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The role of α2u-globulin in ochratoxin A induced renal toxicity and tumors in F344 rats

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Abstract

The mycotoxin ochratoxin A (OTA) was shown to be a potent kidney carcinogen in rats demonstrating a marked sex difference in the response. Compared to female rats, male rats had a 10-fold higher incidence of kidney carcinomas. The objective of this study was to investigate whether this sex difference in tumor response is due to an exacerbation of effect resulting from the interaction of the male rat specific urinary protein α2u-globulin (α2u) with OTA. Male and female rats were treated by oral gavage with OTA (1 mg/kg per day), D-limonene (dL; 1650 mg/kg per day) as a positive control or corn oil for 7 consecutive days. OTA induced severe renal lesions predominantly in the P_3 region of the proximal tubules. The lesions consisted of necrotic cells and cell exfoliations. No hyaline droplets were found in the P_2 segment following OTA treatment, whereas dL induced the expected accumulation of droplets. The results suggest that OTA induced kidney lesions are in all characteristic points different from the known α2u-nephropathy induced by dL. Based on these experiments the male rat specific protein α2u does not seem to be involved in the mechanism(s) leading to the high tumor incidence observed in OTA exposed male rats.

Keywords: Ochratoxin A; α2u-Globulin; Renal pathology; Cell proliferation; Sex differences

1. Introduction

Ochratoxin A (OTA) is a naturally occurring low molecular weight mycotoxin produced by a variety of *Aspergillus* and *Penicillium* species (van der Merwe et al., 1965; Steyn, 1984). OTA con-

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sists of a 5-chloro-8-hydroxy-dihydro-3-methyl isocoumarin which is linked through the 7-carboxy group by an amide bond to L,β-phenylalanin. The mycotoxin has been found as a natural contaminant in a wide variety of feed, food, animal tissues and consequently also in human blood (Krogh, 1987; Kuiper-Goodman and Scott, 1989). The presence of OTA in moldy feed was assumed to be the most important cause of spontaneous mycotoxic porcine nephropathy (Krogh et al., 1974) and nephropathy in poultry (Krogh et al., 1976; Harwig et al., 1983). Human Balkan nephropathy was also attributed to OTA exposure. However, although there is a considerable amount of data regarding this disease, it is still controversial whether OTA plays a causative or only a subordinate role in the induction of the human Balkan nephropathy (Berndt and Hayes, 1979; Radovanovic, 1989, 1991; IARC, 1993). Laboratory studies with rodents, pigs, poultry and fish have demonstrated that the kidney is the primary organ for OTA induced toxicity (Fuchs, 1988).

