996/ATEB). The study followed the principles of laboratory care as outlined in national laws for animal caring by the Hungarian Ministry of Health. All the animals were maintained on ad libitum water and Purina rat chow until killed.

Twenty-five Fisher (F) 344-type 4 to 6-month-old adult rats of both sexes were injected with a single dose of 50 mg/body weight alloxan (Sigma) i.v. The rats had an initial weight of 299.84 ± 59.35 g, which was maintained to the end of the 12 weeks of observation. They were then killed under ether anesthesia. Five animals died before the end of the experiment and were excluded from further investigation because of autolysis.

Twenty-five F344-type, 4 to 6-month-old rats of both sexes were treated with Fe-NTA i.p. twice a week for 6 weeks. The dose of Fe-NTA corresponded to 10 mg iron/kg/day. The initial weight of the rats was 289.1 ± 83.55 g and remained unchanged at the end of 8 weeks of observation following the last injection. Only 16 animals remained alive and were the subject of the study.

Thirty-six newborn (48 hours) F344 rats of both sexes with an initial weight of 6.58 ± 0.94 g received a single injection of STZ (Upjohn) i.p. in a dose of 90 mg/body weight. The animals were killed at the thirty-sixth week, at which point they weighed 295.73 ± 71.26 g. Thirty-five separate F344 newborn rats (48 hours) were treated with 4 U insulin (Ultralente MC, Human) s.c. 48 hours after the STZ injection. They were killed at the thirty-sixth week. The initial weight of the animals was 6.79 ± 0.53 g, which reached 288.34 ± 59.33 g at the time of death.

Thirty F344-type 4 to 6-month-old adult rats weighing 294.67 ± 65.34 g and 15 newborn (48 hours) F344-type rats weighing 6.82 ± 0.47 g served as controls and were given physiologic salt solution into the tail vein or intraperitoneally. The final weight of the adult rats did not change, while that of the newborn rats was 291.07 ± 54.38 g. The animals were killed at the same time-point as their treated counterparts.

The serum or skeletal muscle[1] (soleus) concentration of sodium, potassium, chloride, and blood glucose were determined if available with an Abbott Spektrum multichannel chemical analyzer following the manufacturers instructions.

Histochemical procedures

Kidneys were removed, fixed in 4% paraformaldehyde and embedded in paraffin or snap-frozen in liquid nitrogen for cryostat sectioning. Sections were stained with hematoxylin and eosin (HE). To characterize the tubular origin of the cysts we used lectin-binding studies (peroxidase-labeled *Dolichos biflorus* agglutinin (DBA) and *Soybean* agglutinin (SBA) (Dako, 1:100) and rabbit anti-mouse Tamm-Horsfall protein (THP) known to cross-react with rat tissue (a personal gift from Dr V. Thomázy, University Medical School of Debrecen). Immunohistochemical detection was carried out by the avidin-biotin-peroxidase complex (ABC) method. Briefly the sections were deparaffinized, washed in PBS (pH 7.4) and both endogenous peroxidase and endogenous biotin were blocked by 1.5% H_2O_2 in methanol and by an Avidin-Biotin Blocking Kit (Dako) respectively. To detect THP we used peroxidase-labeled secondary antibody (anti-rabbit IgG, 1:100, Dako). Reactions were visualized by Horseradish peroxidase (HRP)-linked streptavidin and vector red (Dako) as chromogen. For demonstrating apoptosis we used propidium iodide (Sigma) staining in a concentration of 4 mg/l in the presence of 100 mg/l RNAse, and an Apoptag Kit (Oncor). Cell proliferation was examined with anti-proliferating cell nuclear antigen (PCNA, prediluted, Dako) after microwave pretreatment. Bcl-2 oncoprotein (3F11, 1:100, a personal gift from Z. Oltvai-Nagy, Northwestern University Medical School, Chicago) was demonstrated on cryostat sections fixed in cold methanol. Biotinylated anti-hamster IgG was used as secondary antibody and the reaction was visualized by the ABC method and vector red as chromogen.

Quantitative evaluation of proliferation and apoptosis

The number of apoptotic and proliferating cells was calculated by counting 1000 cells in randomly chosen fields (magnification 40×) with the index being the percentage of positively stained nuclei. The proliferation–apoptosis ratio was determined from the average values. Data were analyzed by Fisher's *F*-test and Student *t*-test as statistical methods and the results were regarded as significant if $P < 0.05$.

