Cell Proliferation Induced by Uracil-Calculi and Subsequent Development of Reversible Papillomatosis in the Rat Urinary Bladder

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ABSTRACT

The sequence of cellular alterations in urinary bladder epithelium associated with uracil-induced reversible urolithiasis was investigated in male F344 rats. Initial changes were submucosal edema with occasional mucosal erosion or ulceration which appeared on Day 2 of uracil administration. Simple hyperplasia of bladder epithelium was already evident at this time and calculus formation was noted as early as Day 4. Labeling indices in the bladder epithelium assessed by bromodeoxyuridine incorporation were about 32% on Day 4 and then gradually decreased to 6% at Week 8 and 4% at Week 25 of chronic treatment. Histologically, a direct progression from simple hyperplasias, through papillary hyperplasias to papillomatosis, accomplished by Week 5, was evident. Dysplastic lesions were also apparent by Week 25. Topographically, papillomatosis was composed of marked interconnecting mucosal ridges of relatively uniform width. No polypl-like protrusions were present and the vascular pattern revealed by resin perfusion casting demonstrated that these mucosal lesions were supported by a uniform plexus of capillary vessels. After withdrawal of uracil from the diet the labeling index dropped dramatically to 0.002% after 1 week and urolithiasis and papillomatosis had disappeared by Weeks 2 and 3, respectively.

The findings suggest that papillomatosis associated with uracil-calculi is a hyperplastic rather than a neoplastic response and that induction of putative neoplastic lesions is directly related to prolonged vigorous cell proliferation.

INTRODUCTION

The urinary bladder epithelium of rodents has been shown to readily proliferate in response to mechanical irritation such as the presence of stones (1–5), freeze ulceration (6), chemicals (7), or even physiological saline (8). Lalich (9) reported a high incidence of urolithiasis in rats given uracil orally, and recently, we found that a dietary supplement of uracil at a concentration of 3% induced mucosal papillomatosis in the urinary bladder of all rats treated (10, 11). Although urolithiasis and papillomatosis were severe and extensive, they disappeared when the treatment with uracil was stopped. Histological and transmission electron microscopic examination revealed that the epithelial cells of papillomas had essentially normal differentiation with the exception of numerous short uniform microvilli, ropy or rounded microridges, and occasional pleomorphic microvilli on the surface (10).

It was also demonstrated that even a short period of uracil administration strongly promoted tumour development in the urinary bladder of rats pretreated with a nitroso bladder carcinogen (11). Because of the lack of mutagenicity (12) and the reversibility of induced lesions, urothelial proliferation in rats given uracil was considered to be due to continuous irritation by the calculi formed. Thus, uracil-induced proliferative lesion development in rat urinary bladder can serve as a good experimental tool for studying regulation of epithelial growth and cell differentiation.

The present experiment was performed to clarify the sequence of morphological and proliferative changes associated with administration and withdrawal of uracil. A combined histopathological, BrdUrd incorporation, and scanning electron microscope approach was adopted to allow comparison with earlier findings after administration of unequivocal urinary bladder carcinogens. An investigation of vascular support by resin casting was also included.

