Virchows Arch (1996) 427:589–594

ORIGINAL ARTICLE

Shinji Yamamoto · Satoshi Yonezawa · Masao Ichinose · Kazumasa Miki · Tsuneo Masui Shoji Fukushima · Hanae Inoue · Masae Tatematsu

Immunohistochemically demonstrated variation in expression of cathepsin E between uracil-induced papillomatosis and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced preneoplastic and neoplastic changes in rat urinary bladder

Received: 22 September 1995 / Accepted: 24 October 1995

Abstract Expression of rat urinary bladder cathepsin E in benign papillomatosis induced by uracil and various stages of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN)-induced carcinogenesis was investigated immunohistochemically. Seven-week-old, male F344/DuCrj rats were used. In the normal urothelium of control rats, cathepsin E stained in all layers of cells, although in umbrella cells and some basal cells the reaction was relatively weak. In rats given a diet containing 3% uracil for 5 weeks immunoreactivity of cathepsin E in uracil-induced papillomatosis was consistently homogeneous in all layers, but weaker than in normal urothelium. In rats given 0.05% BBN in drinking water for 12 weeks and subsequently maintained without treatment for 48 weeks cells with little cathepsin E, never observed in normal urothelium, appeared at 5 weeks above the basement membrane in the earliest stage of BBN-induced urinary bladder cancer (simple hyperplasia). Throughout the neoplastic process, groups of cells with a little cathepsin E were randomly distributed, with expression in the urothelium being markedly unstable. Almost all areas of squamous cell proliferation in TCC were negative for cathepsin E. Instability of cathepsin E expression in rat urothelium therefore appears characteristic for carcinogenesis and offers the possibility of using this feature as an early biomarker for urinary bladder carcinogenesis.

Key words Cathepsin E · Uracil · *N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine · Rat urinary bladder carcinogenesis · Papillomatosis

S. Yamamoto · T. Masui · H. Inoue
Masae Tatematsu (✉)
Laboratory of Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya, 464 Japan

S. Yamamoto · S. Fukushima
First Department of Pathology, Osaka City University Medical School, 1-4-54, Asahi-machi, Abeno-ku, Osaka, 545 Japan

S. Yonezawa
Department of Embryology, Institute for Developmental Research, Aichi Prefectual Colony, Kasugai, 480-03 Japan

M. Ichinose · K. Miki
First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113 Japan

Introduction

Cathepsin E is a major intracellular aspartic proteinase in mammals, present in a limited distribution [5, 6, 13, 15–17]. In man cathepsin E concentration is highest in gastric mucosal cells, followed by the urinary bladder, thymus, spleen, bone marrow, red blood cells, and neutrophils [3, 6, 8]. In gastric mucosal cells, it has been suggested that cathepsin E may have important roles in protein degradation or host defence mechanisms in the gastric mucosal barrier [5]. Cathepsin E in human erythrocytes may act in a self-destruction mechanism for removing senescent or damaged cells from the circulation [7]. Generally, despite many attempts to explain the localization and concentrations of cathepsin E in various organs, only limited information is available, in contrast to the situation with intracellular proteinase cathepsin D, which has been well characterized [7, 8].

Various experimental models using rodents have been established to study the various stages of chemical carcinogenesis [1, 2, 4, 12]. In some animal models, use of putative preneoplastic lesions have been established to predict the subsequent development of neoplastic changes: for example, immunohistochemically demonstrated glutathione *S*-transferase placental form (GST-P)-positive foci and pepsinogen isozyme 1 (Pg1)-altered pyloric glands (PAPG) are applied for rat hepatocarcinogenesis [2] and stomach carcinogenesis [12] respectively. Histopathologically, those precancerous lesions often have relatively normal appearances under the light microscope

and therefore attention has been concentrated on their identification by immunohistochemical procedures. The premise that there is a good correlation between incidence or number of these histopathological biomarkers and cancer induction in the long term has been confirmed [2, 12].