Results

Blood glucose was significantly elevated ($P < 0.001$) in all treated animals versus controls while insulin substitution prevented hyperglycemia. Serum ion concentration of animal models did not really differ from control values (Table 1).

Microscopic findings

The leading microscopic changes in the kidneys were segmental with diffuse dilation of the tubules within the cortico-medullary junction (Fig. 1) and in the superficial cortex. Large solitary cysts also developed, especially in STZ-treated rats (Fig. 2). Dilated tubules contained an eosinophilic, amorphous substance in their lumen. Tubules of the medulla were dilated in Fe-NTA-treated animals. Glomerular distension was seen with alloxan induction (Fig. 3). Fe-NTA caused focal cortical necrosis in some cases. The interstitium became fibrotic in four STZ-treated rats (Fig. 4) and focal lymphocytic infiltration was also noticed, but not around the dilated or cystic tubules. The epithelium of the distended nephrons and of the cysts was flattened, while the large cysts in STZ-injected animals became cuboidal or large columnar with pale, clear cytoplasm. Lectin-binding studies and THP reaction indicated great variability in

[1] Streptozotocin-treated animals, with or without insulin, were also the subject of an experiment evaluating the role of diabetes on muscle contractility by T. Bányász.

Table 1 Serum concentration of sodium, potassium, chloride and glucose of rats

	Na (mmol/l)	K (mmol/l)	Cl (mmol/l)	Glucose (mmol/l)
Control[1]	144 ± 7.81	4.29 ± 0.8	106.23 ± 3.61	3.71 ± 0.44
Fe-NTA	153.97 ± 15.4	6.04 ± 1.3	108.17 ± 2.54	7.82 ± 1.12***
Alloxan	149.07 ± 2.78	5.47 ± 0.27	105.04 ± 1.63	12.89 ± 7.83***
STZ	28.4 ± 1.3[2]	n.a.	n.a.	21.2 ± 0.89***
STZ + insulin	20.88 ± 3.1[2]	n.a.	n.a.	4.2 ± 1.08

[1] As values were not significantly different, control animals of all models made one group
[2] Concentration of sodium was measured in musculus soleus
Fe-NTA ferric nitrilotriacetate, *STZ* streptozotocin, *n.a.* no available data. *** significant difference control ($P < 0.001$)

