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Bladder epithelial cell proliferation of rats induced by terephthalic acid-calculi

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Abstract

Objective: Urinary bladder hyperplasia associated with terephthalic acid (TPA) treatment was examined with concomitant use of sodium bicarbonate ($NaHCO_3$) or hydrochlorothiazide to allow assessment of the relationship among bladder stones, epithelial hyperplasia, and corresponding cell cycle checkpoint gene expression in Sprague–Dawley (SD) rat.

Methods: A total of 112 weanling male SD rats that divided between six groups were given basal diet (control), diets containing 5% TPA or in combination with either 4% sodium $NaHCO_3$ or 0.02% hydrochlorothiazide. After 90-day feeding, bladder samples were collected for histopathological diagnoses, and immunohistochemical method was used to characterize the expression of $p16^{Ink4a}$, cyclin D_1, CDK_4, EGFr and cyclin E in relation to that of proliferating cell nuclear antigen (PCNA).

Results: In TPA treatment groups, bladder stone incidence was 40% (21/52) with 14 cases of proliferative bladder. In control and other groups, neither stone nor epithelial cell proliferation was diagnosed. PCNA-positive focal hyperplasic lesions involved all epithelial layers. Overexpressions of cyclin D_1, CDK_4, EGFr are found in the corresponding lesion. $p16^{Ink4a}$ nuclear staining reduced in proliferative bladders especially with a great quantity of stone. In addition, no positive expression was detected on cyclin E.

Conclusion: The present study provides a strong evidence of a link between induction of bladder hyperplasia, deregulation of the $p16^{Ink4a}$-cyclin D_1/CDK_4 pathway, and abnormal EGFr mediated signal transduction pathway.
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Keywords: Bladder hyperplasia; Terephthalic acid; Calculi; Immunohistochemistry; Cell-cycle proteins; PCNA

1. Introduction

Occupational exposures, cigarette smoking, bladder calculi and/or schistosomal infections are important factors for bladder cancer induction (Jung and Messing, 2000). Among them, bladder stone is a well-documented risk factor for tumor development in human and rodent (Stonehill et al., 1996; Ogasawara et al., 1995; Burin et al., 1995; Cohen, 1995a; Clayson et al., 1995). The development of calculi-induced bladder tumor depends on the extent of the stimulus, its initial change is focal of simple hyperplasia, following papillary and nodular hyperplasia, then papillomas or diffuse papillomatosis, and ultimately transitional cell carcinomas (Clayson et al., 1995). However, molecular pathway(s) underlying the processes above remain unclear.

Abbreviations: EGFr, epidermal growth factor receptor; IHC, immunohistochemistry; LI, labeling index; PCNA, proliferating cell nuclear antigen; PN, papillary or nodular; SD, Sprague–Dawley; TPA, terephthalic acid

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Terephthalic acid (TPA), one of the most commonly produced chemicals in the world, had been extensively used for the synthesis of certain crystalline polyester resins, films, and fibers. Oral administration of TPA at dietary of 3–5% for two weeks has been shown to induce bladder stone accompanied by hyperplasia of the bladder transitional epithelium, and this lesion was not detected in rats that ingesting large doses of TPA and in the absence of uroliths (Chin et al., 1981; Wolkowski-Tyl et al., 1982a; Heck and Tyl, 1985). Heck and Tyl (1985) demonstrated that bladder carcinomas were induced following TPA administration to rats for two years and the incidence was also related to the presence of calculi in the urinary bladder. Thus, either the hyperplasia or the carcinomas induced by TPA in rat urinary bladder appears to result from calculus formation. However, the concentration of TPA to attain super saturation in the urine was necessary to form the stone, and the dose of TPA intakes was approximately 2.0 g/day (Wolkowski-Tyl et al., 1982b), which was obviously impossible for human to absorb of such large quantities.

Increased cell proliferation can account for the carcinogenicity of non-genotoxic chemicals (Fukushima et al., 1992). It has been demonstrated that TPA-induced bladder stone disappeared after coadministration of sodium bicarbonate ($NaHCO_3$) or chlorothiazide (Wolkowski-Tyl and Chin, 1983; Wolkowski-Tyl et al., 1982a). This phenomenon may be the key to understanding the general mechanism of TPA-induced epithelium proliferation. In the present study, we evaluated the proliferation of TPA by its administration to rat for 90 days, and the expressions of key proteins at the G_1-S checkpoint, which may be involved in the bladder tumor development with TPA exposure. We also examined the effect of administration of TPA with $NaHCO_3$ and hydrochlorothiazide to determine the mechanism for the proliferation of TPA in the rat urinary bladder.