The urinary bladder is one of the major target organs of toxic and/or carcinogenic chemicals [4]. The initial uracil-induced rat benign proliferative urothelial lesions have been described as simple hyperplasia with later papillary or nodular (PN) hyperplasia being replaced by papillomatosis [9, 10]. In contrast, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN)-induced urinary bladder carcinogenesis, while featuring simple and PN hyperplasia, progresses to papillomas and cancers [1]. It is very difficult to distinguish benign reversible hyperplastic changes from preneoplastic lesions [11], which may act as precursors for true tumours and a reliable marker for preneoplastic changes in the urinary bladder is required [4,14].

In the present study, we investigated the immunoreactivity of rat cathepsin E in reversible uracil-induced papillomatosis and control tissues, comparing the results with findings for the various stages of BBN-induced carcinogenesis.

Materials and methods

Experimental protocols

BBN and uracil, 2,4-dioxypyrimidine were obtained from Tokyo Kasei Kogyo, Tokyo, Japan. Seven-week-old, F344/DuCrj male rats (Charles River Japan, Kanagawa) were used in the study. At the beginning, they were randomly divided into three groups, housed five to a plastic cage. The room temperature was controlled at 22±2°C, and the relative humidity at 55±10%.

Experiment 1 was a control study of cathepsin E expression in various organs. Animals were maintained without chemical supplement for 30 weeks. At autopsy, forestomach, glandular stomach, liver, kidney, spleen and urinary bladder tissues were taken for evaluation. In experiment 2, rats were given a diet containing 3% uracil, prepared by mixing weight quantities of uracil and Oriental MF powdered diet with 2% corn oil in a stainless-steel mixer for 30 min, for 5 weeks. After completion of the uracil treatment all rats were given basal diet for 11 weeks. Subgroups of three rats were killed at days 2, 4, 6, 8 and weeks 2, 3, 5 of this period. In experiment 3, rats were treated initially with 0.05% BBN in their drinking water for 12 weeks. After the completion of BBN treatment, animals were given basal diet and water without any chemical supplement for 48 weeks. Subgroups of three to five rats were killed at weeks 1, 5, 9, 12, 18, 24, 30 and 60. During each experiment, the animals had free access to feed and tap water ad libitum. Animals were killed by ether anaesthesia, gross examinations were performed, and detailed examinations of the urinary bladder were carried out after fixation. Normal tissue in experiment 1 and most of the abnormal tissue in experiments 2 and 3 were fixed in 10% phosphate-buffered formalin, but some tissues were also fixed in ice-cold acetone or sublimated formaldehyde, made from 6% $HgCl_2$ in a mixture of 5% acetic acid and 4% formaldehyde. For microscopical examination, tissues were embedded in paraffin, sectioned, and routinely stained with haematoxylin and eosin (HE). Pathologically diagnosed urinary bladder lesions induced by uracil and BBN were classified as described previously [1, 9]. In the latter case these comprised simple hyperplasia, PN hyperplasia and papilloma or carcinoma.