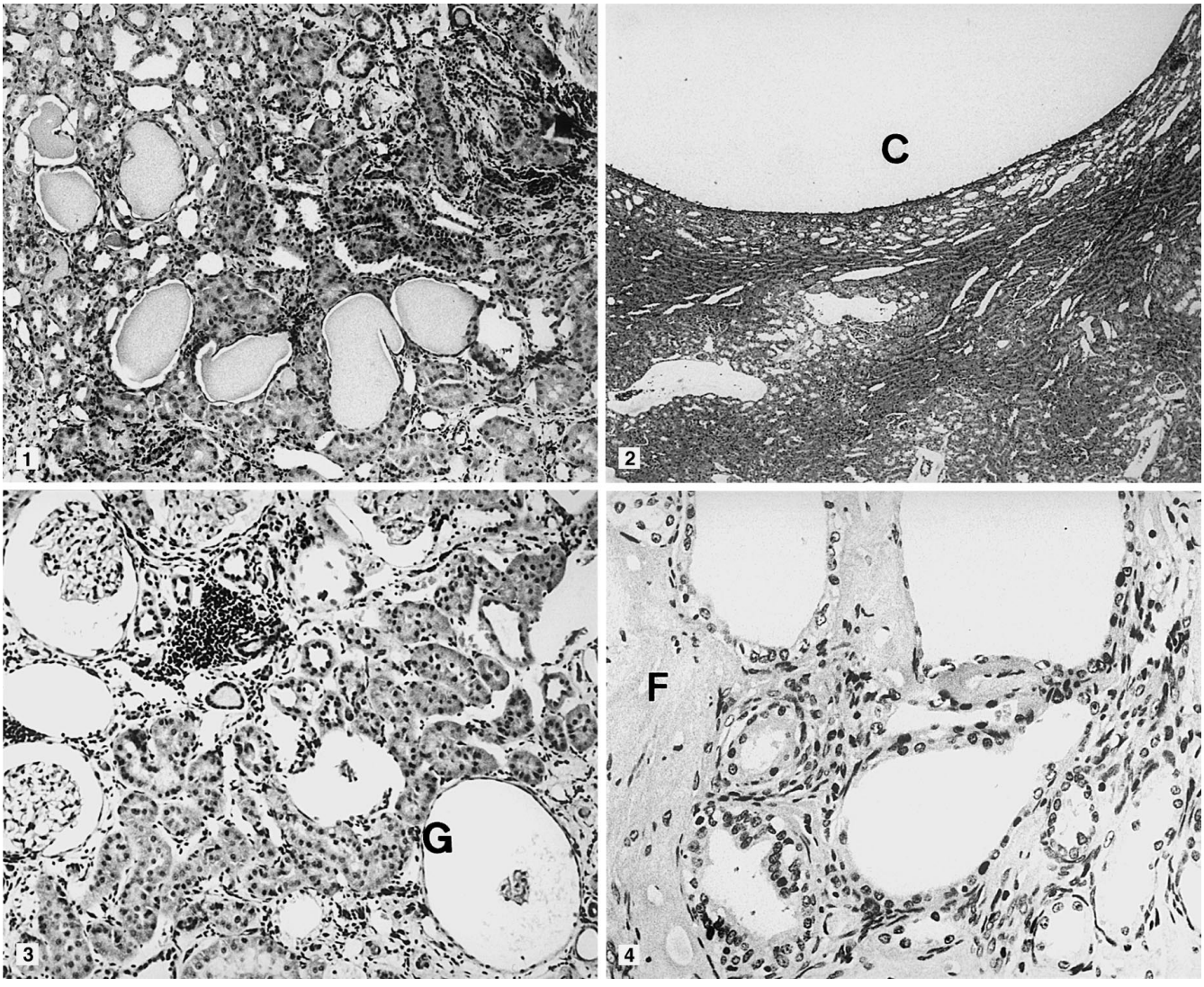


Fig. 1 Dilated tubules at the cortico-medullary junction of a kidney from streptozotocin (STZ)-treated rat. HE; ×200

Fig. 2 Large, unilocular cyst (*C*) in a rat kidney induced by STZ. HE; ×100

Fig. 3 Distended glomeruli (*G*) of a rat kidney with alloxan treatment. HE; ×200

Fig. 4 Cysts surrounded by dense fibrous tissue (*F*) in STZ-treated rat kidney. HE; ×200

tubular origin. STZ-and alloxan-treated rats showed a positive reaction with the collecting duct marker lectins (Fig. 5) while THP remained negative. Epithelium of the dilated tubules of Fe-NTA injected animals were negative for both lectins and THP antibody.

