Scanning Microscopy

Volume 7 | Number 1 Article 39

1-25-1993

Application of Scanning Electron Microscopy and X-Ray Analysis to Urinary Tract Cancer in Animals and Humans

Martin Cano
University of Nebraska, Omaha

Toru Suzuki
Dokkyo University

Samuel M. Cohen
University of Nebraska, Omaha

Scanning Microscopy, Vol. 7, No. 1, 1993 (Pages 363-370)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/93$5.00+.00

APPLICATION OF SCANNING ELECTRON MICROSCOPY AND X-RAY ANALYSIS TO URINARY TRACT CANCER IN ANIMALS AND HUMANS

Martin Cano[1,*], Toru Suzuki[2], and Samuel M. Cohen[3]

[1,3]Department of Pathology and Microbiology, and [1,3]The Eppley Institute,
University of Nebraska Medical Center, Omaha, NE 68198,
[2]Department of Urology, Dokkyo University School of Medicine, Tochigi, Japan.

(Received for publication April 24, 1992, and in revised form January 25, 1993)

Abstract

Scanning electron microscopy (SEM) has proven useful in various aspects of urinary bladder carcinogenesis research and these are reviewed as they pertain to our research involving sodium saccharin in the rat. Sodium saccharin-carcinogenesis in rats requires administration at high doses beginning at birth or earlier. Administration beginning at ages of 5 weeks or later results in much lower incidences of bladder tumors. Methods were developed for examining the rat fetal and neonatal bladder to further evaluate effects at these critical ages. Several significant differences were found by SEM between the fetal bladder compared to the adult. The typical polygonal superficial cells of the bladder with asymmetric unit membrane were present before birth, but the slow turnover rate of the adult bladder did not occur until 3-4 weeks of age. Sodium saccharin causes increased proliferation rates and hyperplasia of the urothelium which is dose-dependent. SEM was found to be more sensitive than either light microscopy or labeling indices to detect the earliest lesions induced by sodium saccharin. More recently, amorphous and crystalline material in the urine of rats fed high doses of sodium saccharin were detected by SEM examinations which contained silicon as well as calcium, phosphate, and magnesium as detected by energy dispersive X-ray analysis (EDS) with the SEM. These parameters may be relevant to differences between rats and humans and pertain to extrapolations regarding risk assessment.

Key Words: Scanning electron microscopy, silica, saccharin, urine, X-ray, bladder.

*Address for correspondence:
Martin Cano
University of Nebraska Medical Center
600 South 42nd Street
Omaha, NE 68198-3135