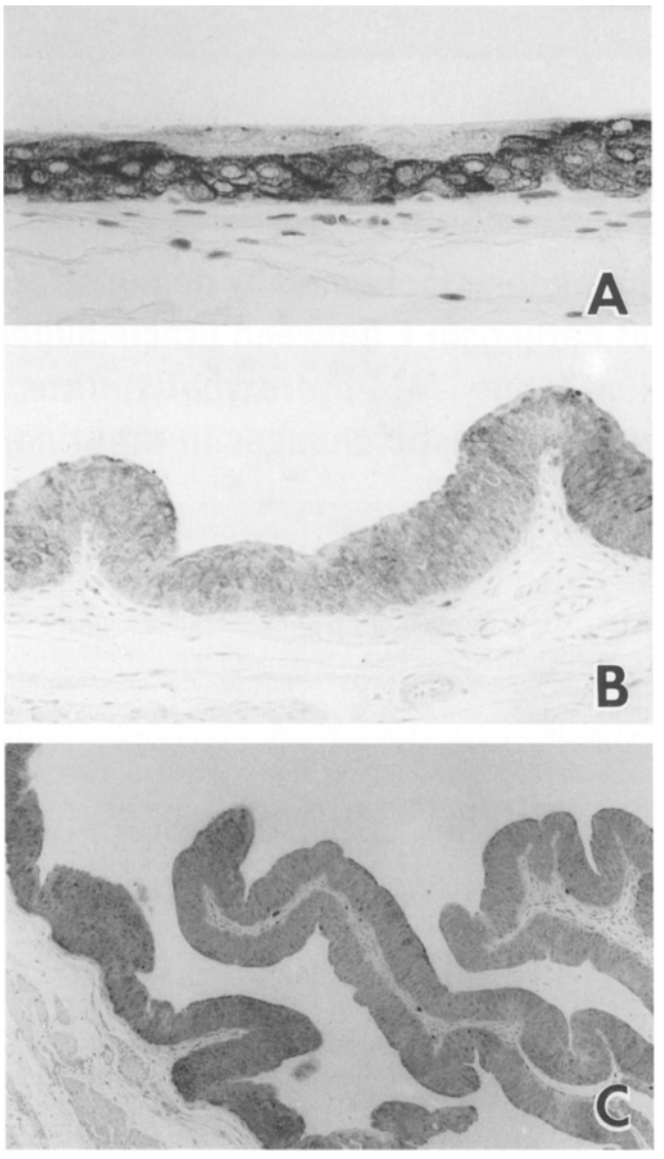

Fig. 1 **A** Normal urothelium without any chemical supplement. Cathepsin E is expressed relatively homogeneously in the cytoplasm of all epithelial cells. Expression in umbrella cells is weak but cathepsin E negative cells are absent. Basement membrane and submucosal tissues are negative. **B, C** Reversible changes in bladder epithelium induced by uracil. Cathepsin E is expressed homogeneously and relatively weakly in the reversible lesions, uracil-induced simple hyperplasia (**B**) and papillomatosis (**C**). Fixed in sublimated formaldehyde; **A** ×400, **B** ×200, **C** ×100

Immunohistochemical staining

Anti-rat cathepsin E antibody was prepared as described previously [17]. The avidin-biotin-peroxidase complex (ABC) method was used to determine the location of cathepsin E in each tissue sample. After deparaffinization, sections were treated with rabbit anti-rat cathepsin E (1:8000). The sites of peroxidase binding were demonstrated by the diaminobenzidine method. Sections were then counterstained with haematoxylin for microscopical examination.