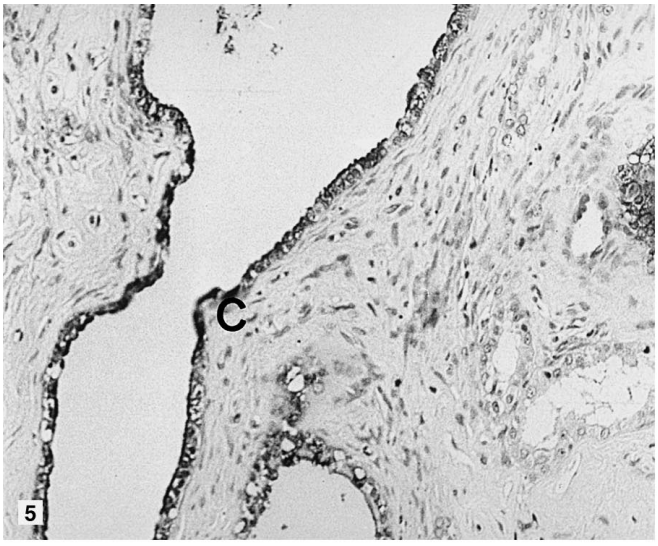
Cell proliferation and apoptosis

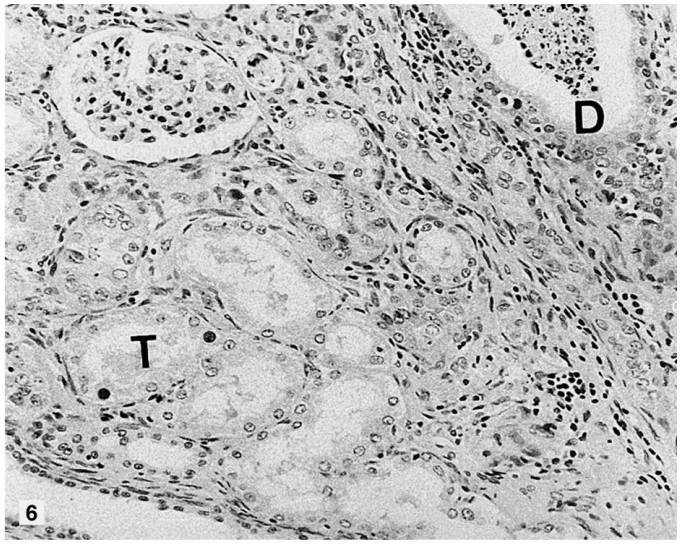
All chemicals induced a significant ($P < 0.001$) increase in proliferating activity (Table 2) of the epithelium of

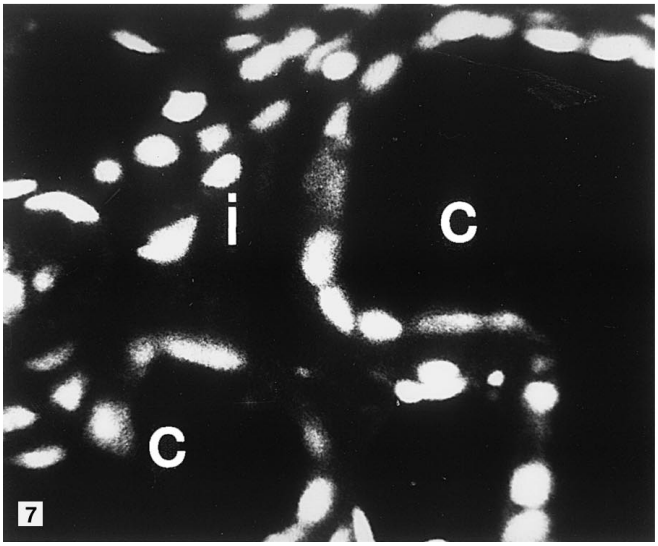
Table 2 Analysis of cell proliferation and apoptosis in the cystic kidneys of animal models. Values mean percent of positively stained nuclei by counting 1000 cells in randomly selected fields

	Cell proliferation	Apoptosis	Proliferation–apoptosis ratio
Control[1]	0.45 ± 0.28	0.41 ± 0.28	1.07
Fe-NTA	6.61 ± 0.47***	1.68 ± 0.31***	3.93
Alloxan	3.14 ± 1.82***	2.86 ± 1.01***	1.09
STZ[2]	2.73 ± 11.29***	15.62 ± 3.28***	0.17
STZ + Insulin[2]	2.24 ± 1.04***	0.79 ± 0.16***	2.84

[1] Controls of all induction types formed one group as the values did not differ
[2] Differences in the values of apoptosis between STZ and STZ + insulin treatment are significant ($P < 0.001$) but not of cell proliferation. *** significant difference ($P < 0.001$) versus control







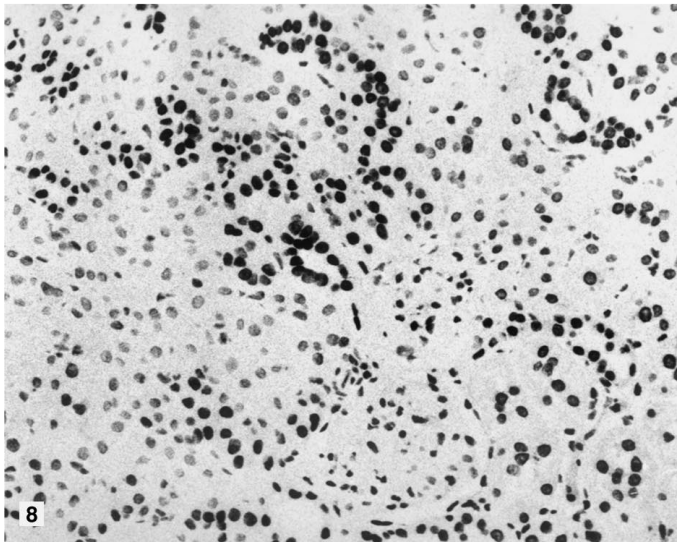


Fig. 5 Peroxidase-labeled soybean agglutinin stains cell membrane of renal cysts (*C*) in STZ-treated rats. ×200