OTA was also shown to be teratogenic, to suppress immunefunction and protein synthesis and to alter a number of biochemical parameters, e.g. mitochondrial respiration or carbohydrate metabolism (Berndt and Hayes, 1979; Dirheimer and Creppy, 1991). However, these effects were observed only at high dose levels, i.e. within the range of the respective LD_{50}'s of the experimental animals used (DFG, 1990). No genotoxic activity could be found in various microbial and mammalian gene mutation test systems (Kuiper-Goodman and Scott, 1989; DFG, 1990). On the other hand, OTA was reported to induce DNA single-strand breaks in vivo in rat kidney and liver (Kane et al., 1986), and a mutagenic effect was observed in primary hepatocyte cultures after metabolic activation (Hennig et al., 1991). Using the ^{32}P-postlabeling technique, gavaging of male mice with 2.5 mg/kg OTA was reported to induce DNA-adduct-formation in the kidney and to a lesser extent in the liver and spleen (Pfohl-Leskowicz et al., 1993). However, most recent preliminary experiments from two independent laboratories failed to reproduce the latter data and thus failed to confirm the reported genotoxic activity of OTA (Dietrich and Turesky, personal communication). A two year mouse and rat bioassay (Bendele et al., 1985; Boorman et al., 1992), however, demonstrated that OTA is a potent renal carcinogen. These studies, revealed also a pronounced sex and species difference in kidney tumor induction, i.e. no tumors were found in female mice at the high dose of 40 ppm OTA in feed (approximately 4.8 mg/kg body weight), while a high number of tumors were found in male mice (Bendele et al., 1985). In contrast, an approximately 20-fold lower dose (210 μg/kg body weight) than that used in the mouse study, induced a 89% renal tumor incidence in male rats. The incidence of renal carcinomas was 28%. Female rats, on the other hand, were approximately 10-fold less responsive although not refractive to renal tumor induction (Boorman et al., 1992). Although several studies have been carried out to elucidate the mechanism(s) involved in OTA induced nephrotoxicity and carcinogenicity, little is known about the mechanism(s) leading to the observed sex and species differences. Metabolism and toxicokinetic studies comparing male and female rats and mice respectively have not been able to demonstrate significant differences between males and females of either species (Størmer and Pedersen, 1980; Størmer et al., 1981; Støren et al., 1982; Appelgren and Arora, 1983; Størmer et al., 1983; Hagelberg et al., 1989; Galtier, 1991; Li et al., 1997). The latter observations in conjunction with the previously mentioned lack of overt genotoxicity could suggest that one or more nongenotoxic mechanisms are involved in OTA renal toxicity and carcinogenicity in rodents. As male rats respond with the highest renal tumor and carcinoma incidence at extremely small OTA doses, the question is raised whether at least one or more of the mechanisms involved in OTA induced renal tumors in male rats are male rat specific. The existence of such a male rat specific mechanism involved in chemicals induced renal tumors has been demonstrated and shown to be causally related to the presence of the 18.7 kDa male rat specific urinary protein α2u-globulin (α2u) (Dietrich, 1995). One of those chemicals is D-limonene (dL), a natural terpene, occurring in ethereal oils of lemon, orange, caraway, dill and

bergamot, which is used industrially as a solvent, wetting and dispersing agent and as a scent. dL does not induce tumors of any organ of rodents with the exception of renal tumors in the male rat (NTP, 1990). Using the α2u deficient male NBR rat it was possible to demonstrate that the dL induced renal tumors were causally related to the presence of this male rat specific urinary protein (Dietrich and Swenberg, 1991a).

OTA was shown to bind to serum albumin and to a yet unidentified 20 kDa rat serum protein with high affinity (Stojkovic et al., 1984; Hagelberg et al., 1989; Fuchs and Hult, 1992), and that the largest amounts of OTA found in the kidney accumulated in the proximal tubules, where kidney lesions appear to be predominantly located (Elling, 1977; Lee et al., 1984; Fuchs, 1988; Breitholz-Emanuelsson et al., 1991; Fuchs and Hult, 1992). Therefore the question was asked whether OTA binding to α2u and subsequently increased cell proliferation could exacerbate the tumorigenic response in male rats and thus be responsible for the higher tumor incidence observed in male than in female rats.

2. Materials and methods

2.1. Animals

Fifteen male and female F344 rats (from SAVO, Kisslegg im Allgäu, Germany) 5–6 weeks of age were quarantined for 2 weeks prior to the onset of the experiments. The animal rooms were maintained at 21 ± 1°C, and a 12-h light/dark cycle. Tap water and feed (Nafag 890 from Nafag AG, Gossau, Switzerland) were given ad libitum throughout the study.

2.2. Experimental procedure

The rats were assigned to three groups of five male and five female rats each. Control rats received corn oil only (control: 2 ml/kg, Mazola, Knorr-Nährmittel AG, Thayngen, Switzerland), while the dL group received dL dissolved in corn oil (1650 mg/kg/day, Sigma, St. Louis, USA) according to a previously established protocol demonstrating high tolerance of these high doses (Dietrich and Swenberg, 1991b). Analogous to the dL group the OTA group received OTA dissolved in corn oil (1 mg/kg per day, Sigma, St. Louis, USA). All animals were gavaged daily for 7 consecutive days and killed 24 h after the last treatment as described below.