Phone No.: (402) 559-4175
Fax No.: (402) 559-6018

Introduction

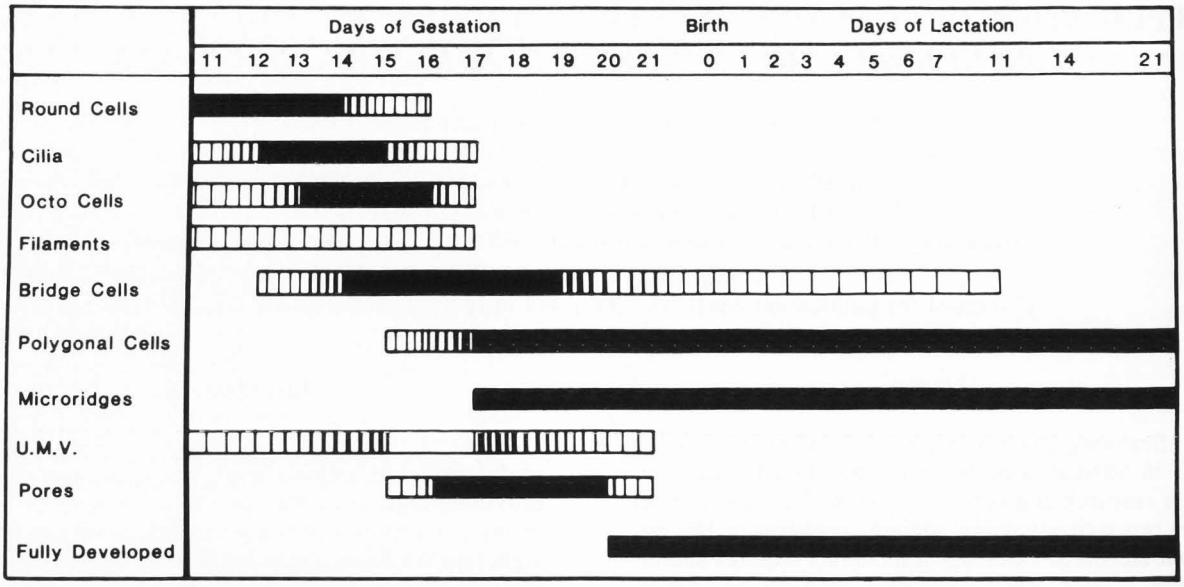
Scanning electron microscopy (SEM) has been useful in the examination of biological specimens by providing high resolution as well as high magnification of the surfaces of various structures, giving greater insight into the three dimensional structure of tissues than is generally possible by examination by light microscopy or transmission electron microscopy (15). Combined with energy dispersive X-ray analysis (EDS), detailed examinations can be performed for specific elements in biological specimens. Our investigations have utilized the rat model of urinary bladder carcinogenesis as well as extensive findings from this model and from other species, including humans. Although none of these procedures have progressed to the level of routine procedures in clinical medicine, several of them have proven useful in various research investigations into the pathogenesis of bladder cancer as well as characteristic features of increased cell proliferation and neoplasia. In this presentation, we will briefly review some of the morphologic alterations which occur during embryonic and fetal development, hyperplasia, and carcinogenesis, as well some recent research on the role of silicates in the pathogenesis of increased cell proliferation following administration of various organic sodium salts.

Fetal Development of the Rat Urinary Bladder

Until 1985, little was known about *in utero* urinary bladder embryogenesis and the development of the urothelium of laboratory animals (1). To provide a more detailed examination of bladder embryogenesis, especially early in gestation, several light, transmission, and scanning electron microscopic methods were combined and modified to trace normal differentiation of the rat urinary bladder at the earliest possible state of development to more fully understand the possible susceptibility of toxic insult to the bladder. This initial study provided for the preparation, isolation and identification of the fetal bladder as early as day 11 of gestation (2).

The epithelium at day 11 of gestation is composed of small, loosely connected, round cells with short uniform microvilli. Polygonal cells characteristic of the

Table 1. Diagrammatic representation of the times of appearance of the various features fetal and neonatal periods of the developing rat urinary bladder.



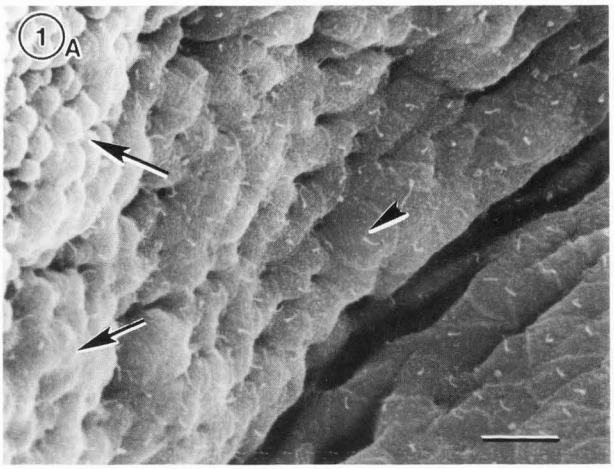
adult bladder begin to appear by day 15, but the microridges with asymmetric unit membrane are not apparent until day 17. By day 20, the epithelium appears morphologically similar to the adult bladder. Several morphological cellular features were observed at different stages of gestation which are not seen in the normal adult bladder, but have been observed in bladder tumors. During days 12-15, most of the luminal lining cells of the bladder epithelium have a single central cilium (Fig. 1). Cells that appeared to form bridges between cells were observed on day 14 of gestation and continued to be present through day 11 post partum (Fig. 2). It was apparent from these studies that the bladder epithelium is a rapidly changing, proliferating tissue *in utero*, continuing for a brief period after birth (5) (See Table 1).