MATERIALS AND METHODS

A total of 115 male F344 rats, 6 weeks old and weighing about 120 g at the beginning of experiments, were purchased from Charles River Japan Inc., Kanagawa, Japan. Animals were housed in plastic cages with hard wood chip bedding, in an air-conditioned room with a 12-h/12-h light/dark cycle, and given food (Oriental MF; Oriental Yeast Co., Tokyo, Japan) and water ad libitum. Uracil (Wako Pure Chemical Co., Osaka, Japan) was added to the basal diet at a final concentration of 3.0%. 102 rats were initially placed on diet containing uracil for up to 25 weeks. 37 of them were returned to a uracil-free diet after 8 weeks of treatment. 13 rats given only the basal diet served as nontreated controls. For histological evaluation, four to six rats continuously given uracil were sacrificed on Days 2, 4, and 7, and at Weeks 2, 3, 5, 8, 12, and 25 of continuous administration, and groups of four or five rats which received uracil for 8 weeks and were then returned to basal diet were sacrificed at 1, 2, 3, 6, and 17 weeks after the cessation of uracil administration as indicated in Tables 1 and 2. Nontreated control rats were killed 0, 8, 14, and 25 weeks after the beginning of the experiment. To assess levels of DNA synthesis in the urothelium, three rats per experimental group at each time point were given a single i.p. injection of bromodeoxyuridine at a dose of 120 mg/kg b.w., 40 min before sacrifice. BrdUrd (Sigma Chemical Co., St. Louis, MO) was dissolved in physiological saline at a concentration of 2.4% immediately before use. Injection of BrdUrd was always performed between 9 and 10 a.m. to avoid circadian variation. At sacrifice, the urinary bladder, ureter, and renal pelvis of rats were removed, inflated, and fixed in buffered 5% formalin for 3 days. They were routinely processed for histological examination and paraffin sections cut at 3 μm were stained with hematoxylin & eosin. Incorporation of BrdUrd into the nuclei was visualized immunohistochemically using the avidin-biotin-peroxidase complex method (13) with a monoclonal antibody against BrdUrd as described previously (14). Labeling indices were obtained by counting the number of labeled cells among at least 1000 epithelial cells per urinary bladder and expressed as percentage values.

Fixation of tissues with a mixture of 1% glutaraldehyde and 4% formalin (pH 7.2) at 4°C for longer than 48 h was applied in 23 rats for subsequent scanning electron microscopic examination, with postfixation in 1.0% osmic acid (one or two rats at each time point as described for the histological evaluation). Vascular casts of the urinary bladder were prepared as described previously (15) by perfusion with saline followed by injection of Mercox CL-2B (Dainippon Ink and Chemicals, Inc., Tokyo, Japan). This procedure was performed on two rats at each time point for the first 8 weeks of uracil administration. Vascular casts of the entire bladder were examined by scanning electron microscopy after tissue digestion.

1 The abbreviations used are: BrdUrd, bromodeoxyuridine; PN hyperplasia, papillary or nodular hyperplasia.

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RESULTS

Development of Urolithiasis and Cell Proliferation. Table 1 summarizes the results of histopathological examination of the urinary bladders, ureters, and kidneys according to the duration of the treatment with uracil. There was no urolithiasis on Day 2 of uracil administration. Some rats had hematuria from around Day 4. A few small calculi of <1 mm diameter, were evident in the urinary bladders of 10 rats killed on Day 4. After Day 4, all rats had calculi and the number of calculi and their size increased with increasing duration of treatment with uracil until after 3 weeks, the urinary bladders became enlarged and filled with numerous yellowish-white calculi varying from 1 to 3 mm in diameter. No further increase in size of calculi was evident thereafter. Thickening of the bladder wall with dilatation of the major blood vessels was also evident. Hydrouretere and hydronephrosis were first observed on Day 4 of uracil administration, becoming severe with increasing duration of the treatment. Sometimes variation between the right and left side kidneys and ureters was evident. Calculi were also found present in the ureter and kidney pelvis, and their appearance and incidence correlated with the development of calculi in the urinary bladder. However, they tended to be smaller than those found in this latter organ.

Histologically, on Day 2 of the treatment, inflammatory cell infiltration (neutrophils with a small number of lymphocytes) with edema was evident in the submucosal layer of all cases. Erosion or ulceration of the bladder epithelium was sporadically present. These lesions were not observed on Day 14 and thereafter. Mitotic figures were most frequent on Day 4 (Fig. 1a).