2.3. Cell proliferation studies

At the beginning of the experiment, i.e. immediately following the first time of gavaging, the animals were anesthetized with metofane® (Pitman-Moore, Washington Crossing, USA) and 7-day osmotic mini-pumps (Alzet®, model 2ML1, Lot No. 52965, flow rate 10.77 μl/h, 7 day delivery, Alza, Pato Alto, USA) were implanted subcutaneously. The mini-pumps contained 2 ml BrdU solution (20 mg BrdU/ml sterile PBS, pH 7.4, Sigma 5002).

2.4. Histopathology and immunohistochemistry

Twenty-four h after the last dose the animals were anesthetized with metofane and perfused via the abdominal aorta as described by Dietrich and Swenberg (1991b). Briefly, the animals were perfused with 185 mOsm (pH 7.4) sodium phosphate buffer followed by fixative solution (1% glutaraldehyde and 2% paraformaldehyde in 185 mOsm (pH 7.4) sodium phosphate buffer). During the perfusion with sodium phosphate buffer and prior to perfusion with the fixative solution, the left kidney was removed for immunological characterization of α2u by Western blot analysis. The fixed right kidney was further immersed in fixative solution over night at 4°C and embedded in paraffin thereafter. Hyaline droplet evaluation and general pathology was performed on sections (4–5 mm thick) stained with hematoxylin–eosin. For measurement of cell proliferation in the proximal tubules, the kidney sections were stained immunohistochemically for incorporation of BrdU into DNA as described by Dietrich and Swenberg (1991a). Briefly, a monoclonal primary antibody (anti-BrdU IgG; Becton Dickinson, Research Triangle Park, USA) was detected with a secondary link antibody (biotinylated goat anti

IgG), which links the primary antibody to a label (streptavidin) coupled with alkaline phosphatase (BioGenex, San Ramon, USA). Fast red was used as the chromogen and the sections were counterstained with hematoxylin.

For quantification of labeled and unlabeled P_2-cells, a minimum of 500 P_2-cells in at least 15 randomly chosen microscopic fields of the kidney cortex were counted (magnification 400 $\times$). The P_2-cell labeling index was calculated as the percentage of labeled cells over the total number of cells counted for each animal analyzed. The labeling index of each group was expressed as the arithmetic mean $\pm$ S.D. The presence of α2u was assessed via an α2u-specific immunostain. The staining procedure involved a monoclonal primary antibody (anti α2u IgG (Dietrich and Swenberg, 1991b)) and the same link and label that were used for the BrdU-immunostaining. α2u-Immunostained sections from each animal were assigned a score based on the intensity and area of positive staining. The grade scale ranged from 0 (negative) to 5 (markedly severe).

2.5. SDS-Page and immunoblotting

The left kidney was rinsed in PBS, weighed and homogenized in a glass homogenizer in ice cold PBS at a tissue:buffer ratio of 1:2 (w/v). The insoluble material was removed by centrifugation (200 $\times$ *g*, 10 min). The supernatants were diluted 1:10 with buffer. The protein content was measured by the method of Bradford (1976) using bovine serum albumin as the standard. The protein samples were separated by SDS-polyacrylamide gel electrophoresis using a 4% stacking gel and a 12% separating gel (Laemmli, 1970). Subsequently the proteins were transferred electrophoretically to a 0.2 μm-pore nitrocellulose membrane (Schleicher and Schuell, Rhien, Switzerland). The membranes were rehydrated in PBS after transfer and incubated for 1 h at room temperature in blocking solution (5% dry milk, 0.02% sodium azide in PBS). After rinsing, the membranes were incubated with primary antibody (anti α2u IgG (Dietrich and Swenberg, 1991b); diluted 1:6000 in blocking solution) overnight at 4°C. After washing, the membranes were incubated with a secondary horseradish peroxidase (HRP)-linked anti mouse antibody diluted 1:100 in PBS for 45 min at room temperature. Detection was performed with an ECL detection kit (Amersham, Buckinghamshire, UK) and electrochemical luminescence was recorded on autoradiography film.

