

Ochratoxin A: Apoptosis and Aberrant Exit from Mitosis due to Perturbation of Microtubule Dynamics?

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Ochratoxin A (OTA) is a potent nephrotoxin and causes high incidences of renal tumors in rodents. The molecular events leading to tumor formation by OTA are not well defined. Early pathological changes observed in kidneys of rats treated with OTA *in vivo* include frequent mitotic and abnormally enlarged cells, detachment of tubule cells, and apoptosis within the S3 segment of the proximal tubule, suggesting that OTA may interfere with molecules involved in the regulation of cell division and apoptosis. In this study, treatment of immortalized human kidney epithelial (IHKE) cells with OTA (0–50 μ M) resulted in a time- and dose-dependent increase in apoptosis and activation of c-Jun N-terminal kinase. At the same time, OTA blocked metaphase/anaphase transition and led to the formation of aberrant mitotic figures and giant cells with abnormally enlarged and/or multiple nuclei, sometimes still connected by chromatin bridges. Immunostaining of the mitotic apparatus using an α -tubulin antibody revealed defects in spindle formation. In addition, OTA inhibited microtubule assembly in a concentration-dependent manner in a cell-free, *in vitro* assay. Interestingly, treatment with OTA also resulted in activation of the transcription factor nuclear factor kappa B (NF κ B), which has recently been shown to promote cell survival during mitotic cell cycle arrest. Based on these observations, we hypothesize that the mechanism by which OTA promotes tumor formation involves interference with microtubuli dynamics and mitotic spindle formation, resulting in apoptosis or—in the presence of survival signals such as stimulation of the NF κ B pathway—premature exit from mitosis. Aberrant exit from mitosis resulting in blocked or asymmetric cell division may favor the occurrence of cytogenetic abnormalities and may therefore play a critical role in renal tumor formation by OTA.

Key Words: ochratoxin A; carcinogenicity; apoptosis; mitosis; microtubule.

The mycotoxin and food contaminant ochratoxin A (OTA) (N-[[[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl]-carbonyl]-3-phenyl-L-alanine) is a potent nephrotoxin and causes high incidences of renal tumors in rodents (Boorman

et al., 1992; NTP, 1989). The molecular events leading to tumor formation by OTA are not well defined, and both genotoxic and nongenotoxic mechanisms may be involved. Chemically induced renal tumors in rats frequently occur as a result of direct interaction of the compound or its metabolite with DNA, generation of reactive oxygen species leading to oxidative DNA damage, or sustained regenerative cell proliferation in response to cytotoxicity (either directly or mediated via α_2 -globulin accumulation) (Lock and Hard, 2004). However, the mechanism by which OTA functions to induce tumors does not appear to fit into any one of these categories. Several studies using radiolabeled OTA indicate that OTA does not form covalent DNA adducts (Gautier *et al.*, 2001; Gross-Steinmeyer *et al.*, 2002; Mally *et al.*, 2004; Schlatter *et al.*, 1996), and although OTA has been shown to induce DNA strand breaks presumably via oxidative stress, the contribution of these lesions to renal tumor formation remains unclear (Mally *et al.*, 2005b). Furthermore, accumulation of α_2 -globulin, sustained cytotoxicity, and compensatory cell proliferation do not play a role in OTA carcinogenicity (JECFA, 2001; Rasonyi *et al.*, 1999).

Kidney tumors in OTA-exposed rats arise from the straight segment of the proximal tubule and are characterized by a relative rapid onset, aggressive behavior, tendency toward an uncommon anaplastic phenotype, and high potential to metastasize (JECFA, 2001; Lock and Hard, 2004). Early histopathological alterations, which occur even after short-term OTA treatment, consist of disorganization of the tubule arrangement and a high incidence of apoptosis and mitosis (Mally *et al.*, 2005c). Abnormally enlarged and aberrant mitotic figures, karyocytomegaly, and increasing polyploidy were frequently observed in kidneys of OTA-exposed animals, indicating that DNA replication occurred in the absence of cytokinesis (Maaroufi *et al.*, 1999; Mally *et al.*, 2005c). These changes were most prominent in tubule cells located in the outer stripe of the outer medulla and appear to be a specific feature of OTA toxicity. Moreover, the presence and severity of nonneoplastic changes were found to correlate with tumor incidence in the 2-year bioassay, suggesting that these early changes may play a critical role in tumor formation mediated by OTA (Boorman *et al.*, 1992). However, the molecular

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events leading to these histopathological alterations remain unclear.

In vitro, OTA has been shown to induce apoptosis in a variety of cell lines (Gekle *et al.*, 2000; Gennari *et al.*, 2004; Horvath *et al.*, 2002; Kamp *et al.*, 2005; Schwerdt *et al.*, 1999a; Scibelli *et al.*, 2003). Mitogen-activated protein (MAP) kinases, which are involved in a wide variety of cellular events including cell death by apoptosis, have been implicated in OTA toxicity. Results from several studies demonstrate that apoptosis induced by OTA is associated with activation of the stress-activated protein kinase c-Jun N-terminal kinase (JNK) and p38 (Gekle *et al.*, 2000; Horvath *et al.*, 2002; Sauvant *et al.*, 2005a). In addition, treatment of kidney epithelial cells with OTA resulted in activation of the extracellular signal-regulated kinase (ERK) 1/2, which is generally activated in response to mitotic stimuli and is thought to promote cell survival (Horvath *et al.*, 2002; Sauvant *et al.*, 2005a). It has, therefore, been suggested that OTA toxicity may be mediated at least in part by modulation of MAP kinase signaling, yet little is known with respect to the initial damage which triggers activation of these pathways.