2. Materials and methods

2.1. Chemicals

Terephthalic acid (TPA), white powder, purity ⩾99.99%, obtained from Yi Zheng Chemical Fiber Co. (Jiang Su, China); p16^{Ink4a} mouse monoclonal IgG_{2a} antibody, mouse anti-proliferating cell nuclear antigen (PCNA) antibody, epidermal growth factor receptor (EGFr) rabbit polyclonal antibody, CDK_4 rabbit polyclonal antibody, cyclin D_1 mouse monoclonal IgG_{2b} antibody are purchased from Santa Cruz, mouse SP kit and rabbit SP kit from Beijing Zhongshan Biotechnology Co, Ltd; cyclin E rabbit polyclonal antibody from Wuhan Boster Bio Tech Ltd. All other chemicals used were of the highest purity commercially.

2.2. Animals

A total of 112 weanling male Sprague–Dawley (SD) rats were obtained from Shanghai B&K laboratory animal Co. Ltd (Shanghai, PR China). They were quarantined for 1 week before starting of the experiment. The rats were housed at a rate of 2 animals/cage in stainless steel wire mesh cages and placed in a controlled environment with a 12 h light-dark cycle (lights on 7:00–19:00) at of 22 ± 3 °C and 60 ± 10% humidity. Rats were ad libitum fed throughout the experiment. The Institutional Animal Care and Use Committee of Nanjing Medical University approved all conducted experiments.

2.3. Experimental design

SD rats were sampled to six dietary groups randomly. The experimental groups were administered with 5% TPA (52 rats), 5% TPA plus 4% $NaHCO_3$ (12 rats), 4% $NaHCO_3$ (12 rats), 5% TPA plus 0.02% hydrochlorothiazide (12 rats), and 0.02% hydrochlorothiazide (12 rats) in powdered basal diet, respectively. The control group (12 rats) was maintained on the basal diet without any treatment. The treatment schedule was continued for 90 days. During the experiment, a daily health check was performed, body weight measured each week, and food consumption recorded daily.

2.4. Urine analysis

Fresh urine samples (100–1000 μl) were collected by holding the rats directly over a 10 ml plastic tube and firmly squeezing the tail from 12 rats of each group in the morning (7:00–9:00) three days before sacrificed. Urinary pH were immediately detected with MI 129 IS-FET pH meter (Switzerland), and then stored at −20 °C. Two days before sacrificed, 12 rats of each group were kept separately in metabolic cages and urine was collected every 24 h for volume measurement.

2.5. Histopathology

Bladder, liver and kidney from each rat were removed and fixed in 10% formalin solution. Particular care was taken with the urinary tracts for stone observation. Tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). Histopathologically, the urinary bladder lesions were classified into simple hyperplasia and papillary or nodular (PN) hyperplasia according to the criteria described previously (Shirai et al., 1986, 1989).

2.6. Von Kossa staining

7 μm sections of bladder samples were deparaffinized and hydrated, immersed in 2% silver nitrate for 60 min

under ultraviolet light, then changed to 5% sodium hyposulfite for 2 min, and counterstained with 1% neutral red.

2.7. Immunohistochemical detection of $p16^{Ink4a}$, cyclin D_1, CDK_4, cyclin E

Serial sections of 4 μm thickness were cut and spread on APES coated slides. Paraffin sections were immersed in two changes of xylene and hydrated using a graded series of ethanol. Endogenous peroxidase activity was quenched by applying 3% hydrogen peroxide for 20 min. Antigen retrieval was performed routinely by microwave heating. Sections were immersed in citrate buffer (0.01 M, pH 6.0) in a glass container and were heated to boiling temperature repeatedly and then cooled down to room temperature. Non-specific binding was blocked by incubating sections with non-immune serum at room temperature for 20 min, and then sections were incubated overnight at 4 °C in a humidified chamber with respective primary antibodies: $p16^{Ink4a}$ 1:50, cyclin D_1 1:50, CDK_4 1:100 and cyclin E 1:50, following incubation with appropriate biotinylated secondary antibodies. Slides were then incubated with streptavidin peroxidase. Expression of $p16^{Ink4a}$, cyclin D_1, or CDK_4, cyclin E was localized by a final incubation with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and counterstained with light hematoxylin.