3. Results

3.1. Pathological changes

Treatment of male and female rats with 1 mg/kg OTA induced severe lesions predominantly involving P_3 epithelial cells of the proximal tubules, i.e. located at the junction of the cortex and the outer stripe of the outer medulla. These lesions were characterized by necrotic and regenerating single epithelial cells or multiple cells and tubules (Fig. 1). In addition, desquamation and exfoliation of whole cells into the tubular lumen as well as cells seemingly undergoing apoptotic necrosis (Majno and Joris, 1996; Levin, 1998) were observed (Fig. 2). Some of the epithelial cells of severely affected tubules presented with enlarged nuclei and an increased nucleus to cytosol ratio (Figs. 1 and 2). Although the lesions were similar in appearance and location in male and female rats, the lesions in the male rats appeared

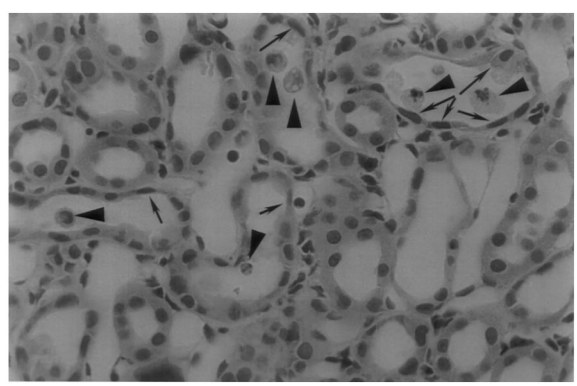

Fig. 1. Hematoxylin–eosin stained kidney section of a male rat, treated with 1 mg/kg OTA for 7 days, demonstrating a high number of necrotic (—►) exfoliated or regenerative (→) epithelial tubules cells in the inner part of the cortex (magnification $\times$ 400).

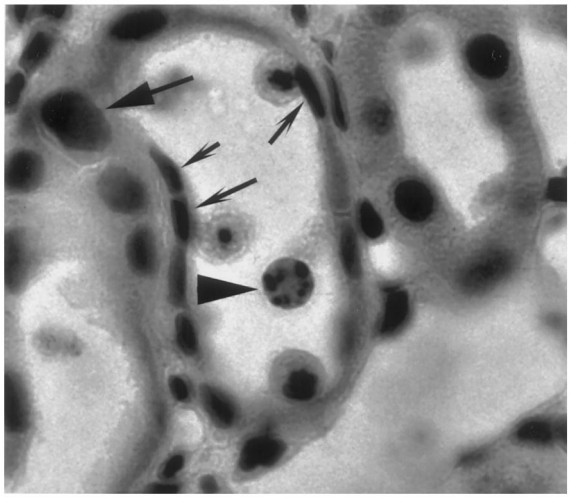

Fig. 2. Hematoxylin–eosin stained kidney section of a male rat, treated with 1 mg/kg OTA for 7 days, demonstrating exfoliated cells seemingly undergoing apoptotic necrosis (►). Regenerative epithelial cells (→) as well as cells (—►) with giant nuclei can be observed within the same affected tubule (magnification x 800).

more frequent (involving more tubules per area as judged from the cross-sections) and more severe than those in the OTA treated female rats. No such changes were observed in control male and female rats (Fig. 3). As expected the typical lesions associated with α2u-nephropathy (Dietrich, 1995) were seen in male rats treated with 1650 mg/kg dL: hyaline droplets, necrotic (oncotic necrosis (Majno and Joris, 1996; Levin, 1998)) proximal tubule epithelial cells of the outer cortex of the kidney and exfoliation and regeneration of epithelial cells, predominantly of the P_2 region (Fig. 4). In contrast, neither an enhanced formation of hyaline droplets nor necrotic cells were detected in the kidney cortex of female rats treated with dL. However, an increased number of regenerative epithelial cells were seen in the region of the outer cortex of the kidney suggesting some treatment associated cytotoxicity.