Proliferative lesions of the urothelium were classified as described previously (10, 16). In the early stage of uracil administration, the urinary bladder mucosa demonstrated simple hyperplasia (Fig. 1a) involving a diffuse increase in epithelial cell layers (five to six cells). This stage was followed by papillary hyperplasia (Fig. 1b) where short finger-like growths of the epithelium tissue supported by fine fibrovascular connective tissue protruded into the lumen. Papillary hyperplasia was occasionally accompanied by nodular hyperplasia (inverted growth of hyperplastic epithelium). After 3-week treatment with uracil, papillary projection of the urinary bladder epithelium became marked and generally distributed throughout the bladder wall of all animals treated, a condition referred to as papillomatosis (Fig. 1c). Thereafter, papillomatosis was present for as long as uracil was given. Single or multiple diverticuli lined with hyperplastic urothelium were observed in the urinary bladder of all rats given uracil for longer than 5 weeks. They all showed luminal formation and were normally located in the submucosa and muscle layer (Fig. 1d), although some extended

### Table 1 Sequential changes in development of calculi and in the histology of the bladder, ureter, and kidney tissues in rats given uracil for up to 25 weeks

<table>
<thead>
<tr>
<th>Duration of uracil treatment (days)</th>
<th>No. of rats examined</th>
<th>Urinary bladder</th>
<th>Ureter hyperplasia</th>
<th>Kidney hydronephrosis</th>
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<td>2 (1)</td>
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<td>7 (1)</td>
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<td>35 (5)</td>
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<td>84 (12)</td>
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<td>175 (25)</td>
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* PN hyperplasia, papillary or nodular hyperplasia.

* Dysplasia was found in three of five rats.

* No change; ±, trace; +, slight; ++, moderate; +++, marked.

Fig. 1. Proliferative epithelial lesions of the bladder in response to uracil treatment. a, simple hyperplasia with submucosal cell infiltration (on Day 4) (× 200). Four mitotic figures are evident; b, papillary hyperplasia (at Week 2) (× 100); c, papillomatosis (at week 5) (× 40); d, a diverticulum (at Week 8). Sections were stained with H & E.
into the subserosa. Diverticuli became large and cystic with prolonged administration of uracil and calculi were occasionally present. Of the five rats which received uracil for 25 weeks, three were found to have localized dysplastic lesions with distinct cellular atypia within the areas of papillomatosis (Fig. 2). Variability in cell and nuclear size and loss of nuclear polarity compared to the surrounding epithelium were pronounced.

The ureter epithelium also became hyperplastic and demonstrated development of uniform papillary structures. Papillary hyperplasia of the renal pelvic epithelium was first observed in rats given uracil for 25 weeks.

Disappearance of the Lesions. Withdrawal of uracil after 8 weeks treatment was associated with gradual regression of almost all the lesions induced (Table 2). The first dramatic change was disappearance of the calculi. Only a few tiny calculi could be seen in all five rats 7 days after the cessation of treatment and none were found after another 7 days. Epithelial cells within areas of papillomatosis demonstrated shrinkage and many basophilic bodies and vacuoles suggesting pyknotic cells or cell debris were observed in the mucosa (Fig. 3a). Papillary projection of the epithelium appeared to collapse (Fig. 3b). Within 3 weeks, almost all papillary growths of the epithelium had disappeared and the mucosa was composed of simple hyperplastic epithelium (simple hyperplasia) with a few areas of papillary or nodular hyperplasia (PN hyperplasia). Both lesions were observed in all rats. An increase of collagen fibers and occasional dilated blood vessels were evident in the submucosa. Diverticuli became smaller and smaller and finally disappeared or remained as tiny glandular cavities within the muscle layer. Dilated and thickened urinary bladder and ureter walls gradually returned to the normal appearance although papillary hyperplasia of the ureter did not regress as quickly as that in the urinary bladder.

The time sequence of development and disappearance of calculi and proliferative epithelial lesions in the bladder is illustrated in Fig. 4.