Fig. 6 Proliferating cells in the epithelium of non-dilated tubules (*T*) caused by ferric-nitrilotriacetate. Dilated tubules (*D*) are negative. PCNA; ×200

Fig. 7 Apoptotic cells lining the cysts (*C*) and also within the interstitium (*i*). Propidium iodide staining; ×200

Fig. 8 Almost diffuse expression of p53 oncoprotein in the nuclei of non-dilated tubular cells. ×200

non-dilated tubules (Fig. 6), of glomerular mesangial cells, and of interstitial cells. Dilated tubules and cysts represented a moderate cell proliferation or were even negative. Fe-NTA induced the highest proliferating reaction while there was no significant difference between the others. Insulin treatment did not change the proliferating activity compared with STZ alone (Table 2).

The number of apoptotic cells (Table 2) was significantly increased ($P < 0.001$) in rats treated with FE-NTA, STZ and alloxan. The most striking increase in

apoptotic cells was noticed with the administration of STZ. Apoptotic cells were found mainly in the interstitium, mesangium and also in the dilated and cystic tubules (Fig. 7). Detached apoptotic cells were found in the lumen of cortical tubules of Fe-NTA-treated animals. Insulin significantly ($P < 0.001$) decreased the number of apoptotic cells compared with STZ treatment and even with the control animals.

We calculated the proliferation–apoptosis ratio from the average values (Table 2). This ratio did not differ from the control group in alloxan treatment and remained around 1. In Fe-NTA-induced animals proliferation was predominant. STZ administration resulted in a very low value indicating the excess of apoptosis. The ratio proved to be higher than 1 with insulin substitution as proliferation was predominant compared to apoptosis.

Oncoproteins

Bcl-2 was present only in the parietal epithelium of Bowman's capsule, while the epithelial cells of normal and dilated tubules remain negative. p53 reaction was diffuse (Fig. 8) in the tubular epithelial cells of the Fe-NTA-treated rats, and could also be detected in STZ-injected animals. Alloxan did not lead to the expression of this oncoprotein and the reaction remained negative with insulin substitution.

Discussion

Renal cysts develop in various conditions in humans. As well as in autosomal dominant polycystic kidney disease, acquired cysts are frequently seen in renal failure and dialysis. Cysts occur with high incidence with benign and malignant tumors. The microscopic findings in cysts and tumors in experimental animals seem to be similar to those in human [4]. Chemicals used in cyst induction frequently resulted in tumors of the kidney and of other organs, suggesting that the mechanism of carcinogenesis and cystogenesis is the same, possibly involving cell proliferation, inhibited apoptosis, oncogene and growth factor expression. This theory was supported by those results which indicated that increased cell proliferation, oncogene over expression and a defective prepro-epidermal growth factor gene expression were detected in inherited polycystic kidney disease [2]. Although STZ and Fe-NTA are known to induce tumors in rats and mice [7, 10, 15], no neoplasia was found in our animals, probably due to the relatively short duration of the experiment.