JNK is known to be activated in response to a wide variety of stimuli including proinflammatory cytokines, UV radiation, reactive oxygen species, genotoxicity, and cellular stress. Recent evidence indicates that loss of cell anchorage may also provide a strong stimulus for JNK activation, suggesting a role of JNK in detachment-induced apoptosis (Frisch *et al.*, 1996a). Apoptosis induced by loss of cell adhesion has been shown to occur in renal epithelial cells in response to nephrotoxicants *in vitro* (van de Water *et al.*, 1999) and has been put forward as an interesting hypothesis to explain apoptotic cell death caused by OTA, particularly since detachment of tubule cells from the basement membrane was frequently observed in kidneys of rats treated with OTA *in vivo* (Mally *et al.*, 2005c; Rasonyi *et al.*, 1999). In COS cells, treatment with OTA resulted in cell detachment and inhibition of focal adhesion kinase (FAK), a central component of integrin-mediated survival signaling, suggesting that disruption of cell adhesion may contribute to JNK activation and apoptosis in response to OTA (Scibelli *et al.*, 2003). However, other prominent changes induced by OTA treatment *in vivo* such as karyomegaly and aberrant mitosis may not be easily explained by loss of cell adhesion, and the possibility that disruption of cell-matrix interactions may be the consequence rather than the cause of OTA toxicity needs to be considered. In this study, we have utilized a human kidney epithelial cell line derived from the proximal tubule to characterize early effects of OTA on cell division, cell adhesion, and cell death by apoptosis to gain further insights into the mechanism of OTA toxicity and carcinogenicity.

MATERIALS AND METHODS

Chemicals. Acetone, ethanol, and methanol were purchased from Merck, Darmstadt, Germany. OTA was purchased from Sigma-Aldrich, Taufkirchen,

Germany (lot-no. 38H4120) and from Prof. Peter Mantle, Imperial College of Sciences, London, United Kingdom. Purity of OTA was > 99.9% as assessed by HPLC with UV and fluorescence detection. Primary antibodies used were monoclonal mouse anti-phospho-JNK (Thr 183/Tyr 185) (Cell Signaling, Beverly, MA), mouse anti-phospho-FAK (Tyr 397) (BD Biosciences, Heidelberg, Germany), mouse anti-phospho-ERK (Santa Cruz, Heidelberg, Germany), mouse anti- α -tubulin (Sigma, Taufkirchen, Germany), and polyclonal rabbit anti-nuclear factor kappa B (NF κ B) (p65) (Santa Cruz, Heidelberg, Germany). Unless otherwise indicated, all other chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany).

Cell culture. Immortalized human kidney epithelial (IHKE) cells (originally obtained from S. Mollerup, National Institute of Occupational Health, Norway) were kindly provided by Dr Evelyn O'Brien, University of Konstanz, Germany. This cell line, established from normal human embryonic kidney epithelial cells by chemical transformation with nickel(II), has been demonstrated to display morphological and physiological characteristics of the proximal tubule (e.g., presence of apical brush border and various transport systems, and gap junction intercellular communication) (Hirsch *et al.*, 1998, 1999; Jessen, 1994; Jessen *et al.*, 1994, 1996; Rivedal *et al.*, 1996) and has previously been used in a number of studies to assess OTA toxicity *in vitro* (Benecic *et al.*, 2000; Eder *et al.*, 2000; Schwerdt *et al.*, 1999a,b). Cells were cultured under standard cell culture conditions (37°C, 5% CO₂) in Dulbecco's modified Eagle medium (DMEM)/Ham's-F12 (1:1) supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Colbe, Germany), 15mM HEPEs, 2mM glutamine, 0.1 μ M hydrocortisone, 5 mg/l human apotransferrin, 5 mg/l bovine insulin, 10 μ g/l human epidermal growth factor, 5 μ g/l sodium selenite, 2000 U/l penicillin, and 2 mg/l streptomycin. OTA was freshly dissolved in ethanol and diluted in DMEM supplemented with 5% FCS and 2mM glutamine.

Cytotoxicity. Cells were seeded in 96-well plates at a density of 1×10^4 cells per well, allowed to grow for 24 h in OTA-free medium (~ 75% confluency), and subsequently incubated with OTA at concentrations between 0 and 100 μ M for up to 24 h. For the determination of numbers of cells per well, cells were washed in PBS, 50 μ l of 0.2% crystal violet in 0.1M citric acid was added to each well, and stained nuclei were counted using a hemocytometer. Release of lactate dehydrogenase (LDH) into the medium was determined using the Cytotox 96 nonradioactive cytotoxicity assay (Promega, Mannheim, Germany) according to the manufacturer's instructions. The MTT-based *in vitro* toxicology assay kit (Sigma) was used to measure the activity of living cells via mitochondrial dehydrogenases. The general principle of this cytotoxicity assay is the conversion of the tetrazolium salt (MTT) to the colored product formazan, which can be measured photometrically at 570 nm, by intact mitochondria. Each assay was performed in at least two independent experiments carried out in triplicate.