Cell Kinetics. Fig. 5 illustrates the changes in labeling indices of bladder epithelial cells during and after treatment with uracil. The index was expressed as the percentage of anti-BrdUrd-positive cells and the value for normal bladder epithelium before

![Fig. 2. Dysplasia of the bladder epithelium observed in a rat given uracil for 25 weeks (H & E, × 200).](image)

![Fig. 3. Epithelial changes of the bladder after withdrawal of uracil treatment, a, note vacuoles containing cell debris and pyknotic nuclei in the epithelial layer (H & E, × 400); b, collapse of the luminal protrusions at Week 2 (× 200).](image)

![Fig. 4. Illustration of the time sequence in the development and disappearance of calculi and epithelial proliferative lesions. S; simple hyperplasia, PN; papillary and/or nodular hyperplasia, P; papillomatosis.](image)

![Table 2. Disappearance of calculi and proliferative lesions in the bladder and ureter after cessation of uracil treatment](table)

<table>
<thead>
<tr>
<th>Weeks after uracil treatment</th>
<th>No. of rats examined</th>
<th>Calculi</th>
<th>Simple hyperplasia</th>
<th>PN hyperplasia</th>
<th>Papillomatosis</th>
<th>Atrophy of epithelium</th>
<th>Degeneration of epithelium</th>
<th>Diverticula</th>
<th>Ureter epithelial hyperplasia</th>
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* PN hyperplasia, papillary or nodular hyperplasia.

*-, no change; ±, trace; +, slight; ++, moderate; +++, marked.
the treatment with uracil commenced was about 0.06%. The labeling indices sharply increased on Days 2 and 4 of uracil treatment; the maximum labeling index was 31.5 ± 13.5% on Day 4 (Fig. 6a), followed by a sharp decrease to 15.0 ± 7.9 on Day 7 (at Week 1) and 11.2 ± 4.1 at Week 2. Thereafter, the mean labeling indices slowly declined to 6.4 ± 1.3 at Week 8 (Fig. 6b) and then 3.8 ± 1.9% at Week 25. Most labeled cells were located in basal and intermediate layers of the epithelium. A few were also observed in the surface layer in the early days of the treatment (Fig. 6a). Later most labeling was confined to the basal cells (Fig. 6b). One week after the treatment with uracil was stopped at Week 8, the labeling index dropped from 6.4 to 0.0024% (see Fig. 6c).

Surface Topography of the Urinary Bladder (Scanning Electron Microscopy). Scanning electron microscope studies revealed that the papillary mucosal proliferations observed in histological sections were winding, interconnected mucosal ridge-like protrusions (Fig. 7, a and b). The widths of mucosal ridges were relatively constant (50–75 μm) and there were no single polyp-like mucosal protrusions suggesting the presence of papilloma. There were sometimes hollows within the mucosa suggesting previous presence of calculi. Fine ridges, which are the normal surface structure of urothelial cells, became short, uniform microvilli and/or ropy or leafy microridges shortly after the start of uracil treatment. Pleomorphic microvilli were sometimes observed but did not increase at least within 16 weeks of uracil administration.

Vascular Casts (Scanning Electron Microscopy). The capillary bed of untreated urinary bladder was characterized by a loose plexus of vessels with uniform diameter present at a low density with the network of capillary vessels distributed in a relatively flat plane. As mucosal cells proliferated in response to treatment, irregular capillary ridges developed from the base of the capillary bed in a pattern corresponding to that of the mucosal ridges (Fig. 8, a and b). Capillary ridges were composed of loops of capillary vessels of relatively uniform diameter.

DISCUSSION

The results of the present experiments confirmed our previous findings that uracil at a concentration of 3% in diet readily induces urolithiasis and papillomatosis in rats and that these lesions, at least up to 15-week treatment, are reversible (10). Sequential analysis revealed that the development of urolithiasis and proliferation of the urothelium begins very shortly after the commencement of treatment with uracil with a very rapid, marked increase in DNA synthesis in the epithelial cells of the urinary bladder. Although DNA synthesis decreased from the peak of 32% observed on Day 4 of uracil administration, the level of labeling remained very elevated, being still 6.4% at Week 8, a value more than 100 times the normal level. Thus continuous vigorous cell proliferation throughout the bladder leads to formation of papillary growths of the epithelium, and since the maximum thickness of normal epithelium without fibrovascular proliferation seems to be seven or eight cell layers, over-proliferation inevitably induces mucosal exophytic growth which requires the support of fibrovascular tissue resulting in the formation of a diffuse papillary structure.

