

Expression and Localization of Ornithine Decarboxylase in Reversible Papillomatosis Induced by Uracil in Rat Bladder

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Direct mechanical irritation by uracil calculi formed following feeding of 3% uracil in the diet to male rats produces severe papillary hyperplasia (papillomatosis, which is reversible) of bladder epithelium. To evaluate the mechanism of the appearance of uracil-induced papillomatosis, we examined the changes of the enzyme activity and the localization of ornithine decarboxylase (ODC), as well as polyamine biosynthesis, and epithelial proliferation, that accompany the sequential bladder epithelial changes following administration and withdrawal of uracil. Moreover, expression of ODC mRNA was investigated using northern blotting and localization of ODC mRNA was demonstrated using *in situ* hybridization. ODC activity during uracil administration was maintained at a high level compared to that in normal epithelium, but sharply decreased after cessation of uracil treatment. The accumulation of ODC protein was observed in the proliferating bladder epithelium by immunohistochemical examination and western blotting analysis, and even after cessation of treatment, the protein binding to anti-ODC antibody remained mildly elevated. Sequential changes of proliferating cell nuclear antigen (PCNA)-positive cells in the epithelium during the development and disappearance of papillomatosis correlated with ODC activity. ODC mRNA was expressed strongly in the proliferating epithelium in rats treated with uracil and weakly in normal epithelium, in accordance with the location of ODC protein. Consequently, our data demonstrate that cell proliferation in the development of papillomatosis is closely associated with polyamine metabolism, and moreover suggest that ODC activity is up-regulated at a post-translational step.

Key words: Ornithine decarboxylase — *In situ* hybridization — Rat bladder papillomatosis

Ornithine decarboxylase (ODC), a rate-limiting enzyme of polyamine biosynthesis, increased with epithelial cell proliferation of skin or bladder when carcinogenic promoting agents were administered to rodents.^{1,2)} We previously reported that ODC and spermidine/spermine *N*¹-acetyltransferase (SAT), a rate-limiting enzyme of biodegradation of polyamines, increased with epithelial cell proliferation in rat bladder *in vivo*.³⁾

The bladder epithelium of rodents readily proliferates in response to mechanical irritation, such as the presence of urinary calculi,⁴⁻⁸⁾ freeze-ulceration,⁹⁾ chemicals,¹⁰⁾ or even intravesical instillation of physiological saline.¹¹⁾ Lalich¹²⁾ reported a high incidence of urolithiasis in rats given uracil orally. Shirai *et al.*^{13, 14)} found that a dietary supplement of uracil at a concentration of 3% quickly induced bladder mucosal papillomatosis secondary to the formation of urinary calculi in all treated rats. Surprisingly, although urolithiasis and bladder papillomatosis were severe and extensive, they disappeared when the uracil treatment stopped; the bladder mucosa returned to normal.

Thus, uracil calculi induced reversible hyperplasia of the bladder epithelium. In contrast, long-term oral administration of uracil to rats induced urinary calculi and bladder carcinomas.^{15, 16)}

The present study was performed to clarify the sequential changes in cell proliferation and polyamine metabolism of rat bladder epithelium associated with administration and withdrawal of uracil. In particular, the expression and localization of ODC protein were examined by western blotting and immunohistochemical studies, and the expression and localization of ODC mRNA in bladder epithelium were investigated by northern blotting and *in situ* hybridization.

MATERIALS AND METHODS

Test chemical Uracil (2,4-dioxypyrimidine) was obtained from Yamasa Shoyu Co., Chiba. The purity of the preparation was more than 99.9%.

Animals and their maintenance Seventy male F344 rats obtained at 5 weeks of age (Charles River Japan, Inc., Hino) were housed in an air-conditioned room at a temperature of 23±1°C and relative humidity of 36±6%, with a 12 h light-12 h dark cycle. The animals had free access

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to feed (Oriental MF powdered diet; Oriental Yeast Co., Tokyo) and tap water.

Experimental procedure The experimental design is shown in Fig. 1. Uracil was added to the powdered diet at a concentration of 3% by weight. Forty-five rats, 6 weeks old at the commencement of the experiment, in group 1 received the diet containing uracil for 8 weeks and were then returned to the diet without uracil. Five rats from this group were killed at each of weeks 2, 4, 8, 9, 10, 11, 12, 14, and 20 after the beginning of the experiment. Five rats in group 2 received the diet containing uracil for 9 weeks, and then were killed. Twenty non-treated control rats (group 3) received the diet without uracil, and five were killed at each of weeks 0, 8, 9, and 20 after the beginning of the experiment.