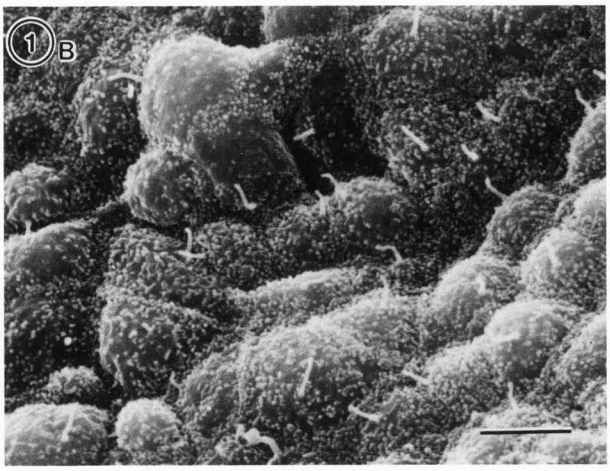
Urinary Bladder Carcinogenesis in the Rat

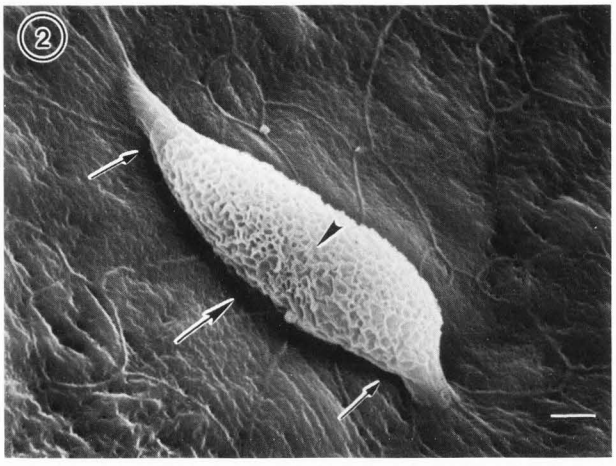
Cancers of the urinary bladder in rats, as well as humans, are predominantly transitional cell carcinomas arising from the highly specialized urothelium of the lower urinary tract extending from the renal pelvis to the urethra (15). Numerous animal models have been developed for the detailed study of the changes occurring from normal to invasive carcinoma, including the administration of a variety of different chemicals either alone or in combination (simultaneously or sequentially) which produce urinary bladder cancer in a period of six months to two years depending on the chemical and the dose (4). By light microscopy, the epithelium goes through various changes beginning with simple hyperplasia, and then progressing to papillary and nodular hyperplasia, papilloma, and ultimately transitional cell carcinoma. Invasive lesions tend to occur much later, and metastases are infrequent.

In all of these models, the changes occurring in the urothelium are generally the same, especially when observed in detail by SEM (15). By SEM, the normal adult bladder epithelium has a luminal surface composed of large, uniform, polygonal cells which are covered with a fine, microridge system due to the highly specialized asymmetric unit membrane lining the luminal surface with the interplaque, and the symmetric unit membrane portion forming the ridges. Rapidly following the administration of a bladder-specific, chemical carcinogen, the urothelium develops pleomorphism of cellular size and shape, frequently associated with increased exfoliation of some of the superficial cells exposing the underlying intermediate cells. As the proliferation continues, the repair process is inadequate to maintain full differentiation, and there is the appearance on the surface of the more rounded intermediate cells underlying the superficial cell layer. These are covered with uniform microvilli. With continued administration of the carcinogen, there is the appearance of numerous pleomorphic microvilli on the surface, which are pleomorphic in both size and shape, with frequent clubbing of the tips of the microvilli. These changes appear throughout the bladder with administration of potent, genotoxic bladder carcinogens such as N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide or N-butyl-N-(4-hydroxybutyl)nitrosamine.

Although pleomorphic microvilli were initially thought to be characteristic of neoplastic transformation, it became apparent that they also occurred on the urothelial surface anytime there was rapid proliferation (Fig. 3), whether secondary to carcinogen administration or whether secondary to a regenerative response to injury, such as surgical wounding, ulceration, or calculus formation (13, 15).