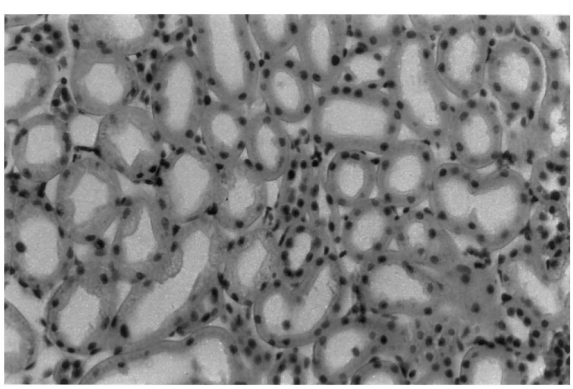

Fig. 3. Hematoxylin–eosin stained kidney section of a control male rat (magnification × 200).

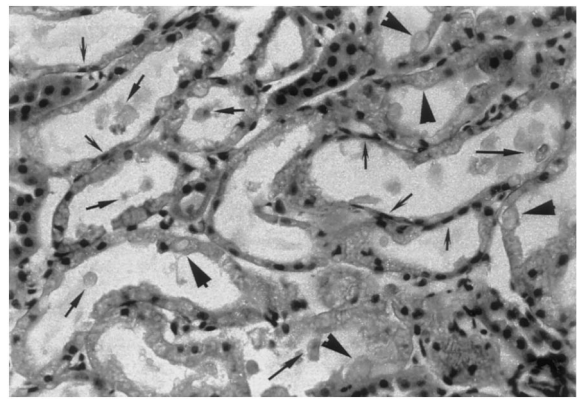

Fig. 4. Hematoxylin–eosin stained kidney section of a male rat, treated with 1650 mg/kg dL for 7 days, demonstrating an increase in hyaline droplets accumulation (►), necrosis (—►) and regeneration (→) of epithelial cells in the P_2 segment of the proximal tubules (magnification × 200).

3.2. α2u-Immunohistochemistry and immunoblotting

OTA treatment of male rats did not significantly increase the area nor intensity of α2u-immunostaining in the proximal tubule epithelial cells (immunostains not shown). Female rats, treated or control, were negative for α2u-immunostaining, whereas all male rat kidney sections stained positive for α2u. Male rats treated with 1650 mg/kg dL demonstrated a massive increase in α2u-staining area and intensity (Table 1). This marked increase in α2u-accumulation corresponded to those areas of the renal cortex in which dL induced pathological changes were observed.

Immunoblotting analysis of α2u in kidney homogenates corroborated the immunohistochemi-

Table 1
α2u-Immunostaining intensity and cell proliferation rates (labeling index) of P_2 cells in kidney sections of control and treated male and female F344 rats

Group	α2u-Immunostaining		Labeling index (%)	
	Male	Female	Male	Female
Control	1–2	0	7.0 ± 1.2	7.0 ± 0.7
dL	4–5	0	20.0 ± 6.6*	22 ± 5.0*
OTA	1	0	7.0 ± 1.9	6.0 ± 2.0

Note: grade scale for area and intensity of α2u-immunostaining: 0, absent; 1, present; 2, mild; 3, moderate; 4, severe; and 5, markedly severe.
* Significantly different ($P < 0.05$ two-way ANOVA) from controls and OTA group.

cal observations. Indeed, no α2u was detected in the female rat kidney homogenates of all groups, whereas 2 bands positive for α2u, representing the native protein (18.7 kDa) and a 16.6 kDa degradation product (Saito et al., 1991; Lehman-McKeeman and Caudill, 1994), were detected in all male rat kidney homogenates (dotted and full arrow in Fig. 5). Although the Western blots were evaluated only qualitatively, a higher amount of the native and degradation product of α2u was observed in the dL treated male rats when compared to the corresponding controls. In contrast, OTA treatment appeared to reduce the α2u content of both the native form and the degradation product present in the kidney homogenates.