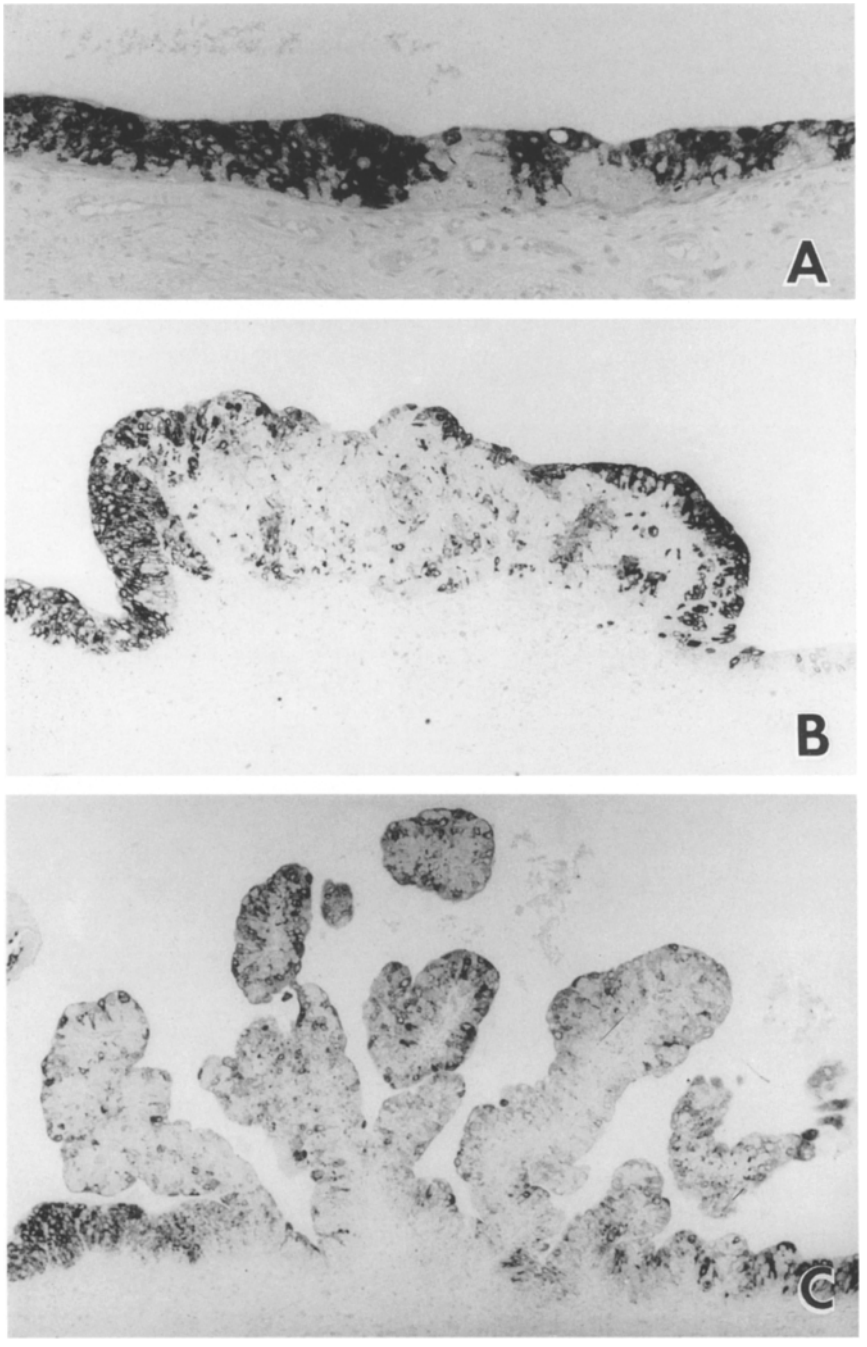

Fig. 2A–C Precancerous lesions induced by BBN. **A** At week 9 areas of cells with little cathepsin E were presented. Some individual cells with little cathepsin E appeared right above the basement membrane. **B** Nodular hyperplasia and **C** transitional cell papilloma were observed during weeks 12–18 with contrasting groups of cells (**C**). Fixed in formalin; ×100

Results

Experiment 1

In normal gastric mucosa, cathepsin E was found to be strongly expressed in parietal cells and foveolar epithelial cells, although both oesophagous and forestomach squamous epithelium were negative. Strong immunoreactivity in the pyrolic glands was evident, especially in sections from material fixed in sublimated formaldehyde. In contrast to the generally negative reaction of kidney tubules for cathepsin E, normal urothelium of the renal pelvis consistently demonstrated an intensity of expression as strong as in the gastric mucosa.

In normal urinary bladder epithelium of animals without any chemical treatment, cathepsin E stained the cytoplasm of all layers of cells. However, immunoreactivities of umbrella cells and some basal cells were weaker than those in the middle layers (Fig. 1A). Basement membrane and submucosal tissues were negative for cathepsin E.

Experiment 2

A large number of small stones filled the rat urinary bladders in the first week and simple hyperplasia was then observed (Fig. 1B). One week thereafter, flat hyper-