Tissue processing The rats were killed by exsanguination under ether anesthesia and the bladders were cut into two equal parts with a razor blade. One was fixed in buffered Carnoy fixative (Ishizu Seiyaku Ltd., Osaka) for immunohistochemical staining for ODC, and proliferating cell nuclear antigen (PCNA), and hematoxylin and eosin staining for histological examination. In the other half, the bladder mucosa was mechanically stripped off and frozen in liquid nitrogen to measure the ODC activity, the protein and mRNA of ODC, and the concentration of polyamines, and part of the piece was fixed in 4% buffered paraformaldehyde for *in situ* hybridization.

Measurement of ODC activity and polyamine contents ODC activity was measured by the method of Otani *et al.*¹⁷⁾ The frozen piece of rat bladder epithelium was suspended in 0.5 ml of 50 mM Tris (pH 7.5) containing 0.25 M sucrose and disrupted in a homogenizer for a few minutes. The homogenized suspension was centrifuged at 100,000g for 30 min, and the supernatant was assayed for ODC activity by measurement of radioactive putrescine formed from [5-¹⁴C]ornithine.

The frozen pieces were excised and homogenized in 4 volumes of ice-cold 5% trichloroacetic acid and centrifuged for removal of the protein. The concentration of polyamines in the acid extract was analyzed by HPLC (Shimadzu LC-6A, Shimadzu, Kyoto) with a fluorescence

detector.¹⁸⁾ The separation of polyamines was carried out on an STR ODS-II column (4.6×150 mm, particle size 5 μM, Shimadzu Techno-Research, Inc., Kyoto). A 10 mM 1-hexanesulfonic acid sodium salt/100 mM sodium perchloric acid solution was used as solvent A and solvent A/methanol (1:3) as solvent B. Elution was carried out with solvent A for 3 min, and then with a linear gradient from 4% to 55% of solvent B in solvent A over 22 min at a flow-rate of 0.7 ml/min. The polyamine content values were expressed as nmol/μg DNA. The amount of DNA was determined by the method of Burton.¹⁹⁾ Calf thymus DNA (Sigma Chemical Co., St. Louis, MO) was used as a standard.

Preparation of ODC antibody A *lacZ/odc* fusion protein was used as the antigen for antibody preparation. The *HincII-PvuII* fragment of mouse ODC cDNA²⁰⁾ was ligated to an *Escherichia coli* (*E. coli*) expression vector, pUEX-2²¹⁾ linearized with *SmaI* or *BamHI* and *PstI*. This fusion protein was produced in *E. coli* and purified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. It was injected subcutaneously into 8-week-old JW rabbits. Rabbit immunoglobulin G (IgG) containing ODC-specific antibody was purified from anti-sera by affinity chromatography on an *E. coli* galactosidase (Sigma Chemical Co.) conjugated activated CH-Sepharose gel (Pharmacia Biotech, Co., Uppsala, Sweden) and a protein G-Sepharose gel (Pharmacia Biotech, Co.).

Immunohistochemical staining for ODC and PCNA The avidin-biotin-peroxidase complex (ABC) method described by Hsu *et al.*²²⁾ was used. After deparaffinization, bladder sections were treated sequentially with normal goat or horse serum, rabbit-anti ODC antibody or mouse-anti PCNA antibody (Dako Japan Co., Ltd., Kyoto), the secondary antibody, and ABC. The sites of peroxidase binding were demonstrated by the diaminobenzidine method. Sections were then counterstained with hematoxylin for microscopic examination. PCNA-positive cells were obtained by counting the number of positive cells among at least 1000 epithelial cells per urinary bladder. Negative control slides were established for each immunohistochemical staining.

Western blotting for ODC We examined the expression of ODC protein in bladder epithelium of control rats at week 0, and rats treated with uracil for 2 weeks.