Mitosis and apoptosis. The frequency of mitosis and apoptosis was determined by morphological evaluation of cells grown on coverslips and stained with 4',6-diamidino-2-phenylindole (DAPI) as described below (immunofluorescence). Cell preparations were analyzed at $\times 63$ magnification using a Leitz Diavert inverted fluorescence microscope fitted with a Nikon DXM 1200 digital camera. Apoptotic cells were identified based on chromatin condensation and fragmentation, morphological features characteristic for apoptosis. Mitotic cells were further categorized into prometaphase/metaphase and anaphase/telophase. Cells with irregular and abnormally enlarged nuclei were classified as aberrant cells. Apoptosis was also determined by flow cytometry after Annexin V/propidium iodide (PI) staining as previously described (Mally *et al.*, 2006).

Immunofluorescence. Cells were seeded at a density of 2.5×10^4 cells per well onto coverslips in 12-well plates and allowed to grow for 2 days until they reached ~ 75% confluency. Following OTA treatment, cells were washed in PBS, fixed in 10% neutral buffered formalin, and permeabilized by treatment with 0.2% Triton X-100 for 5 min. For immunostaining of α -tubulin, cells were fixed in methanol at - 20°C. After fixing, cells were washed in PBS, blocked in 10% goat serum in PBS, and subsequently incubated with primary antibody (1:100 to 1:500 in 1% bovine serum albumin in PBS overnight at 4°C) and

FITC-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, Alabama) or goat anti-rabbit IgG (Sigma, Taufkirchen, Germany) (1:50 dilution, 1 h at room temperature [RT]). Coverslips were mounted with Vectashield mounting medium containing DAPI (Vector, Burlingame, CA). Duplicate slides were prepared for each treatment.

Western blot. Cells were seeded into 60-mm cell culture dishes at a density of $3\text{--}4 \times 10^5$ cells per plate, allowed to grow to subconfluency (~75%) and exposed to OTA for up to 24 h. To obtain total cell lysates, cultures were washed twice with ice-cold PBS and lysed in 500 μ l RIPA buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1% Nonidet P40, 0.24% Na-deoxycholate, 1mM EDTA, 1mM Na_3VO_4 , 1mM NaF, and 1 μ l/ml protease inhibitor cocktail [Sigma, containing 104mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 80 μ M aprotinin, 2mM leupeptin, 4mM bestatin, 1.5mM pepstatin A, and 1.4mM E-64]). Following incubation on ice for 15 min, cell lysates were centrifuged for 15 min at 4°C and 10,000 rpm. Protein concentrations in the supernatant were determined using the Bio-Rad DC protein assay (Bio-Rad, München, Germany). To determine subcellular localization of NF κ B, nuclear and cytosolic fractions were prepared by lysing cells in 10mM HEPES, pH 7.5, 150mM NaCl, 0.6% Nonidet P40, 1mM EDTA, 1 μ l/ml protease inhibitor cocktail (Sigma, Taufkirchen, Germany), 1mM Na_3VO_4 , and 1mM NaF. After incubation for 20 min on ice, nuclei were pelleted by centrifugation at 4°C and 1500 rpm for 15 min and homogenized in nuclear extraction buffer (20mM HEPES, pH 7.9, 420mM NaCl, 1.5 MgCl_2 , 0.2mM EDTA, 25% glycerol, 5mM dithiothreitol, 1 μ l/ml protease inhibitor cocktail, 1mM Na_3VO_4 , and 1mM NaF). Protein concentrations in the cytosolic fraction (supernatant) were determined using the Bio-Rad DC (detergent compatible) protein assay (Bio-Rad, München, Germany), while protein concentrations in the nuclear fraction were measured by the Bio-Rad protein assay (Bio-Rad, München, Germany) due to the high glycerol content of the nuclear extraction buffer, which is not compatible with the Bio-Rad DC protein assay.

Equal amounts of protein (10 μ g) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat, dry milk in TBST buffer (50mM Tris, 150mM NaCl, 0.1% Tween 20, pH 7.4) for 2 h at RT and subsequently incubated with primary antibody (overnight at 4°C) and secondary goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP (both Amersham Biosciences, 1:2000, in 5% nonfat, dry milk in TBST, 2 h at 4°C). Dilutions of primary antibodies were 1:200 (anti-phospho-ERK, anti-NF κ B), 1:1000 (anti-phospho-FAK), and 1:2000 (anti-phospho-JNK) in 5% nonfat, dry milk in TBST. Membranes were developed using the ECL western blotting detection system (Amersham Biosciences, Freiburg, Germany) and exposed to Hyperfilm (Amersham Biosciences, Freiburg, Germany). Densitometry was performed using the Bio-Rad Gel Doc 2000 system running with Molecular Analyst software (Bio-Rad, München, Germany). Changes in protein expression in response to OTA treatment are presented as percentage of control.