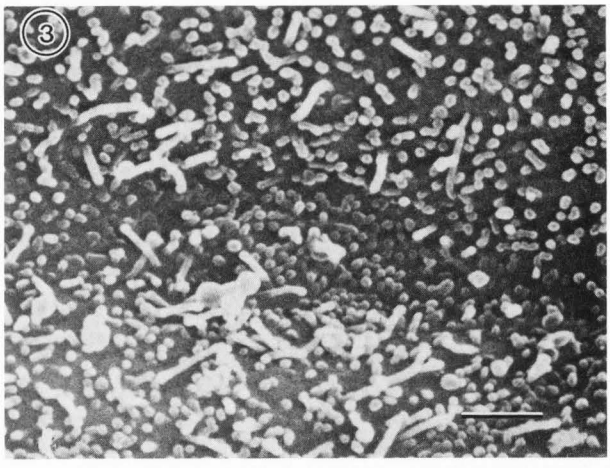


Figure 1. (**A**) SEM of bladder epithelium from a rat at 15 days of gestation. The left portion of the figure shows rounded epithelial cells (arrows) with uniform microvilli gradually merging into the more polygonal-shaped cells, each also having a single, centrally-located cilium (arrowhead). Bar = 0.1 mm. (**B**) Higher magnification of day 15 gestational rat fetus. Bar = 10 μm.

Figure 2. Bladder epithelium from a rat at day 19 of gestation showing a cell that appears to bridge (arrows) across two areas of the bladder epithelium. The cell surface is covered with leafy and ropy microridges (arrowhead). Bar = 5 μm.

Figure 3. Bladder epithelium of a rat fed 7.5% sodium saccharin for 10 weeks. Note that cells have short uniform microvilli and also pleomorphic microvilli. Bar = 10 μm.

In humans, numerous features have been observed on the surfaces of tissue and cytologic specimens from patients with transitional cell carcinoma of the bladder, including many of those that were observed in the animal model, especially pleomorphic microvilli (13, 15). Pleomorphic microvilli have been particularly notable on low-grade, papillary carcinomas, and in some instances this feature has been helpful in the early detection of these otherwise cytologically normal-appearing cells (3, 21). These cytologic specimens can be collected from either urine or bladder washing, and easily observed by SEM techniques. Additional features have been described (21), and quantitative differences have been detected (3).

Sodium Saccharin Effects on Rat Urinary Bladder

Sodium saccharin administered in the diet at high doses (5%) produces bladder cancer in rats (11). However, the chemical must be administered beginning at birth or earlier, whereas administration beginning at 6-8 weeks of age and continuing for 2 years appears insufficient to produce a significant incidence of bladder tumors. Also, the male rat appears considerably more susceptible than the female. In mice, hamsters, and monkeys, sodium saccharin administered at high doses for long periods of time appears to have no effect on the urothelium. In contrast to typical, genotoxic carcinogens, sodium saccharin is not metabolized to a reactive electrophile (it is nucleophilic, with a pKa of approximately 2.8), it does not bind to DNA, and it is not mutagenic. However, it does produce increased cell proliferation, and using various computer modeling techniques we demonstrated that the various protocols involving sodium saccharin carcinogenesis in the rat can be explained in terms of increased cell proliferation without invoking a direct genotoxic mechanism (6, 10, 11). In tumorigenicity and short-term cell proliferation studies, there is an apparent no-effect level at the 1% dose of the diet. The main question to be addressed is whether sodium saccharin has a tumorigenic effect on the human urinary tract, and if so, "Is there the possibility of a true threshold effect?" To be able to answer this question, some understanding of the mechanism involved for saccharin to produce its effect in the rat is necessary, and an explanation as to why the other species are resistant to the effect must be understood. The question then can be addressed as to whether the human is more like the sensitive species, the rat, or the resistant species, the mouse.