Rat bladder epithelium disrupted with 10 nmol/liter Tris-HCl (pH 7.5) containing 1% Nonidet P-40 (Sigma Chemical Co.), 0.5% sodium deoxycholate, 0.1% SDS, 0.15 mol/liter NaCl, 1 mmol/liter EDTA, and 2 mmol/liter phenylmethylsulfonyl fluoride for 30 min at 4°C. Cells were pelleted by centrifugation at 100,000g for 30 min and boiled for 3 min in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. PAGE in the presence of SDS was performed according to the method of Laemmli.²³⁾ Protein concentrations were measured with a Bio-Rad

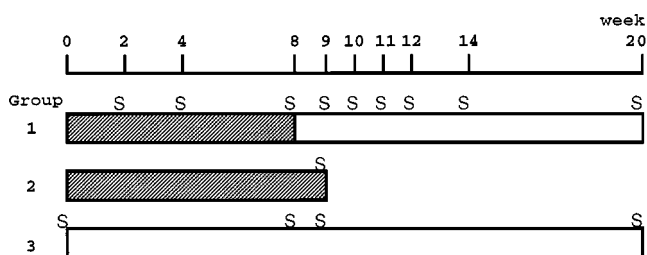


Fig. 1. Animals, 6-week-old male F344; powdered diet with uracil at a final concentration of 3%; S, animals killed.

protein assay kit (Bio-Rad Lab., Richmond, CA) using bovine serum albumin (BSA) as a standard. Forty micrograms of protein was run on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membrane. After electrophoretic transfer, the membrane was blocked overnight at 4°C with 0.5% casein. Then it was washed once with Tris-buffered saline containing 0.05% Tween 20 (TBST) (5 min) and incubated in anti-ODC antibody (1:500 in TBST and 1% BSA). After incubation with the primary antibody, the PVDF membrane was washed three times with TBST (5 min each) and incubated with anti-rabbit IgG conjugated with horseradish peroxidase (1:2500 in TBST). After extensive washing with TBST, the blots were developed with the ECL chemiluminescent detection system. The relative amounts of ODC protein were calculated by densitometric analysis.

Probe preparation We prepared a cDNA clone corresponding to a 758 bp fragment (865–1622) of mouse ODC mRNA.²⁰⁾ This cDNA was obtained by reverse transcription, followed by polymerase chain reaction, and subcloning into pBluescript II SK⁺.

RNA extraction and northern blotting Total RNA was extracted from the rat bladder epithelium treated with uracil for 0, 2, 4, 8, 9, and 10 weeks by the method of Chirgwin *et al.*²⁴⁾ For northern blotting, 20 µg of total RNA was fractionated on a 1% agarose gel and transferred to a Hybond N nylon membrane. Membranes were prehybridized and then hybridized with [³²P]dCTP-labeled probes, according to the manufacturer's instructions. After hybridization, the membranes were washed and signals were detected by autoradiography. The relative amounts of ODC mRNA were calculated by densitometric analysis.

In situ hybridization for the detection of ODC mRNA *In situ* hybridization was performed in bladder epithelium of rats treated with uracil for 2 and 4 weeks. Details of the *in situ* hybridization technique used have been described previously.²⁵⁾ Digoxigenin-labeled single-strand RNA probes were prepared for hybridization using a DIG RNA Labeling Kit (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacturer's instructions. Hybridization of ODC mRNA was performed at 60°C for 16 h, and the signals were detected using a Nucleic Acid Detection Kit (Boehringer Mannheim Biochemica). The controls included: 1) hybridization with the sense probe, and 2) RNase treatment before hybridization.

Statistical analyses Statistical analyses of observed values were performed using Student's *t* test.

RESULTS

Development and disappearance of the bladder epithelium Table I summarizes the results of histopathological examination of the bladder epithelium following adminis-

Table I. Sequential Changes of Histopathological Findings of the Bladder Following the Administration and Withdrawal of Uracil in Group 1

Weeks	No. of rats	Urinary bladder		
		calculi	simple hyperplasia	papillary hyperplasia
2	5	+++	++	+
4	5	+++	+++	++
8	5	+++	+	+++
9	5	–	+	+
14	5	–	+	+
20	5	–	+	–

Symbols: – no change, + slight, ++ moderate, +++ marked.

tration and withdrawal of uracil in group 1. From weeks 2 to 8, all rats developed calculi, and the number of calculi and their sizes increased with increasing duration of treatment with uracil. The bladders became enlarged and were filled with numerous yellowish-white calculi varying from 1 to 3 mm in diameter. In group 1, withdrawal of uracil after the 8-week treatment was associated with rapid regression of induced lesions. The first dramatic change was disappearance of the calculi. No calculi were found in any rat 1 week after the cessation of treatment (at week 9). Within 1 week, almost all papillary growth of the bladder epithelium disappeared, and the mucosa was composed of simple hyperplastic epithelium with a few areas of papillary hyperplasia. Papillary hyperplasia completely disappeared by week 20.