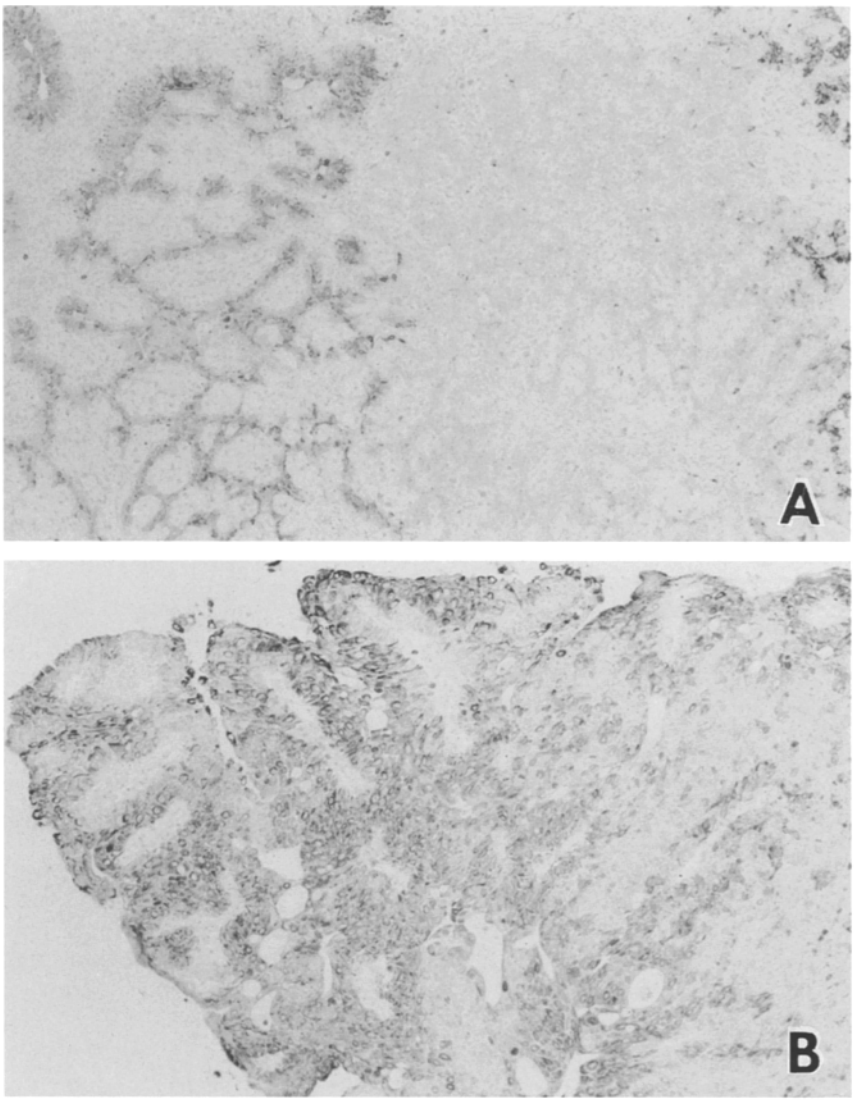

Fig. 3A, B *Transitional cell* carcinoma (TCC) observed after week 24. **A** In well-differentiated (grade 1) TCCs, large number of cells formed groups with different cathepsin E expression. Fixed in formalin; ×25. **B** Immunoreactivities became more unstable and varied in individual neoplastic cells in high-grade (grade 3) tumours. Fixed in acetone; ×25