3.3. Cell replication

OTA treatment had no effect on P_2 cell proliferation in either sex of rats (Table 1). dL treatment, on the other hand, led to the expected 4–5-fold increase in P_2-cell proliferation rate subsequent to treatment of male rats with a chemical reversibly binding to α2u (Dietrich, 1995). However, treatment with this rather high dose of dL also increased P_2 cell proliferation rates in female rats. It was surprising that dL exposed female rats also developed an increase in P_2-cell proliferation. It is assumed that the high dose of 1650 mg/kg body weight applied for 7 consecutive days may have led to cytotoxicity and regeneration of proximal epithelial cells independent of α2u accumulation. In contrast, no cytotoxicity or regenerative replication was reported in NBR rats exposed to 1650 mg dL/kg body weight applied for 4 consecutive days (Dietrich and Swenberg, 1991b) nor in male NBR rats and female F344 rats exposed to 150 mg dL/kg body weight for 6 months and 2 years, respectively (NTP, 1990; Dietrich and Swenberg, 1991a).

4. Discussion

The renal lesions induced by OTA differed in all characteristic points from those induced by dL. OTA induced lesions appeared to be primarily located in the P_3 segment of the proximal tubules i.e. at the outer stripe of the medulla, while cells of the P_2 segment, which are primarily affected by α2u-inducing agents such as dL, presented with little or no pathological changes. Indeed, P_2-proximal tubule cells of OTA treated rats showed neither an accumulation of α2u, overt signs of single cell exfoliation, necrosis nor regeneration of individual epithelial cells. This observation is cor-

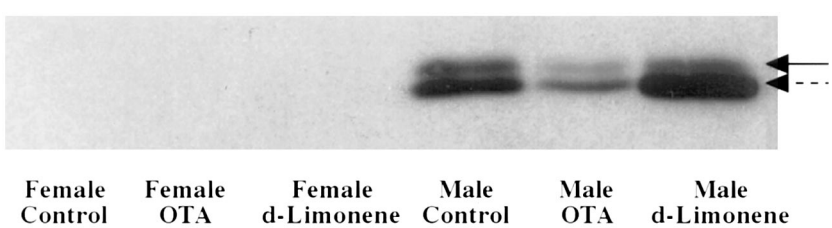

Fig. 5. Western blot analysis of kidney homogenates (5 μg protein/lane) of female and male rats treated with OTA (1 mg/kg body weight), dL (1650 mg/kg body weight) or corn oil for 7 consecutive days. Arrows (→) indicate the native form and the degradation product of α2u globulin detected with the monoclonal antibody.

roborated in that no differences were observed in the P_2 cell proliferation response between OTA treated male and female rats, indicating that OTA has neither a cytotoxic nor mitogenic activity in P_2 cells. Furthermore, OTA treatment led to an overall decrease of the native form and the degradation product of α2u present in the kidneys of male rats (Saito et al., 1991; Lehman-McKeeman and Caudill, 1994). This reduction apparently affected the amount of both α2u forms present in the kidneys equally (Fig. 5). α2u Is a low molecular weight protein that is synthesized in large amounts at the onset of puberty under multihormonal control in the liver of male but not female rats (Lehman-McKeeman et al., 1989; Swenberg et al., 1989; Lehman-McKeeman et al., 1990; Dietrich and Swenberg, 1991a,c; Swenberg, 1991; Lehman-McKeeman, 1993; Lehman-McKeeman and Caudill, 1994; Dietrich, 1995). Because of its low molecular weight (187 kDa) α2u is freely filtered by the glomerulus. Forty percent of the filtered α2u is excreted via the urine while the rest is reabsorbed primarily by P_2 cells of the renal cortex proximal tubules where it is enzymatically hydrolyzed via lysosomal digestion. In view of the limited pathological changes observed in the P_2 cells of the proxima, the observed decrease in the amount of α2u present in the renal cortex following OTA treatment of male rats may be possibly due to a general reduction of liver protein synthesis via competitive inhibition of phenylalanin–tRNAPhe synthetase by OTA (Creppy et al., 1984; Röschenthaler et al., 1984) rather than to a decreased reabsorption of α2u by the proximal tubules.