Microtubule polymerization assay. Microtubule proteins were prepared from horse brain by two polymerization-depolymerization cycles according to the method of Shelanski *et al.* (1973). The protein pellet was resuspended in assembly buffer (100mM morpholinoethanesulfonic acid, 0.5mM MgCl_2 , 1mM EGTA, pH 6.4), and the protein concentrations were determined using the Bio-Rad protein assay. For the assembly assay, OTA was dissolved in DMSO and added to 0.5 ml of assembly buffer containing 1 mg/ml microtubule protein to give a final concentration of 50–400 μ M. After a 15-min incubation at 37°C, microtubule assembly was started by the addition of GTP (final concentration 0.5mM) and the increase in turbidity in the samples measured at 340 nm for 30 min. Disassembly at 4°C and a second assembly cycle were carried out to distinguish between normal microtubule formation and aggregation. Control incubations were carried out in which OTA was omitted and were used as reference (100% assembly). Colchicine at a final concentration of 2 and 4 μ M was used as a positive control.

Statistical analysis. Data are expressed as mean \pm SD. Statistical analysis was performed by ANOVA and Dunnett's multiple comparison test. Values significantly different from control are indicated as * p < 0.05 and ** p < 0.01.

RESULTS

Cytotoxicity of OTA in IHKE cells was assessed over a wide range of concentrations using cell number, mitochondrial activity, and LDH release as end points for toxicity. Cell number was found to be the most sensitive end point for OTA cytotoxicity (Fig. 1). A clear decrease in cell viability was observed in response to treatment with $\geq 1\mu\text{M}$ OTA for 24 h. Consistent with previous results obtained in LLC-PK1 cells (Mally *et al.*, 2005a), OTA did not lead to a significant release of LDH into the culture medium, indicating that loss of cell viability under these conditions was not primarily due to necrosis (Fig. 1). Consistent with these findings, a time- and concentration-dependent increase in the number of cells showing chromosome condensation and fragmentation—morphological features characteristic for apoptosis—was evident following exposure to OTA (Fig. 2). The presence of apoptotic cells was further confirmed by flow cytometry of cells stained with Annexin V/PI, which showed a dose-dependent increase in the number of cells in early and late apoptosis, whereas Annexin⁻/PI⁺ cells (necrotic cells) were not increased in response to OTA treatment (data not shown).

Loss of cell adhesion has previously been suggested to be involved in OTA toxicity, and we, therefore, investigated the effects of OTA treatment on cell contact sites. Similar to results obtained in COS cells (Scibelli *et al.*, 2003), treatment of IHKE cells with OTA for 24 h resulted in disruption of cell-cell and cell-matrix contacts. This was associated with decreased phosphorylation of FAK, a central component of integrin-mediated cell adhesion and survival signaling, which was clearly evident at concentrations of 10 μM and above, as shown by immunofluorescence and western blot analysis (Fig. 3). Consistent with the inhibition of FAK and increased apoptosis, treatment of IHKE cells with OTA lead to activation of the

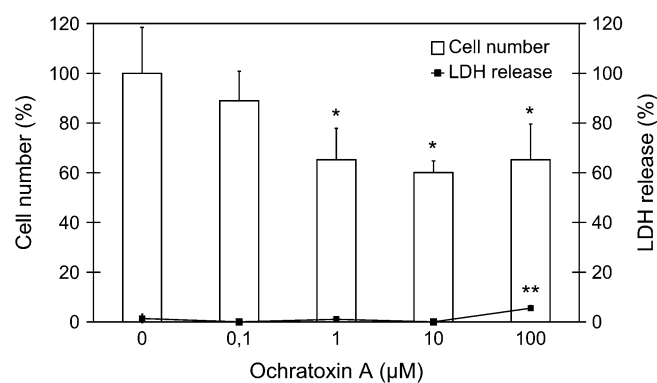


FIG. 1. Cytotoxicity of OTA in human kidney epithelial cells following exposure for 24 h. Cell viability was reduced in OTA-treated cells as assessed by determination of cell number. However, OTA did not induce a significant release of LDH into the culture medium, suggesting that loss of cell viability was not due to necrotic cell death. Data are presented as mean \pm SD. Statistical analysis was performed by ANOVA followed by Dunnett's *post hoc* test. Statistically significant changes compared to controls are indicated as * p < 0.05 and ** p < 0.01.

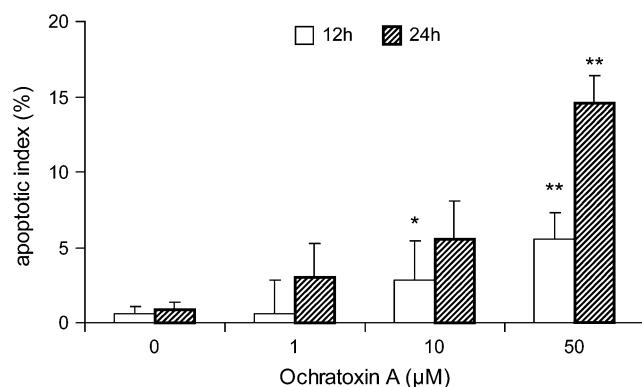


FIG. 2. Treatment of human kidney epithelial cells with OTA resulted in a time- and dose-dependent increase in the number of apoptotic cells as determined by morphological assessment of chromosome condensation and fragmentation. Data are presented as mean \pm SD. Statistical analysis was performed by ANOVA followed by Dunnett's *post hoc* test. Significant changes compared to controls are indicated as * $p < 0.05$ and ** $p < 0.01$.