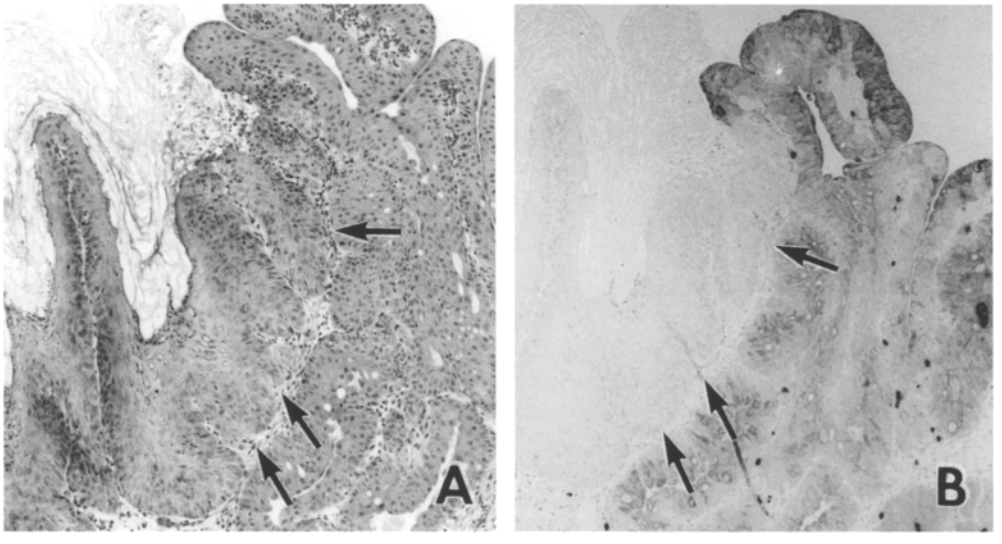

Fig. 4 **A** Transitional cell carcinoma (TCC) with areas of squamous cell differentiation (*arrows*) at week 30. HE ×50. **B** Serial section of **A**. Superficial parts of the tumour tend to have stronger expression. Areas of squamous cell differentiation (*arrows*) in TCC are totally negative for cathepsin E. Fixed in formalin; ×50

plastic epithelial proliferation became papillary (uracil-induced papillomatosis; Fig. 1C). Throughout the experiment, cathepsin E was present in most epithelial cells but its immunoreactivity was consistently low. There was little variation in expression of cathepsin E between layers.

Experiment 3

In a first week of BBN administration, urinary bladder epithelium in HE-stained sections presented almost normal features. However, cathepsin E immunoreactivity of epithelial cells was irregular, most of them demonstrating stronger expression than normal but others becoming weaker. However, cells with very little cathepsin E did not appear until week 5, when the transitional epithelium became thickened to more than 5 layers (simple hyperplasia). Some cells with little cathepsin E initially appeared right above the basement membrane and these cells were independently and randomly distributed.

At week 9 and consistently thereafter, groups of cells with little cathepsin E were present in both normally appearing epithelium and simple hyperplasia (Fig. 2A). PN hyperplasias appeared from week 12 and contrasting expression of cathepsin E between evidently positive and little cathepsin E areas became evident at this stage (Fig. 2B). In most cases, cathepsin E immunoreactivity tended to be stronger in the upper layer, despite the fact that expression of cathepsin E in the upper layer of normal urothelium is usually weak due to the presence of umbrella cells.

After the completion of BBN treatment, cathepsin E expression in papillomas observed in week 18 resembled that in PN hyperplasias (Fig. 2C). At week 24, transitional cell carcinomas (TCC) were observed, most being papillary. Relatively small or well-differentiated tumours consisted of mixed groups of both faintly or weakly positive cells (Fig. 3A). No expression of cathepsin E in the interstitial tissues of papillary tumours was found. The superficial part of tumours tended to have stronger expression. Immunoreactivities of high grade or bulky tumours observed at or after week 30 were relatively weak and unstable; in addition, the heterogeneity of cathepsin E expression became more complicated (Fig. 3B).

In parts of TCCs, small areas of squamous cell differentiation with apparent keratinization were observed and these were totally negative for cathepsin E (Fig. 4A,B). Throughout the experiment, hyperplastic epithelium not directly involved with tumours maintained irregular expression of cathepsin E, both positive and slight immunoreactivities for cathepsin E being present.

Discussion

Cathepsin E is known as a major intracellular proteinase with a limited organ distribution [3, 5, 6, 8, 15–17]. In the present study, immunohistochemical localization of cells with strong cathepsin E expression was also limited, in line with previous reports [7]. According to the previous studies, subcellular localization of cathepsin E in neutrophils suggested its function as a cytosolic enzyme [7]. However, its real role in positive tissues is still unclear. In contrast to the squamous epithelium of the oesophagous and forestomach, which is protected by keratinizing layers, the fundal mucosa is relatively lacking in defence. Since it is the first barrier against ingested food, chemical substances, antigens and bacterias, some defence and/or self-destruction mechanism might be expected and a relationship between the existence of cathepsin E and such mechanisms has been suggested [5].

Urothelium is another significant barrier against bacterial infections. In the normal urinary bladder epithelium of all rats we examined, cathepsin E was expressed in a consistent manner in the middle layers, while umbrella cells were usually weak, in accordance with Sakai's previous report [7]. Although the role of umbrella cells with low cathepsin E expression in urothelium remains to be elucidated, we may speculate that these cells may be structurally significant as part of the barrier system. We have no explanation for this apparent anomaly at present.