The pathological changes observed following treatment of male and female rats with OTA were comparable and involved primarily the P_3 segment of the proxima. The changes included karyomegaly and exfoliation of single cells, oncotic and/or apoptotic necrosis (Majno and Joris, 1996; Levin, 1998) and increased regeneration of single cells as well as of whole tubules (Figs. 1 and 2), corroborating histopathological changes observed previously in the proxima of rats following chronic treatment with OTA (Mantle et al., 1991). Similar pathological changes were reported by Heussner et al. (1998) in primary rat cortex and distal kidney cells as well as in LLC-PK1 cells and by Seegers and coworkers (Seegers et al., 1994) in hamster kidney cells exposed to moderate to high OTA concentrations (10^{-7}–5×10^{-5} M) in vitro. The above findings thus indicate that comparable pathological changes can be observed at high and low doses of OTA in an acute and chronic exposure setting, respectively. This would suggest that similar mechanisms of toxic interactions of OTA lead to the observed acute nephropathy and contribute to renal tumor formation.

Apoptotic necrosis, within the setting of the experiments presented here, could be described using the hematoxylin and eosin stained tissue sections only (Fig. 2). All efforts aimed at demonstrating DNA laddering using DNA extracts from the renal cortex of OTA treated rats failed (data not shown), most likely to the low number of cells affected in proportion to the 'normal' cells present. This interpretation is corroborated by Seegers and coworkers (Seegers et al., 1994) who found a maximum of 5% of hamster kidney cells exposed to OTA in vitro to undergo apoptotic necrosis. In contrast to the findings presented here, the latter authors were able to demonstrate DNA laddering, however, only after synchronizing the hamster kidney cells and then analyzing the detached cells in the supernatant of the exposure dish. It is interesting to note that in the in vivo study presented here, cells undergoing apoptotic necrosis were almost exclusively observed in the lumen of the affected tubules. This may suggest that OTA induced apoptotic necrosis of renal epithelial cells is a late event, i.e. following exfoliation of epithelial cells from the tubules into the lumen. If indeed this were the case, the question must be asked whether the primary interaction(s) of OTA is an adverse interaction with the cellular mechanisms governing cell–basal lamina adhesion or cell–cell recognition.

In comparison to the renal pathological changes observed following OTA treatment, male rats treated with dL (Fig. 4) demonstrated the expected pathological changes characteristic of α2u-nephropathy (Webb et al., 1989; Dietrich and Swenberg, 1991a; Dietrich, 1995), such as accumulation of α2u, necrosis and compensatory cell proliferation of P_2 epithelial cells. Indeed, the

accumulation of the less digestible chemical–α2u complex within the lysosomes of the P_2 segment of the kidney is thought to lead to a lysosomal overload, bursting of lysosomes and subsequent release of lysosomal enzymes to the cytosol, cell necrosis and finally compensatory cell proliferation (Dietrich, 1995). Upon chronic dL exposure this compensatory cell proliferation was demonstrated to promote the formation of renal tumors in male rats but not female or the α2u-deficient male NBR rats (Dietrich and Swenberg, 1991a).

The lack of concordance between the pathological changes as well as α2u-immunostaining and α2u-accumulation in male rats treated with OTA and dL suggested that OTA does not bind to α2u. It is therefore concluded that OTA binding to the male rat specific urinary protein α2u is not the contributory mechanism explaining the higher renal tumor incidences in male than in female rats following exposure to OTA.

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