2.8. Cell proliferation determination by PCNA immunohistochemistry (IHC)

Monoclonal mouse anti-PCNA was used at 1:100 dilution, followed by incubations with biotinylated goat anti mouse antibody and streptavidin peroxidase as described above. PCNA analysis has the potential to identify cell cycle subpopulations (G_1, S, G_2, M) (Fong et al., 2000): dark-staining nuclei represent S-phase cells, light-staining nuclei represent G_1-S and G_2 cells, cells with cytoplasmic staining usually depict mitoses, and non-staining nuclei represent quiescent (G_0) cells. In our study, the spatial and temporal distributions of PCNA-stained nuclei (S phase, G_1-S/G_2) in bladder epithelium were assessed. The scores of labeled cells in the S phase and G_1-S/G_2, dark-staining and light-staining nuclei of the cross section of bladder were counted using light microscopy. PCNA labeling index (LI) was calculated by dividing the number of respective labeled cells by the total number of cells, and the result was expressed as a percentage.

2.9. Epidermal growth factor receptor (EGFr) IHC staining

For EGFr IHC staining, sections were incubated with 3% hydrogen peroxide for 20 min to quench endogenous peroxidase activity, and following with 0.1% trypsin for 60 min at room temperature. EGFr antibody at 1:50 dilution were applied overnight at 4 °C, then by incubations as described above, and membrane expression of epithelial cells were observed.

2.10. Immunohistochemical scoring

The IHC analysis was performed in a blind fashion without knowledge of the rat group. The percentage of positive stained nuclei or membrane was scored in bladder epithelium using the 40×objective. A total of 200 proliferation cells were counted in each slide. Positive staining was defined as the presence of ⩾20% antibody-stained nuclei for cyclin D_1, cyclin E, CDK_4, PCNA, and membrane for EGFr. Negative staining for $p16^{Ink4a}$ is defined as lost of its expression, either in the whole or extended areas of the hyperplasia. Two independent observers did immunohistochemical evaluation.

2.11. Statistical analysis

Statistical analyses of observed values were performed using two-way ANOVA or χ^2 test. A $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. General observations

SD rats exhibited hematuria (Table 1) after two weeks of 5%TPA treatment, whereas other groups of rats appeared healthy. The body weights, weight gains and food consumption showed no significant differences between groups throughout the experimental period. The urinary pH of rats fed with TPA + $NaHCO_3$, or TPA + hydrochlorothiazide, or TPA alone were significantly lower in comparison to that from the control groups, whereas administration of $NaHCO_3$ produced strongly alkaline urine. Animals treated with TPA, TPA + hydrochlorothiazide, $NaHCO_3$ and hydrochlorothiazide showed significantly urine volume elevation, respectively, and combination of $NaHCO_3$ with TPA demonstrated the same urine volume as control.

3.2. Induction of bladder hyperplasia

Rats with 90-days TPA subchronic feeding, total of 21 uroliths cases were detected. One rat was found with 3.171 g stone inside a notable expanded bladder. With *Von Kossa* staining, six cases of micro-calculus were found in the bladders of TPA treatment groups (Fig. 1). With H & E staining, bladder hyperplasia was diagnosed in 5% TPA treated rats. Among 14 cases of

Table 1
Data for urinary bladder histopathological findings

Groups	*n*	Hematuria	Bladder		
			Stone[a]	Simple hyperplasia	PN hyperplasia
Control	12	0	0	0	0
5%TPA	52	17	21	9	5
5%TPA + 4%$NaHCO_3$	12	0	0	0	0
4%$NaHCO_3$	12	0	0	0	0
5%TPA + 0.02%hydrochlorothiazide	12	0	0	0	0
0.02%hydrochlorothiazide	12	0	0	0	0

[a] Observed by optical and *Von Kossa* staining.