MAP kinase JNK as indicated by enhanced immunoreactivity toward an antibody which specifically detects phosphorylated (activated) JNK (Fig. 4). At the same time, exposure to OTA resulted in activation of the (antiapoptotic) transcription factor NF κ B through translocation of the NF κ B subunit p65 into the nucleus, demonstrated by both western blotting and immuno-

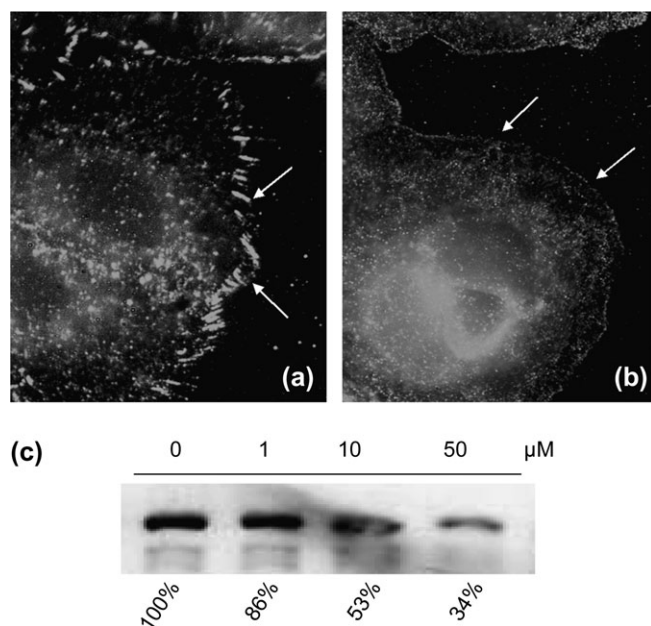


FIG. 3. Effects of OTA on phosphorylation of FAK, a central component of integrin-mediated cell adhesion. The autophosphorylated form of FAK (p-Tyr 397) was detected by immunofluorescence and western blot analysis using a phosphorylation-specific antibody. OTA inhibited phosphorylation of FAK in a concentration- and time-dependent manner. Distinct staining of phosphorylated FAK enriched into punctate adhesive contacts at the cell periphery (arrows) is evident in untreated IHKE cells (a), but is no longer detectable following treatment with 50 μ M OTA for 24 h (b). Representative blot showing a concentration-dependent decrease in FAK phosphorylation after a 24-h treatment with OTA (c).

cytochemistry (Fig. 4). Consistent with previous results in kidney epithelial cells (Horvath *et al.*, 2002; Schramek *et al.*, 1997), increased phosphorylation of ERK1/2 was also observed in cells treated with 50 μ M OTA for 24 h by immunoblotting (Fig. 4).

In evaluating nuclear morphology, we noted that later stages of mitosis (anaphase/telophase) were rarely present in OTA-treated cells in contrast to controls and completely disappeared after incubation with 50 μ M OTA for 24 h (Table 1, Figs. 5a–5c). This was associated with an overall decrease in the rate of mitosis (Table 1). In addition, aberrant metaphases with apparently misaligned chromosomes were frequently observed in the presence of OTA (Figs. 5b and 5d). More importantly, treatment with OTA resulted in the formation of giant cells with abnormally enlarged or multiple nuclei, which were sometimes still connected by chromatin bridges (Figs. 5e and 5f), indicating defects in chromosome segregation and/or cytokinesis. While enlarged cells were infrequently observed in untreated IHKE cells, the number of aberrant nuclei was significantly increased in OTA-treated cultures in a time- and dose-dependent manner (Table 1). Aberrant chromosome segregation and cell division may result from defects in assembly and function of the mitotic spindle, which can be visualized by α -tubulin staining. While untreated cells showed perfectly normal, symmetric spindles, immunofluorescence analysis revealed the formation of irregular, asymmetric, and sometimes multipolar mitotic spindles in the presence of OTA (Figs. 5g–5i).

A cell-free, *in vitro* tubulin polymerization assay was used to determine if defects in mitotic spindle formation were caused by interference with microtubule polymerization. OTA inhibited microtubule assembly in a concentration-dependent manner; however, relatively high concentrations were required to affect tubulin polymerization compared to known microtubule-interfering agents such as colchicine, which was used as a positive control (Fig. 6).

DISCUSSION

OTA is one of the most potent renal carcinogens studied by the NTP to date (Lock and Hard, 2004). However, the primary events leading to tumor formation by OTA are poorly understood. Repeated administration of OTA to rats results in uncommon histopathological alterations which are not consistent with the nonspecific action of reactive intermediates or oxidative stress in the kidney, suggesting that the mechanism of tumor formation by OTA may be highly different from that of most known renal carcinogens (Mally *et al.*, 2005c). Kidney pathology induced by OTA consists of frequent mitotic and abnormally enlarged cells, detachment of tubule cells from the basement membrane, and apoptosis within the S3 segment of the proximal tubule (Boorman *et al.*, 1992; Maaroufi *et al.*, 1999; Mally *et al.*, 2005c). In this study, we found that OTA