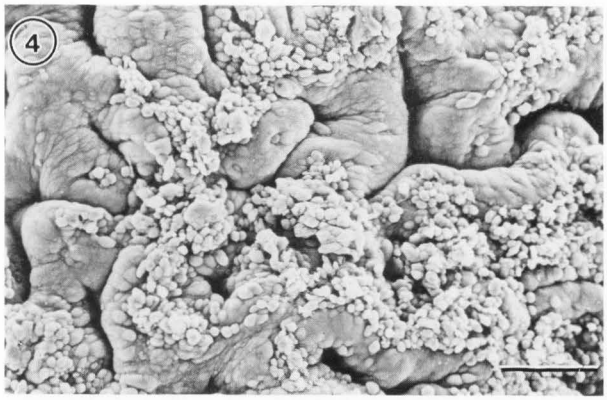
Until about a decade ago, it was assumed that saccharin produced the effect in the rat urinary bladder regardless of what form it was administered. All of the tumorigenic studies until that time had involved the commercially utilized sodium saccharin. In an experiment involving feeding different salt forms of saccharin in the diet at a 5% level for 10 weeks, and then examining the bladder by light microscopy, SEM, and labeling index for DNA synthesis, we observed that the proliferative response is greatest with sodium saccharin, significantly less with potassium saccharin, even less with calcium saccharin, and there was no effect with acid saccharin (12). Experiments with other sodium salts and their corresponding acids have given similar results (11). This includes increased proliferation and tumorigenic effects with compounds such as sodium ascorbate and sodium glutamate, whereas the corresponding acids, ascorbic acid and glutamic acid, gave negative results in the rat. It has also been demonstrated that any treatment which results in acidification of the urine completely inhibits the effects of these compounds. For example, administration of the acid form of the chemicals (acid saccharin, ascorbic acid, glutamic acid) produce an urinary pH less than 6.0 and give negative results in tumorigenicity and proliferation assays (8, 11). Similarly, co-administration of the sodium salt with ammonium chloride produces urinary acidification and inhibits the urothelial effects (8), and the same is observed with administration of sodium saccharin in AIN-76A diet (16), which produces a markedly acidic urine.

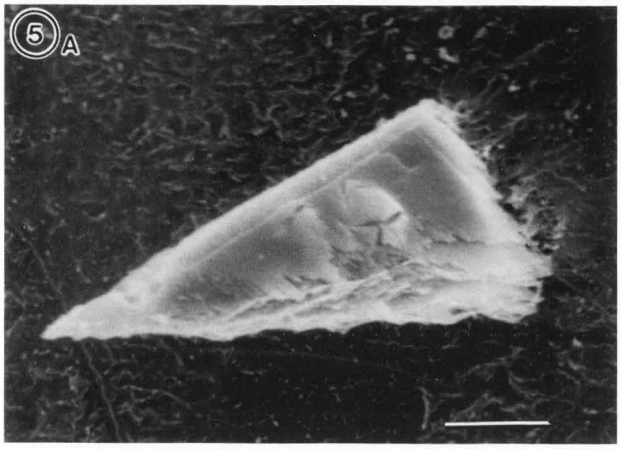
The morphologic changes occurring in the urothelium have been studied in detail following administration of sodium saccharin at high doses to the rat (7). The earlier change is a focal, slight increase in the exfoliation of superficial cells with increased exposure of the underlying intermediate cells, initially predominantly in the bladder dome. This exfoliation increases and becomes more diffuse over the bladder surface. As the exfoliation increases, it leads to a mild regenerative hyperplasia, with the appearance of increasing numbers of the more rounded intermediate cells which are covered by uniform microvilli. Bridge cells are occasionally observed and, there is eventually piling up of these round cells, and the occasional appearance of pleomorphic microvilli after 10 or more weeks of administration of the chemical (Fig. 4).

The urinary concentrations of saccharin do not differ regardless of the chemical form for which saccharin is administered (12). However, there are numerous changes in the urinary milieu with alterations in the concentrations of nearly all of the ions that we have examined in the urine including sodium, potassium, calcium, pH, phosphate, and others. However, none of these alterations cause a change in the chemical structure of saccharin as detected by extensive, detailed nuclear magnetic resonance examinations (22). Also, there does not appear to be a specific cell receptor for saccharin on the urothelium (11).