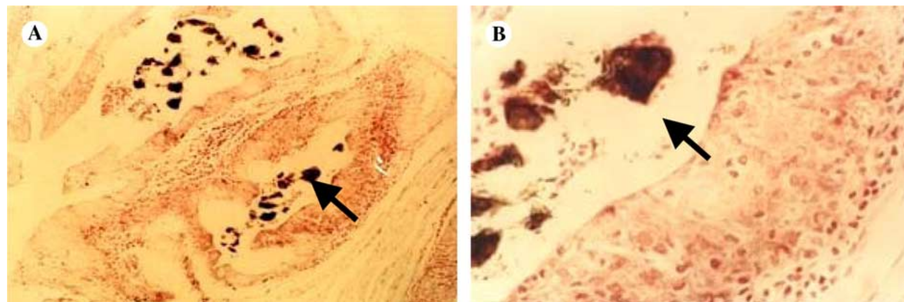

Fig. 1. (A) *Von Kossa* stained bladder section with black calculi inside and epithelium hyperplasia around, 10×; (B) Magnification of A, 40×.

diagnosed bladder hyperplasia, only three cases showed no stone or calculus. $NaHCO_3$ and hydrochlorothiazide treatment successfully abolished the TPA-induced calculi formation and bladder hyperplasia. The link between uroliths and bladder epithelial cell proliferation strongly suggested that chronic calculi stimulation could lead to urothelial cell proliferation.

3.3. Bladder cell proliferation determined by quantitative PCNA immunohistochemistry

Hyperplasia bladders had higher percentage of positive PCNA nuclei staining than normal tissues, and considerably higher LIs for S-phase, and S-phase and G_1-S/G_2 cells than control (Fig. 2).

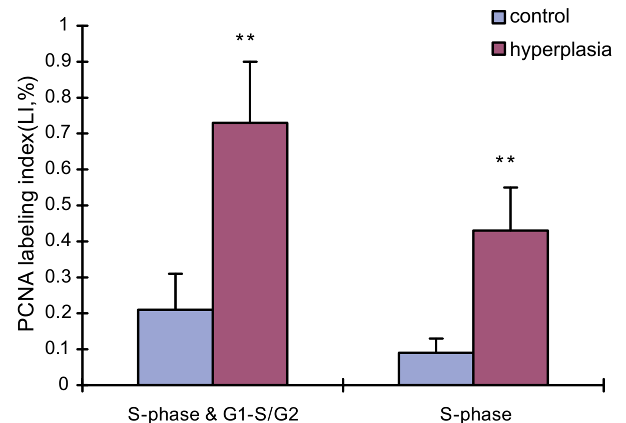

Fig. 2. Epithelial cell proliferation in rat bladder stone model as determined by PCNA immunohistochemistry. LI is calculated by dividing the number of respective labeled cells by the total number of cells, and the result is expressed as a percentage. S phase: experiment versus control, $**P < 0.01$, S phase and G_1-S/G_2: experiment versus control, $**P < 0.01$.

3.4. Expression and localization of cell cycle proteins in urothelial cells

3.4.1. Expression of $p16^{Ink4a}$–cyclin D_1/CDK_4 and cyclin E

In agreement with accepted criteria of $p16^{Ink4a}$ staining (Fong et al., 2000), only distinct nuclear immunoreactivity was considered a sign of positivity. Simple bladder hyperplasia rats showed strong nuclear staining for $p16^{Ink4a}$ in all epithelial cell layers, with the percentage of positively stained cells ranging from 30% to 85%. In contrast, the PN hyperplasia urolithlial cells, especially the cells accompanied with a great quantity of bladder stones, showed absent or very minor staining for $p16^{Ink4a}$ (4/14). Fig. 4A and B depict examples of $p16^{Ink4a}$ staining in simple and PN hyperplasia bladder of rats with and without $p16^{Ink4a}$ expression, respectively. Control group, in addition, exhibited almost no cyclin D_1 and CDK_4 nuclear immunoreactivity in bladder epithelial cells. In contrast, calculi-induced bladder hyperplasia showed positive nuclear staining; the percentage samples detected with overexpression of both proteins in hyperplastic areas were 14% and 36% respectively. Positive expression of cyclin E was not detected either in the experiment or control group.