Uracil-induced papillomatosis histologically resembles the early stage of BBN-induced carcinogenesis but is known to be reversible. After cessation of uracil treatment, stones disappear in a few days and the hyperplastic urothelium returns to normal [9, 10]. Consistent but weak expression of cathepsin E in uracil-induced papillomatosis might be due to immature urothelial cells with a high cellular proliferation rate.

In contrast, the study of cathepsin E expression using our BBN-induced rat urinary bladder carcinogenesis model revealed variation in cathepsin E expression from the earliest to the latest stage of carcinogenesis. After 1 week of administration of BBN, immunoreactivity of cathepsin E in urothelium of normal appearance became stronger in relative terms. This suggests induction as a primary biological reaction. In simple hyperplasia, the earliest stage of urinary bladder carcinogenesis, some cells located right above the basement membrane, but not necessarily basal cells, showed little cathepsin E. In more advanced stages of carcinogenesis, PN hyperplasias, papilloma and cancer, many cells had low or absent cathepsin E expression. This heterogeneity appears to be characteristic for urinary bladder carcinogenesis and was never observed in normal urothelium or uracil-induced papillomatosis, which processes seem to retain normal regulation of cathepsin E. Heterogenous expression of other urothelium-related enzymes in rat urinary bladder carcinogenesis has been demonstrated previously, including the focal reduction in alkaline phosphatase [4] and increase in activity of NADH diaphorase [14].

Whether this change in regulation of cathepsin E has functional relevance to neoplastic cells remains unclear. Here, two speculations about the instability of cathepsin E expression in urothelium can be presented. First, genetic events during urinary bladder carcinogenesis, such

as allelic losses, might be responsible. Second, cathepsin E might be linked to some differentiation state, as shown by its absence in squamous epithelium. Normal epithelial cells basically share the same phenotype, while initiated cells might be unstable in this regard.

As reported in various previous studies [1], urinary bladder lesions with squamous cell metaplasia were occasionally observed. Such squamous differentiation has been thought to be either a reaction against foreign bodies or the result of abnormal differentiation during carcinogenesis. The fact that cathepsin E was not present anywhere in areas of squamous cell proliferation agrees with its absence in normal squamous epithelium in the forestomach and oesophagous [7]. Considering the organotropic distribution of cathepsin E, its expression has been suggested to be highly regulated [7] and, moreover, closely connected to cellular differentiation. Therefore, the absence of cathepsin E in areas of squamous cell differentiation and its unstable expression in BBN-treated transitional epithelium may be due to different mechanisms.

In both normal urinary bladder epithelium and reversible simple hyperplasias, it is accepted that bromodeoxyuridine (BrdU) labelled cells are concentrated in the basal layers [11], indicating that only these cells are responsible for cell division. Cells with little expression of cathepsin E appeared initially directly above the basement membrane in the earliest lesion of bladder carcinogenesis, simple hyperplasia. We speculate that these cells are the source of the primitive neoplastic cells in rat urinary bladder carcinogenesis. If any cells are initiated in the upper layers, they would be expected to disappear with rapid turnover cycles of transitional epithelium. In more advanced stages, small areas of irregular expression of cathepsin E were, in contrast, randomly distributed. These results are in accordance with our previous report [11] that one significant index of irreversible change in rat urinary bladder carcinogenesis is the presence of irregularly distributed areas of BrdU incorporation, independent of basal or surface location. Overall, it is possible that abnormal distribution of cells capable of proliferation is a significant step in early stage of urinary bladder carcinogenesis. This compares well with the finding that cells demonstrating BrdU incorporation in uracil-induced proliferating lesions are arranged in an orderly way in the basal layers [10], in agreement with the regular expression of cathepsin E in these areas.