Although the histopathological pattern of epithelial growths superficially resembles that observed in tumors, the three-dimensional structure of the mucosa was revealed by scanning electron microscopy to be relatively normal. The mucosal ridges were of constant width and were supported by regular vascular...
URACIL-INDUCED REVERSIBLE PAPILLOMATOSIS IN THE RAT BLADDER

networks. In contrast, proliferative bladder lesions induced by carcinogens are topographically very abnormal (17, 18), with mucosal ridges or ruga being irregularly distributed and of varying dimensions. The characteristic sequence of events in carcinogen-treated bladders is the development of polyp-like papillary hyperplasias, papillomas and carcinomas (17, 18). The vascular pattern observed in the present study was similar to that of the Type 3 vascular proliferation in the reversible stage of response to both carcinogens and noncarcinogens (15, 19). Thus the reversibility and morphological characteristics described in the present paper indicate that papillomatosis of the urinary bladder induced by uracil calculi is not a true neoplastic lesion.

The observed induction of vigorous cell proliferation seems to be due to direct mechanical irritation by the calculi formed by uracil. This conclusion is supported by the fact that no other epithelial cells other than those comprising the urothelium were involved and that there was a correlation in the degree between calculus formation and epithelial cell proliferation; the urinary bladder exhibited the most marked cell proliferative response while the epithelium of the ureter or renal pelvis showed only mild proliferative responses. It is well known that rat urinary bladder is very sensitive to various sources of mechanical stimulation (2-5). It is also noteworthy that coadministration of 5% NaCl in the diet with 3% uracil inhibited the formation of uracil calculi, presumably due to threefold increase in urinary volume, and resulted in no hyperplasia of the mucosal epithelium.

Erosion and ulceration of the bladder epithelium observed on Days 2 and 4, therefore, are considered to probably be due to crystals of uracil excreted into the urine rather than being associated with any chemical property or effect of uracil. Since saturation of uracil in the urine is necessary for development of calculi, they would be expected to dissolve and rapidly disappear when uracil is withdrawn from the diet. The decrease in size and number of calculi may account for the rapid regression of papillomatosis, probably because the decreased volume of calculi no longer stimulates the epithelium enough to maintain the hyperplastic condition. During the period of disappearance of papillomatosis, no massive areas of necrosis were found and therefore single cell necrosis observed scattered throughout the epithelium of the papillomas is presumably responsible for the gradual return to normal. This process may be comparable to the controlled cell death referred to as “apoptosis” which was described earlier (20).

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4 S. Fukushima et al., unpublished data.
Since diverticulum formation has also been reported to be present in rat bladder with calculi associated with other agents (2, 5, 21), this is not a lesion specific to uracil-treated bladder. The findings regarding normal differentiation, lack of atypism in the proliferating epithelium of the diverticula and its reversibility indicate that this is not an example of true invasion.

However, in the present experiment, dysplasias were observed in the urinary bladders of rats after 25 weeks of uracil administration. We also observed a transitional cell carcinoma in a rat given uracil for 30 weeks (11) and in a recent additional experiment in our laboratory a high incidence of carcinomas was found in rats given uracil for 36 weeks and killed 4 weeks later (22). Therefore, the dysplastic lesions may be premalignant or early carcinomas. Although the possibility that exposure to unknown carcinogens by way of the urine can not be excluded, the present findings suggest that prolonged cell proliferation in response to mechanical irritation by calculi may itself evoke malignant transformation of the urothelium. This might also explain development of bladder tumors associated with chemical urolithiasis such as that observed with terephthalic acid (21). The reason(s) why simple and PN hyperplasia persisted in the calculus urolithiasis such as that observed with terephthalic acid (21), explain development of bladder tumors associated with chemical irritation also exist.

In conclusion, development of papillomatosis and diverticula in response to uracil or other sources of irritation should be considered to be hyperplastic rather than neoplastic. This model should provide a useful tool for clarification of whether intermediate factors which can stimulate cell growth after mechanical irritation also exist.

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