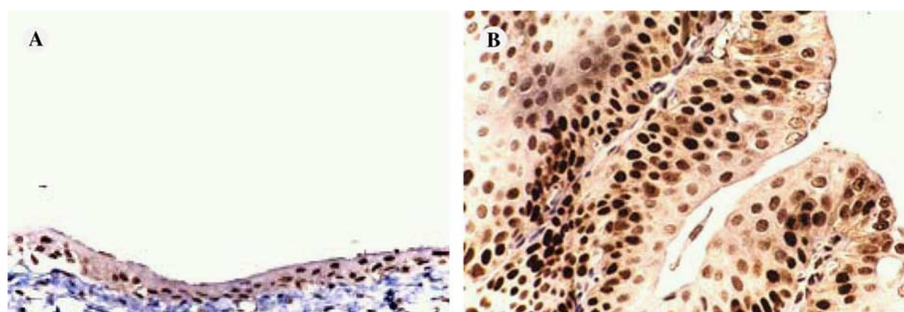

Fig. 3. PCNA expression in rat bladder epithelium (40×). (A) Brown nuclear staining for PCNA in the basal layer of normal epithelium; (B) hyperplasia bladder section stained for PCNA showed intense and extensive nuclear staining.

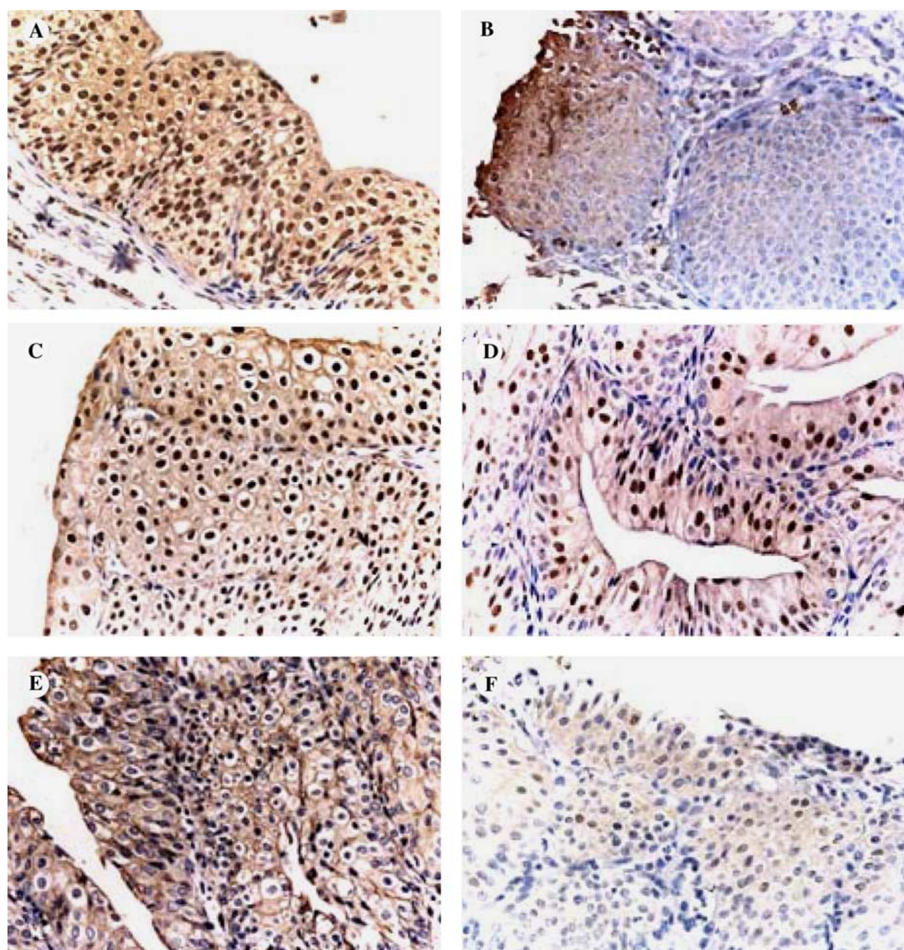

Fig. 4. Immunohistochemical assessment in hyperplasic lesions of the rat bladder ($N = 14$). (A) Positive nuclear staining for $p16^{Ink4a}$, 40×; (B) absent expression of $p16^{Ink4a}$ in highly proliferative epithelium, 40×; (C) overexpression of CDK_4 (brown nuclear, 40×); (D) nuclear staining for cyclin D_1, 40×; (E) positive membrane stained EGFr, 40×; (F) negative nuclei staining of cyclin E, 40×.