In group 2, all rats had many calculi, and their bladders became enlarged up to 9 weeks, similar to the bladder at week 8 in group 1. In group 3, the bladders always showed normal epithelium.

Cell proliferation in bladder lesions Fig. 2 shows that the mean value of PCNA-positive cells rose sharply to a maximum (40.2±8.5%) at week 2. Thereafter, the mean slowly declined to 29.3±5.1% at week 8. The percentage of positive cells was increased significantly in weeks 2, 4, and 8 in group 1, compared to week 0 in group 3. Most positive cells were located in the basal and middle layers of the epithelium. In addition to the increased rate of positive cells, the total number of cells in papillomatosis lesions was markedly increased compared to normal bladder epithelium. One week after treatment with uracil was stopped at week 8, PCNA-positive cells dramatically disappeared, and this continued to week 20. In group 2, the level of PCNA-positive cells in week 9 was 30.5±6.1%, whereas the rate of positive cells at week 9 in group 1 significantly decreased compared to that of group 2. In contrast, the level of PCNA-positive cells was less than 1% in the normal bladder epithelium in all rats of group 3.

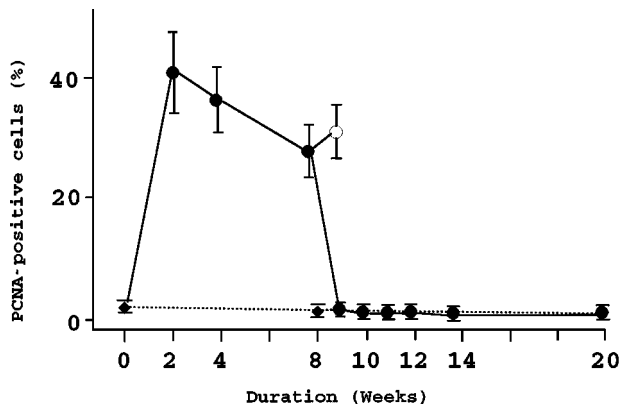


Fig. 2. Sequential changes in the number of PCNA-positive cells in bladder epithelium. ● group 1, ○ group 2, ◆ group 3.

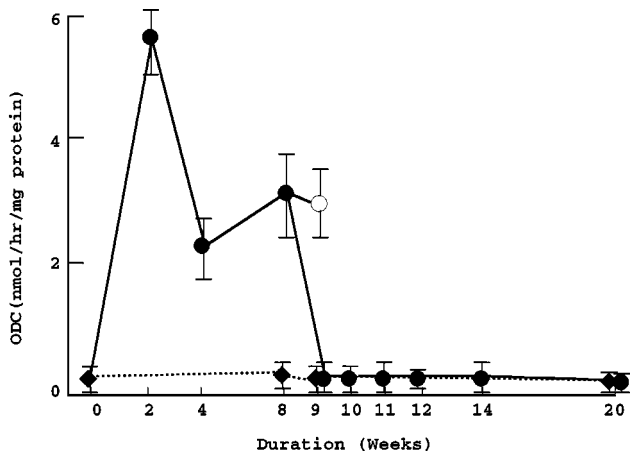


Fig. 3. Sequential changes of ODC activity during the development and disappearance of bladder papillomatosis. ● group 1, ○ group 2, ◆ group 3.

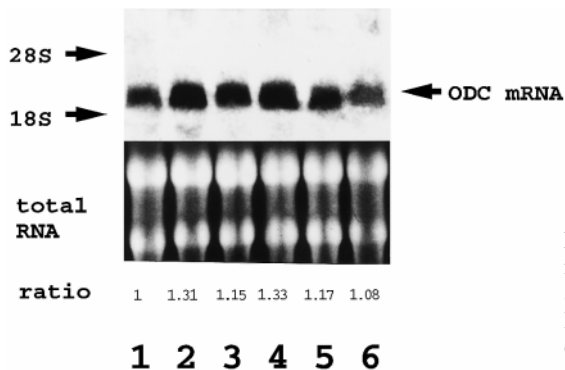


Fig. 5. Northern blotting analysis for the detection of ODC mRNA. Lane 1, week 0 in group 3; lanes 2-6, weeks 2, 4, 8, 9 and 10 in group 1. The relative amounts of ODC mRNA in lanes 2-6 compared to that in lane 1 were calculated from the densitometric results and expressed as a ratio.