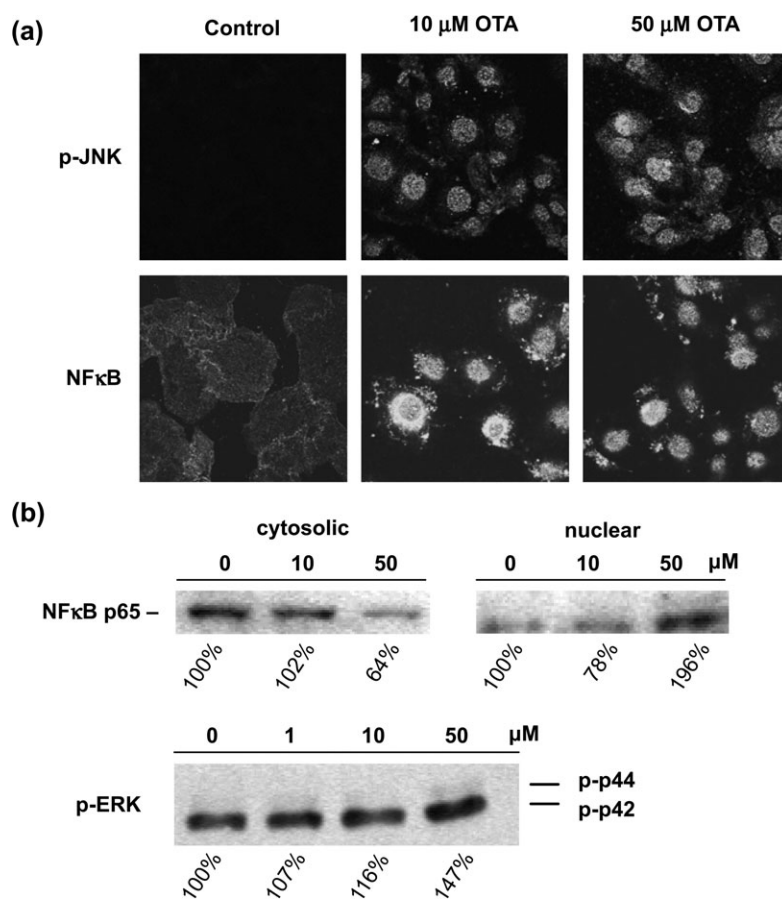


FIG. 4. (a) Representative immunofluorescence images demonstrating increased phosphorylation (activation) of JNK and activation of the transcription factor NFκB. (b) Activation of ERK1/2 and translocation of the NFκB p65 subunit from the cytoplasm into the nucleus indicative of NFκB activation were also confirmed by western blot analysis.

induces morphological alterations in IHKE cells *in vitro*, which closely resemble the effects of OTA in rat kidney, and have utilized this model to gain a better understanding of the primary molecular events involved in OTA toxicity and carcinogenicity.

Although it is difficult to predict intracellular concentrations of OTA in the target cell population *in vivo*, concentrations of OTA which induced apoptosis and aberrant mitosis in IHKE cells in this study were in the same range as OTA plasma

TABLE 1

Concentration-Dependent Effects of OTA on Mitotic Index, Percentage of Cells in Early (prometaphase/metaphase) and Late Stages of Mitosis (anaphase/telophase), and Number of Aberrant Nuclei Determined in Human Kidney Epithelial Cells following Exposure for 12 and 24 h. Data Are Expressed as Mean ± SD. Statistical Analysis Was Performed by ANOVA + Dunnett's Test. Values Significantly Different from Controls are Indicated as * $p < 0.05$ and ** $p < 0.01$

OTA (μM)	Mitotic index (%)	% Cells in prometaphase/metaphase	% Cells in anaphase/telophase	Aberrant nuclei (%)
12 h				
0	3.40 ± 0.32	1.76 ± 0.33	1.64 ± 0.51	1.97 ± 0.61
1	3.46 ± 1.20	2.15 ± 0.85	1.31 ± 0.57	3.26 ± 1.51
10	2.47 ± 1.11	2.15 ± 0.98	0.32 ± 0.30*	4.36 ± 1.15*
50	2.22 ± 0.10*	2.09 ± 0.21	0.13 ± 0.11*	7.25 ± 1.16**
24 h				
0	3.70 ± 1.05	2.53 ± 1.25	1.17 ± 0.21	3.11 ± 1.23
1	2.91 ± 0.43	1.85 ± 0.83	1.06 ± 0.50	3.77 ± 3.30
10	1.79 ± 0.22	1.73 ± 0.21	0.06 ± 0.11*	6.13 ± 1.82*
50	1.12 ± 0.49*	1.12 ± 0.49	0.00 ± 0.00*	11.04 ± 2.01*