The histologic appearance of the kidneys was in accordance with previous findings by others [4, 10, 19]. All the chemicals caused similar changes in renal morphology with segmental tubular dilation at the corticomedullary junction or with large solitary cysts. Although the histologic pattern was rather uniform, the phenotype of the tubules involved seemed to be different. By the consistent negative reaction with anti-THP, the tubular dilation was unlikely to occur in the loop of Henle. The majority of the distended nephrons and cysts were stained with collecting-duct marker lectins; however, negative reaction was also noticed. This finding may indicate that proximal tubules were also involved, or that the toxic effect of the drugs on the epithelial cells changed their phenotype resulting in an altered antigen expression. Others have reported both proximal and distal tubular lesions by the administration of alloxan, STZ and Fe-NTA [7, 10, 19]. The epithelial cells of the Bowman's capsule are suggested to be similar to those of proximal tubules which may explain the formation of glomerular cysts in alloxan-treated rats. It is supposed that tubular dilation could be related to fibrosis or obstruction of the tubules or changes in the elasticity of basement membranes [1]. We did not notice any fibrosis or extracellular fibrosis matrix proliferation around the dilated tubules in our cases except four STZ-injected rats. By the low frequency and by the focal appearance of this fibrosis and especially by remarking that these animals were killed after 9 months we believe this was a secondary reaction and followed rather than preceded the formation of the cysts. We cannot entirely reject the possible role of tubular obstruction at least in the Fe-NTA-treated rats as apoptotic cells were found in the lumen of non-dilated tubules. However, cellular debris was detected in the tubules of the cortex while the dilation occurred at the cortico-medullary junction, which is far below the theorized blockage. The observed distension of the tubules within the medulla is also inconsistent with the presumed obstruction. Insulin substitution prevented the formation of tubular dilation suggesting that these lesions are related to hyperglycemia.

Both cell proliferation and apoptosis were observed to be increased in the epithelium of non-dilated tubules, in the mesangial and interstitial cells due to the administration of different chemicals. Increased cell proliferation has also been observed by others [3, 12, 16]. The proliferation–apoptosis ratio is better demonstrated for understanding the mechanism through which these chemicals acted. The ratio was around 1 in controls indicating a normal regulation. This value remained the same in rats with alloxan treatment. We assume that either toxic or metabolic cellular injury due to the administration of alloxan was followed by a regeneration, which was reported by others [19]. This repair could occur through the stimulated but regulated apoptosis and cell proliferation. That tubular dilation still developed confirms the role of functional defect in the cells, either by direct toxic or by the diabetogenic effect of the drug, resulting in impaired fluid and electrolyte transport.

With this ratio of cell proliferation and apoptosis, Fe-NTA leads to an uncontrolled cell growth. Previously published data mentioned an inhibitory effect of this

chemical on cell proliferation; however, those studies were performed on macrophages and lymphocytes [5]. At the same time the high incidence of renal adenocarcinoma treated with Fe-NTA supports its proliferation-inducing capacity [18]. Increased cell proliferation is extensively mentioned as being ultimately important in cystogenesis. Proliferating cells may block the lumen with the expansion of the adjacent segments. Distension of the tubules possibly inhibits further proliferation, which explains why PCNA reaction was detected only in the non-dilated tubular cells. The increased cell proliferation, which can lead to cyst formation and renal tumors, by Fe-NTA might be related to the expression of mutant p53 as it was diffusely present in tubular cells of rats in this group. Mutant p53 may induce uncontrolled growth [13].

STZ on the other hand demonstrated an opposite response with an increase in the apoptotic potential of the cells. Induction of apoptosis by STZ is known in pancreatic cells [21] and insulin substitution could prevent its action [19]. As the duration of STZ induction could correspond to a later stage of cytogenesis we believe that increased proliferating activity precedes apoptosis, which become dominant thereafter. Although the distribution of anti-apoptotic bcl-2 did not differ from its normal expression we cannot exclude that the absence of this oncoprotein might be responsible for the increased apoptosis. p53 was present in tubular cells of STZ-treated animals but the cell proliferation was only half that of the Fe-NTA-treated animals, therefore the effect of STZ does not seem to be related to the presence of mutant oncoprotein.

From the fact that insulin treatment prevented the formation of tubular dilation and cyst formation we conclude that hyperglycemia or other diabetes-related metabolic cellular defects can be considered as cyst-inducing factors, and cell proliferation with apoptosis are secondary modulators responsible for further expansion either through tubular obstruction or through an intensive tissue loss. While toxic chemicals were effective in renal cyst induction we have to be extremely careful with the interpretation of these findings as relevant in human cystogenesis. Taken together, these results suggest that intracellular defect is the common feature in human and animal models of renal cystic disease. The intracellular defect may act on the translational or post-translational pathway and the nature of the cellular injury or of the damaged cells themselves will determine whether these cells diminish by necrosis/apoptosis or survive and regenerate by proliferation resulting in a new phenotype.

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