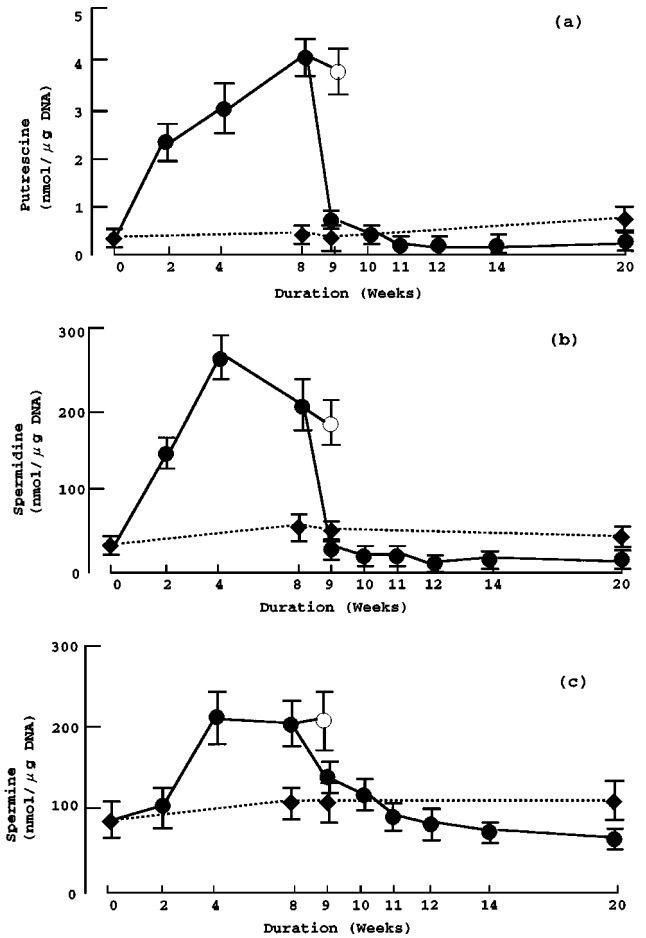


Fig. 4. Sequential changes of polyamine contents during the development and disappearance of bladder papillomatosis. ● group 1, ○ group 2, ◆ group 3. a, putrescine; b, spermidine; c, spermine.

Measurement of ODC activity and polyamine contents Fig. 3 illustrates the ODC activity in the bladder epithelium. In group 1, ODC activity rose sharply to a maximum at week 2, and was again elevated at week 8. After the cessation of treatment, ODC activity at week 9 sharply decreased to the level of normal epithelium. Thereafter, ODC activity was not increased in group 1. In group 2, ODC activity was maintained at a high level at week 9, similar to that in group 1 at week 8. The dramatic decrease of ODC activity at week 9 in group 1 was a significant change compared to group 2. In group 3, ODC activity was not elevated during the experiment.

Sequential changes of polyamine contents in bladder epithelium are shown in Fig. 4. Polyamine biosynthesis increased during the administration of uracil, and the contents of spermidine and spermine reached a maximum at week 4. They sharply decreased to the level of normal epithelium concomitantly with the decrease of ODC activ-

ity at week 9 in group 1, and then remained in the normal range.

Northern blotting analysis and *in situ* hybridization for ODC mRNA The results of northern blotting analysis for ODC mRNA of bladder epithelium are shown in Fig. 5. There was an increase in ODC mRNA level in bladder epithelium of rats treated with uracil for 2 weeks, to about 1.3 times that of normal epithelium at week 0.