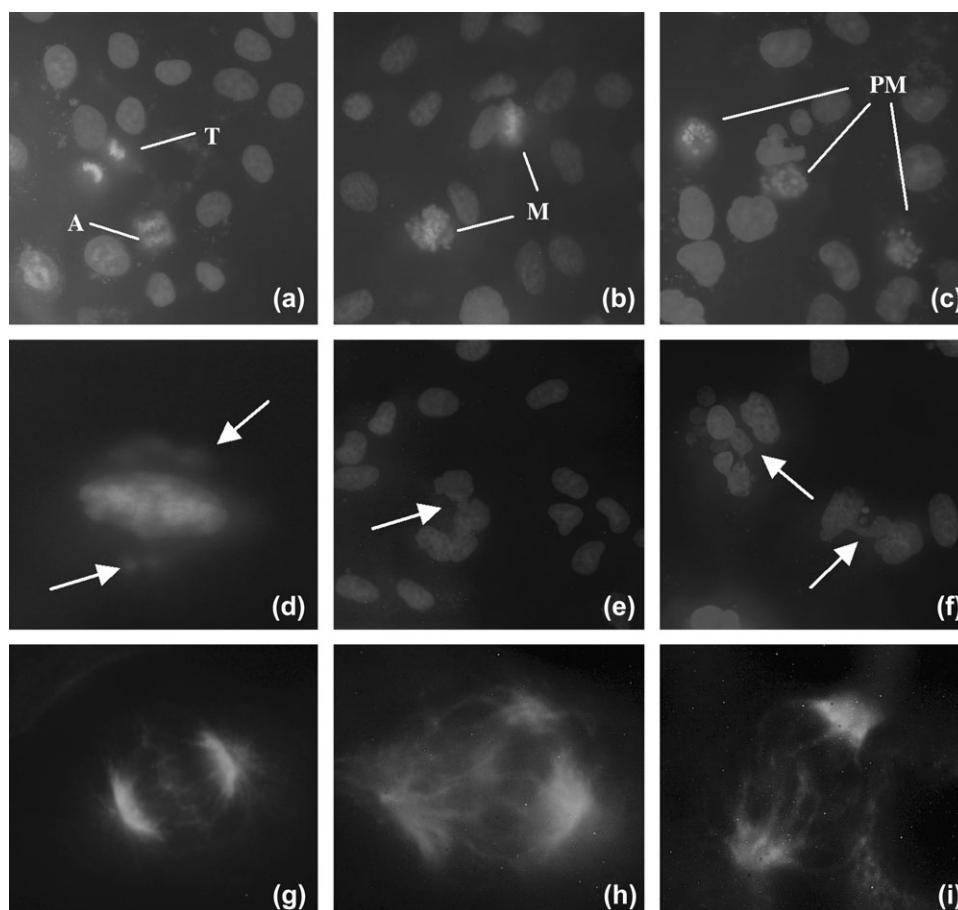


FIG. 5. Disruption of mitosis in human kidney epithelial cells treated with OTA and stained with DAPI. In contrast to untreated cells (a), later stages of mitosis were not detected in OTA-treated cell cultures (b, c), suggesting that OTA blocked metaphase-anaphase transition. In addition, aberrant mitotic figures (d) and cells with abnormally enlarged and multiple nuclei, sometimes still connected by chromatin bridges, (e, f) were frequently observed in cultures incubated with OTA. Immunofluorescence analysis using an anti- α -tubulin antibody revealed the formation of asymmetric, sometimes multipolar, mitotic spindles in the presence of OTA (h, i). In contrast, no spindle defects were observed in control cells (g) (A, anaphase; T, telophase; M, metaphase; PM, prometaphase).

concentrations obtained after repeated administration of OTA to rats at doses known to cause tumors in the chronic rodent bioassay (Mally *et al.*, 2005c), suggesting that the effects observed *in vitro* may be highly relevant to the mechanism of OTA carcinogenicity. In humans, mean OTA plasma concentrations resulting from dietary intake within the European Union are at least three orders of magnitude lower (0.18–1.19 ng/ml corresponding to 0.45–2.95nM) (SCOOP, 2002). However, in some countries such as Tunisia, exceptionally high concentrations of up to 1136 ng/ml (2.8 μ M) have been reported in some individuals suffering from kidney or urinary disorders (Maaroufi *et al.*, 1995). Although a causal link between dietary OTA exposure and human diseases has not been established, it seems reasonable to assume that the morphological and molecular alterations reported here may also occur in individuals exposed to extremely high levels of OTA.

Based on the observation that detachment of proximal tubule cells from the basement membrane was frequently observed in OTA-treated rats and was accompanied by increased apoptosis

(Mally *et al.*, 2005c; Rasonyi *et al.*, 1999), we initially speculated that loss of cell adhesion may contribute to OTA-induced apoptosis. Increasing evidence suggests that cells that lose contact with the extracellular matrix undergo apoptosis because they lack pivotal survival signals normally transmitted by cell-matrix interactions, and activation of FAK by tyrosine phosphorylation has been recognized as an important mechanism by which adhesion-mediated survival signals are transferred into the cell (Frisch *et al.*, 1996b). Similar to recent results in COS cells (Scibelli *et al.*, 2003), we observed decreased phosphorylation of FAK in response to OTA treatment at concentrations which also resulted in activation of JNK and cell death by apoptosis. However, at the same time, OTA stimulated activation of the transcription factor NF κ B and ERK1/2, which are both known to be downstream effectors of activated FAK and mediate signals involved in cell proliferation and survival (Giancotti, 1999). Since activation of these pathways is not consistent with the known effects of FAK inhibition, we concluded that other events must occur in

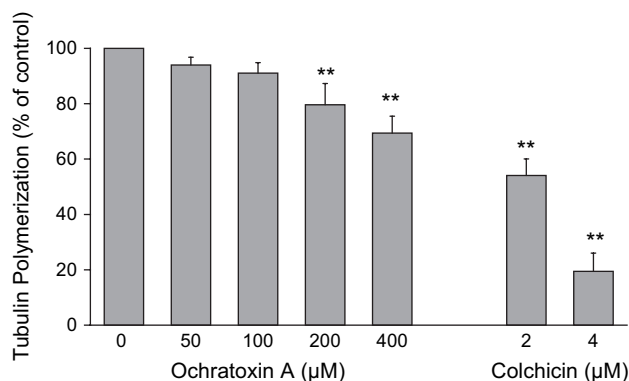


FIG. 6. Inhibitory effects of OTA and colchicine on microtubule assembly under cell-free conditions. Data represent mean \pm SD of four cycles of assembly and disassembly obtained from two independent experiments. Statistical analysis was performed by ANOVA followed by Dunnett's *post hoc* test. Values significantly different from controls are indicated as * $p < 0.05$ and ** $p < 0.01$.

response to OTA treatment which stimulate NF κ B and ERK1/2 survival signaling in OTA-treated cells.