Acknowledgement This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

References

1. Fukushima S, Murasaki G, Hirose M, Nakanishi K, Hasegawa R, Ito N (1982) Histopathological analysis of preneoplastic changes during N-butyl-N-(4-hydroxybutyl)nitrosamine-induced urinary bladder carcinogenesis in rats. Acta Pathol Jpn 32:243–250
2. Ito N, Tsuda H, Tatematsu M, Inoue T, Tagawa Y, Aoki T, Uwagawa S, Kagawa M, Ogiso T, Masui T, Imaida K, Fukushima S, Asamoto M (1988) Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione S- transferase placental form positive foci in rats-an approach for a new medium-term bioassay system. Carcinogenesis 9:387–394
3. Jupp RA, Richards AD, Kay J, Dunn BM, Wyckoff JB, Samloff IM, Yamamoto K (1988) Identification of the aspartic proteinases from human erythrocyte membranes and gastric mucosa (slow-moving proteinase) as catalytically equivalent to cathepsin E. Biochem J 254:895–898
4. Kunze E (1979) Development of urinary bladder cancer in the rat. Curr Top Pathol 67:145–232
5. Muto N, Yamamoto M, Tani S (1987) Comparative studies of two types of acid proteases from rat gastric mucosa and spleen. J Biochem 101:1069–1075
6. Muto N, Yamamoto M, Tani S, Yonezawa S (1988) Characteristic distribution of cathepsin E which immunologically cross-reacts with the 86-kDa acid proteinase from rat gastric mucosa. J Biochem 103:629–632
7. Sakai H, Saku T, Kato Y, Yamamoto K (1989) Quantitation and immunohistochemical localization of cathepsins E and D in rat tissues and blood cells. Biochim Biophys Acta 991:367–375
8. Saku T, Sakai H, Shibata Y, Kato Y, Yamamoto K (1991) An immunocytochemical study on distinct intracellular localization of cathepsin E and cathepsin D in human gastric cells and various rat cells. J Biochem 110:956–964
9. Shirai T, Ikawa E, Fukushima S, Masui T, Ito N (1986) Uracil-induced urolithiasis and the development of reversible papillomatosis in the urinary bladder of F344 rats. Cancer Res 46: 2062–2067
10. Shirai T, Fukushima S, Tagawa Y, Okumura M, Ito N (1989) Cell proliferation induced by uracil-calculi and subsequent development of reversible papillomatosis in the rat urinary bladder. Cancer Res 49:378–383
11. Tatematsu M, Fukushima S, Aoki T, Mera Y, Inoue T, Ito N (1987) Patterns of epithelial proliferation revealed by continuous administration of bromodeoxyuridine during urinary bladder carcinogenesis in rats. Jpn J Cancer Res 78:879–882
12. Tatematsu M, Furihata C, Katsuyama T, Mera Y, Inoue T, Matsushima T, Ito N (1987) Immunohistochemical demonstration of pyloric gland-type cells with low-pepsinogen isozyme 1 in preneoplastic and neoplastic tissues of rat stomachs treated with N-methyl-N′-nitro-N-nitrosoguanidine. J Natl Cancer Inst 78:771–777
13. Tatematsu M, Iwata H, Ichinose M, Kakei N, Tsukada S, Miki K, Imai S, Imaida K (1993) Markers of surface mucous cell type human gastric cancer cells: Galactose oxidase-Schiff reactive mucins, monoclonal antibody SH-9 reactive mucins and cathepsin E. Acta Pathol Jpn 43:500–506
14. Vanderlaan M, Fong S, King EB (1982) Histochemistry of NADH diaphorase and *r*-glutamyltranspeptidase in rat bladder tumors. Carcinogenesis 3:397–402
15. Yonezawa S, Tanaka T, Muto N, Tani S (1987) Immunochemical similarity between a gastric mucosa non-pepsin acid proteinase and neutrophil cathepsin E of the rat. Biochem Biophys Res Commun 144:1251–1256
16. Yonezawa S, Fujii K, Maejima Y, Tamoto K, Mori Y, Muto N (1988) Further studies on rat cathepsin E: subcellular localization and existence of the active subunit form. Arch Biochem Biophys 267:176–183
17. Yonezawa S, Takahashi T, Ichinose M, Miki K, Tanaka J, Gasa S (1990) Structural studies of rat cathepsin E: amino-terminal structure and carbohydrate units of mature enzyme. Biochem Biophys Res Commun 166:1032–1038