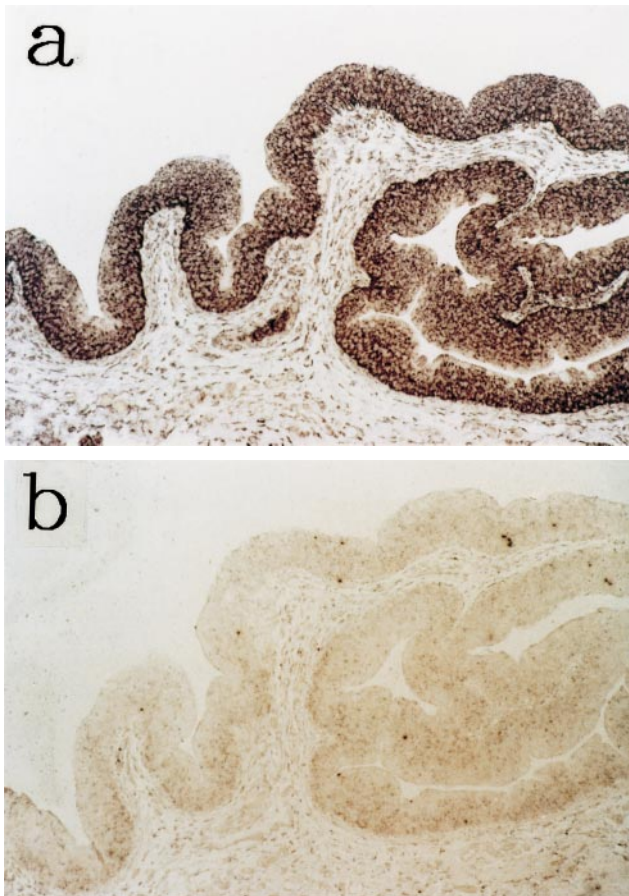


Fig. 6. Localization of ODC mRNA in bladder papillomatosis at week 2 by *in situ* hybridization (group 1). a, antisense probe; b, sense probe.

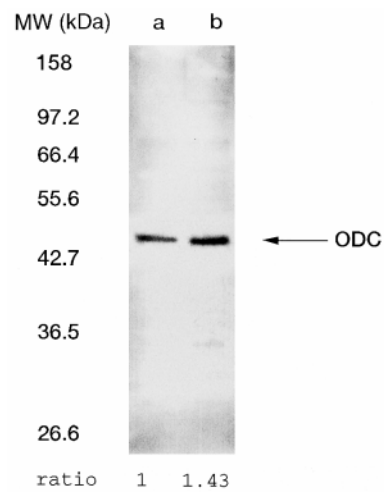


Fig. 7. Western blotting analysis for the detection of ODC protein in the bladder epithelium. Lane a, week 0 in group 3; lane b, week 2 in group 1. The relative amount of ODC protein in lane b compared to that in lane a was calculated and expressed as a ratio.

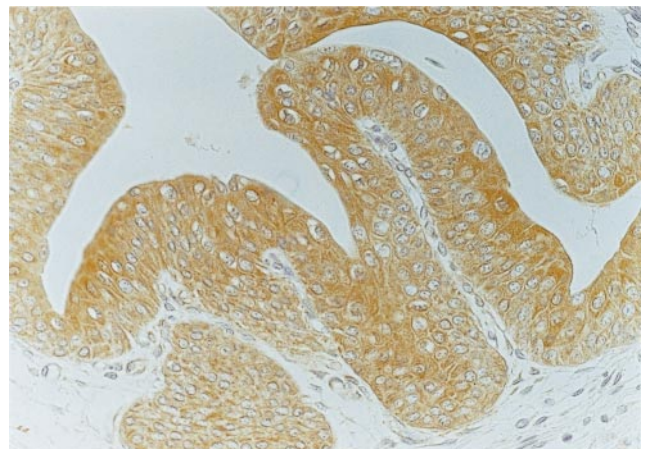


Fig. 8. Immunohistochemical staining with anti-ODC antibody in bladder papillary hyperplasia at week 2 (group 1).

The high levels of mRNA were maintained until week 8, but withdrawal of uracil was associated with a decline of the ODC mRNA level at week 9.

In situ hybridization for ODC mRNA was performed on the bladders of rats treated with uracil at weeks 2 and 4 in group 1 and in control rats at week 0 in group 3. Though all layers of normal epithelium were diffusely labeled for ODC mRNA, all epithelial layers of bladders of rats treated with uracil were labeled more strongly than normal epithelium. Especially in the intermediate and basal layers, ODC mRNA was expressed more strongly than in the surface layer (Fig. 6a). The cytoplasm of epithelial cells expressed ODC mRNA, whereas no stromal cells expressed it. Background labeling with the antisense probe was consistently low, and specific hybridization observed with the sense probe was not recognized in any case (Fig. 6b).

Western blotting and immunohistochemical staining for ODC protein Western blotting analysis for ODC of bladder epithelium is shown in Fig. 7. The expression of ODC protein in bladder epithelium of rats treated with uracil for 2 weeks increased about 1.4-fold, compared to normal epithelium at week 0.