Analysis of the mitotic index showed that the number of cells in mitosis was significantly reduced in the presence of OTA. While the percentage of cells in (pro)metaphase was not significantly altered, the proportion of cells in anaphase and telophase decreased in a time- and dose-dependent manner. These observations suggest that OTA did not block entry into mitosis but inhibited metaphase-anaphase transition, presumably because of defects in chromosome alignment and malfunction of the mitotic apparatus. Numerous natural compounds and anti-cancer drugs including several fungal metabolites exert their antiproliferative effects through perturbation of microtubule dynamics and mitotic spindle formation, resulting in metaphase arrest and subsequently apoptosis of mitotically blocked cells (Koizumi *et al.*, 2004; Pfeiffer *et al.*, 1998), and we, therefore, speculated that OTA may inhibit cell division in a manner similar to microtubule-disrupting agents. In support of this hypothesis, formation of aberrant mitotic spindles and inhibition of microtubule assembly were evident in the presence of OTA. Although the inhibitory effects of OTA on microtubule polymerization were weak compared to other known microtubule inhibitors such as colchicine and required relatively high concentrations, similar effects have been observed with citrinin, another mycotoxin produced by *Aspergillus* and *Penicillium* species (Pfeiffer *et al.*, 1998). While the exact nature of the interaction between OTA and microtubules remains to be determined, it is possible that OTA may inhibit microtubule assembly through mechanisms other than direct binding to tubulin, e.g., through interaction with microtubule-associated proteins.

It is also interesting to note that—in contrast to most microtubule-binding agents—OTA did not induce prolonged mitotic arrest in IHKE cells. Rather, it seems that cells blocked in early mitosis by OTA treatment rapidly commit to apoptosis (mitotic catastrophe) (Castedo *et al.*, 2004) or exit mitosis

without cell division (mitotic slippage), resulting in aberrant cells with increased nuclear content. Although the reasons for this apparent difference remain unknown, the failure of OTA to efficiently block mitosis, thus allowing a subpopulation of cells to escape apoptosis, presents a violation of the “better dead than wrong” principle and may explain why OTA promotes malignant transformation rather than effectively inhibiting mitosis such as microtubule-interfering agents used in cancer therapy. It is becoming increasingly evident that tetraploid cells that arise from failure of chromosome segregation and cytokinesis are genetically highly unstable and frequently progress to become aneuploid (Andreassen *et al.*, 1996). Although little is known regarding the signaling pathways which determine whether a cell transiently arrested in metaphase will exit mitosis rather than commit to apoptosis, NF κ B activation has been shown to suppress apoptosis and promote cell survival in response to microtubule-disrupting agents (Mistry *et al.*, 2004). In addition, NF κ B activation appears to promote cell cycle transition from G1 to S phase (Chen *et al.*, 2001) and may thus facilitate reentry of tetraploid cells into the cell cycle despite failure to divide in the previous cycle, thereby increasing polyploidy and genetic instability. Similarly, stimulation of ERK1/2 survival signaling by OTA may promote mitotic slippage by protecting cells from apoptosis. This is supported by recent findings, which show that simultaneous inhibition of ERK by pharmacological inhibitors potentiates OTA toxicity in opossum kidney tubule cells (Sauvant *et al.*, 2005b).

It is striking that many of the intracellular signaling pathways stimulated by OTA are also known to be activated by microtubule inhibitors (Kook *et al.*, 2000; Mistry *et al.*, 2004; Rosette and Karin, 1995; Wang *et al.*, 1998, 1999, 2000). In addition to stimulation of MAP kinase signaling, disruption of microtubules by nocodazole has been shown to induce degradation of several focal adhesion proteins including FAK (Kook *et al.*, 2000), suggesting that alteration of microtubule function may also provide an explanation for the observed decrease of FAK activity.

In summary, results from this study show that OTA blocks mitosis at metaphase-anaphase transition in human kidney epithelial cells and simultaneously activates pro- and antiapoptotic signaling pathways, resulting in mitotic cell death and aberrant exit from mitosis. Blocked or asymmetric cell division may favor the occurrence of cytogenetic abnormalities and may ultimately lead to tumor formation. Although further work is required to identify the primary cellular targets and fully understand the molecular events and signaling pathways leading to disruption of mitosis in kidney tubule cells exposed to OTA, it should be noted that the proposed mechanism may be able to reconcile most if not all aspects of OTA toxicity, including lack of mutagenicity in bacteria, lack of metabolic activation and DNA adduct formation, induction of micronuclei and chromosomal aberrations, apoptosis and mitotic abnormalities both *in vitro* and in kidneys of OTA-treated rats, and the high incidence and aggressive nature of OTA-induced renal tumors, and

may also provide a reasonable explanation for the apparent non-linear dose response for OTA carcinogenicity.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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