3.4.2. EGFr expression

Normal bladder exhibited weak EGFr membrane immunoreactivity in 10–15% of the basal cells. In contrast, 64% (9/14) cases of hyperplastic bladder showed positive membrane staining in basal, immediate and superficial layers.

4. Discussion

The predominant components of TPA-induced uroliths were TPA, calcium and phosphate (Chin et al., 1981), and its formation requires supersaturation of urine with TPA and calcium (Wolkowski-Tyl et al., 1982a;

Wolkowski-Tyl and Chin, 1983). In this study, we have confirmed that administration of 5% TPA induces urinary calculi which induced epithelial cell proliferation in the bladder of male rats. A clear correlation of bladder calculi and hyperplasia were found with *Von Kossa* staining in 5% TPA-treated rats (Fig. 1). The absence of calculi from the cases of hyperplasia may be ascribed to the passed or lost of stones during processing of tissues for histopathologic examination (Heck and Tyl, 1985). Neither stones nor hyperplasia were observed when ingested TPA was combined with 4% sodium bicarbonate or 0.02% hydrochlorothiazide (Table 1). Combination of $NaHCO_3$ with TPA did not significantly increase urinary pH, although it prevented of TPA-induced urolithiasis. The incorporation of $NaHCO_3$ into the TPA diet did not at an equivalent concentration to that of TPA might be the reason to explain this phenomenon. Hence, the mechanism of $NaHCO_3$ prevention of TPA-induced bladder stone via reduction of aciduria (Wolkowski-Tyl and Chin, 1983) may not be the major way. Wolkowski-Tyl and Chin (1983) reported that dietary 4% bicarbonate ameliorated but did not abolish the 4%TPA-induced aciduria, but enhanced urinary TPA and magnesium levels. Presumably that elevated magnesium may inhibit calcification and nucleation for calcium TPA stone nuclei in this study. Treatment with chlorothiazide is based on its ability to reduce urinary calcium and urinary TPA excretion (Wolkowski-Tyl and Chin, 1983), thus, it can also be speculated that reduced urinary calcium and/or reduction of urinary TPA by hydrochlorothiazide would contribute to the lack of calculi in the bladder.

Although the rat provides an animal model to study the urothelial effects of TPA, there is no evidence that TPA is carcinogenic in humans. In the 2-year bioassay, the bladder stone and neoplasm were only seen in rats ingesting 5%TPA (Heck and Tyl, 1985). These doses are considerably higher than any human exposures to TPA. Due to the anatomical differences between the position of the urinary bladders in rodents and in humans (Desesso, 1995) and the physical differences of urine composition (Cohen, 1995a,b) such as volume, osmolality, quantitative and qualitative differences in protein, rat urinary bladder is more sensitive to various sources of mechanical stimulation than human. These factors also prohibit the direct extrapolation of the rodent findings to human.

The thresholds may be existed in TPA-induced carcinomas, because the most studied mechanisms leading to urothelial toxicity and regeneration are related to the formation of urinary stones such as uracil and chalk (Shirai et al., 1986, 1989; Toyoshima and Leighton, 1975). Arnold et al. (1999) observed that rats fed dimethylarsinic acid in diet at 40 and 100 ppm produces urothelial cytotoxic and regeneration hyperplasia, but not at lower doses, their finding strongly support for the non-linearity of carcinogenic effects.

Bladder epithelium consists of three layers of cells including surface, intermediate and small basal cell. Cellular proliferation, which is regarded as important in the carcinogenic process both for fixation of altered gene information and promotion of tumor development, occurs mainly in the basal cell layer under normal condition. Although the mitotic index in the rat bladder epithelium is normally very low, it may be stimulated after a lag phase into considerably enhanced proliferative activity by physical trauma (Cohen, 1995a; Clayson et al., 1995). Cellular proliferation is involved in all carcinogenesis process including initiation, progress, development and metastasis (Jung and Messing, 2000; Brandau and Bohle, 2001). PCNA overexpression shows a strong correlation with the proliferative activity of the cell, it was applied as a cell proliferation marker in toxicology and as a prognostic marker in human tumors (Dietrich, 1993). It is speculated that the areas of PCNA-positive cells could be responded to mechanical irritation of the calculi (Otori et al., 1997). Our data showed that PCNA-positive cells (most of them in S-phase and G_1-S/G_2) were present at the whole layers of the proliferative epithelium (Figs. 3 and 4). TPA concomitant sodium carbonate or hydrochlorothiazide not only inhibited the formation of stone, but also abolished the lesions of bladder hyperplasia. Because there was no irritation due to calculi, PCNA-positive cells almost disappeared from this urinary epithelium.