Epithelial cells of all layers in the bladder during uracil treatment in group 1 were immunohistochemically stained with anti-ODC antibody, especially at week 2, at which time ODC activity was the highest. They were stained more strongly than at week 0 (Fig. 8). On the other hand, epithelial cells after cessation of uracil treatment, namely from week 9 to 20, were weakly stained. This result indicated that ODC protein exists in the cytoplasm even after uracil treatment. In the bladders of non-treated rats (group 3), epithelial cells were stained more weakly than was the case after the uracil treatment.

DISCUSSION

Our results indicate that ODC played an important role in the severe epithelial proliferation (papillomatosis) of the bladder following mechanical irritation by uracil-induced urinary stones in rats. ODC activity in papillomatosis during the administration of uracil was maintained at high levels compared with normal epithelium, but its activity rapidly decreased to the control level after the cessation of treatment. Moreover, polyamine contents, including putrescine, spermidine, and spermine, were increased concomitantly with ODC activity. The sequential changes of the enzyme activity paralleled the number of PCNA-positive cells.

ODC was observed in the proliferating bladder epithelium during the administration of uracil by western blotting and immunohistochemical staining. The immunohistochemical study revealed that PCNA-positive cells were predominantly located in basal and intermediate lay-

ers of the epithelium and ODC-positive cells were in all layers. Thus, there was a slight difference in distribution between ODC and PCNA. Moreover, ODC activity rapidly decreased even during the period of disappearance of papillomatosis. However, the protein reacting to anti-ODC antibody remained weakly in the bladder epithelium. This antibody might bind to inactivated or partially degraded ODC.

In situ hybridization showed that ODC mRNA was expressed strongly in the cytoplasm of bladder epithelium of rats treated with uracil for 2 and 4 weeks in accordance with the results obtained by immunohistochemical detection of ODC. Northern blot analysis indicated that ODC mRNA was expressed not only in papillomatosis, but also normal epithelium. However, ODC mRNA and protein levels in papillomatosis of rats treated with uracil for 2 weeks were about 1.5-fold higher than that in normal epithelium. On the other hand, the level of ODC activity in papillomatosis was about 40-fold higher than in normal epithelium. There is evidence for increased transcription of the ODC gene in response to some carcinogens, tumor promoters, and oncogenes,²⁶⁻²⁹⁾ but our results suggest that posttranslational up-regulation of ODC occurred in papillomatosis.

Many investigators on chemical carcinogenesis, and in particular, on the characteristics of tumor promotion in two-stage carcinogenesis, have focused on ODC activity in the target organ.¹⁾ In rat bladder carcinogenesis, urine, a tumor promoter in rat heterotopic bladder carcinogenesis, induced elevation of ODC activity.²⁾ Uracil promoted rat bladder carcinogenesis initiated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine.¹⁴⁾ Accordingly, elevation of ODC activity and the accelerated interconversion of polyamines are important steps in the promotion stage of rat bladder carcinogenesis.

In this study, we investigated the cell proliferation associated with polyamine metabolism in urinary bladder papillomatosis induced by uracil administration. Moreover, it was previously reported that apoptosis occurred in the process of papillomatosis regression following withdrawal of uracil treatment.^{30, 31)} Accordingly, the balance between cell proliferation and apoptosis may have an important role in uracil-induced urinary bladder carcinogenesis.

Not only ODC but also SAT can be a rate-limiting enzyme of polyamine metabolism, especially biodegradation. We reported that SAT was a biomarker of epithelial proliferation in rat bladder.³⁾ In another experiment, the sequential change of SAT activity was similar to that of ODC activity (unpublished data). Strongly SAT-positive cells existed in intermediate and surface layers during the administration of uracil. SAT may have been induced by stress due to mechanical irritation by the stones, since it has been reported that many kinds of stress induce SAT activity.³²⁾

In conclusion, the elevation of ODC activity was consistent with the proliferative changes of bladder epithelium induced by the administration and withdrawal of uracil. ODC was detected only in the epithelium by western blotting and immunohistochemical staining, and moreover, ODC mRNA detected by *in situ* hybridization was expressed strongly at the locations of ODC and PCNA, suggesting that uracil-induced papillomatosis develops by cell proliferation associated with polyamine biosynthesis.

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