Multiple studies have demonstrated that TPA belongs to non-genotoxic agents (Heck and Tyl, 1985). Thus, a carcinogenic effect of TPA itself on the rat bladder is highly unlikely. The hyperplasia observed in the present experiment were similar to the toxicity and regeneration seen with bladder calculi induced by feeding of uracil or other chemicals (Shirai et al., 1986, 1989; Toyoshima and Leighton, 1975) result from cell diversion of the epithelium of the urinary tract induced by the mechanical irritant effect of the stones. Prolonged stimulation of excessive proliferation of urinary bladder epithelial cells in rats results in tumor formation (Shirai et al., 1995; Otori et al., 1997). Studies in cell cycle showed that tumor cells typically have acquired gene damages that regulate of G_1-S progression pathway (Bartek and Lukas, 2001). Expression alterative of cell cycle regulators and subsequent deregulation of cell cycle are the most consistently events in bladder cancer (Brandau and Bohle, 2001; Knowles, 2001; Burchardt et al., 2000). To understand how tumors develop from some hyperplastic lesions, we evaluated the biological changes in TPA-induced bladder hyperplasia.

Cyclin D_1 overexpression is related to tumor differation, not to progression (Lee et al., 1997,; Takaba et al., 2000). Oya et al. (1998) demonstrated that cyclin D_1 overexpression drives cell proliferation in the early stage of urothelial tumorigenesis, and it is related to abnormal cell proliferation in bladder carcinomas. How-

ever, abnormal increased of CDK_4 is not a common event in tumorigenesis of human bladder (Simon et al., 2002). Our results showed a consistent correlation between distribution of PCNA-positive cells and cyclin D_1 or CDK_4 overexpression in bladder hyperplasia, that provide a new insight of the interaction of these molecules in the stage of hyperplasia by TPA-induced calculus.

Direct evidence showed that $p16^{Ink4a}$ inhibited of cell growth in $p16^{Ink4a}$-deficient mice (Serrano et al., 1995, 1996). Deletion expression of $p16^{Ink4a}$ protein is an early phenotype in bladder cancer (Friedrich et al., 2001). We measured $p16^{Ink4a}$ deletion expression on PN hyperplasia epithelium, especially in rats with great quantity of bladder stones and severe urothelium injury. The reasons for the decreased expression remain to be elucidated, but it could be due to methylation of the 5′CpG island of the p16 gene (Valenzuela et al., 2002) or mutation of the gene (Miyamoto et al., 1995), however, the data suggested dysfunction of $p16^{Ink4a}$-cyclin D_1/CDK_4 pathway was an early marker in chronic stimulation animal models of bladder hyperplasia.

EGFr expression is normally confined to the basal layer of the urothelium. It became abnormally expressed throughout the entire urothelium in transitional cell carcinomas including those superficial cells directly exposed to urine, and the overproduction of EGFr is related to bladder tumor development and progression (Marjou et al., 2000). Our studies showed that EGFr were expressed on all cell layers including superficial cells that contacted to the calculi. Because ligands that work through EGFrs not only induce mitogenesis but also cellular motility, stimulation of EGFrs in malignant urothelium may encourage transepithelial motility and tumor invasion as well as proliferation (Marjou et al., 2000; Cheng et al., 2002). Hence, abnormal EGFr expression might be an early event in bladder tumorigenesis by TPA.

In conclusion, the bladder with high proliferative rate in TPA treatment rats had altered expression profiles for the genes that control G_1-S progression. Dysfunctions of $p16^{Ink4a}$-cyclin D_1/CDK_4 pathway and overexpression of EGFr is the early molecular mechanism involving in TPA-induced bladder hyperplasia and may play an important role in tumors development.

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