Silicates and Saccharin

The critical urinary changes that appear to influence saccharin-induced proliferation include urinary pH, sodium concentration, and possibly urinary volume. There is some evidence that potassium can substitute for sodium, although the effect appears to be somewhat less, and is pH dependent (14). To explain the effect of these alterations on saccharin-induced proliferation and tumorigenesis, as well as to explain the difference between male and female rats and between species, we (9) have recently proposed a role for the formation of silicate-containing precipitate and crystals in the urine as a mechanism for the effects of saccharin and related organic compounds, such as ascorbate (Fig. 5). Silicate in precipitate and/or microcrystalline form can be cytotoxic and produce the types of changes that are seen in the bladder of rats fed high doses of sodium saccharin. The presence of silicon in the precipitate and crystals is detected by EDS. A sufficiently high magnification by SEM is used to include only the material for which an analysis is desired. Identical changes can be produced by the administration of relatively high doses of various silicates, such as tetraethylorthosilicate (TES) (17).





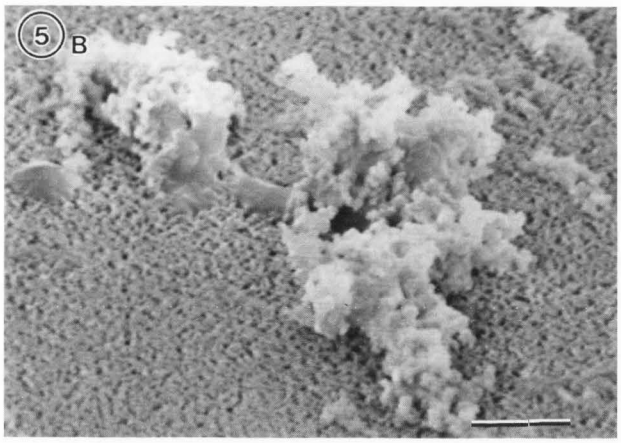


Figure 4. Piling up of small, round cells characteristically observed in bladder cancer of high grade. The cells have numerous microvilli and ropy microridges rather than the leafy microridges of normal superficial cells. Bar = 0.1 mm.

Figure 5. **(A)** Silicate-containing crystal on the surface of the bladder of a rat treated with 7.5% sodium saccharin. Bar = 10 μm. **(B)** Precipitate from voided urine of a rat fed high doses of sodium saccharin in the diet. Bar = 10 μm.

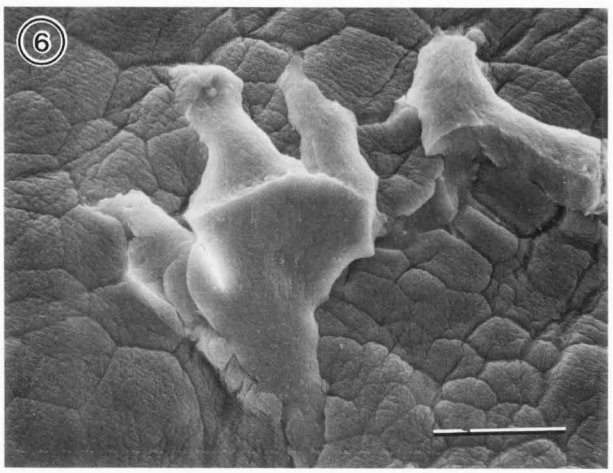


Figure 6. Sharp silicate-containing crystal penetrating the bladder epithelium of a rat fed 7.5% sodium saccharin. Bar = 10 μm.

Other silicates, such as sodium silicate, appear to be considerably less effective, if at all. Male rats fed high doses of sodium saccharin produce a significant amount of precipitate in the urine which contains protein, saccharin, and silicates, as well as other substances being incorporated. These occasionally yield microcrystals which are relatively sharp and abrasive (Fig. 6).

For SEM and X-ray analysis of urine from rats, freshly voided urine is required and a constant time of day is required for collection since there is marked diurnal variation in concentrations of most components of the urine, including pH and sodium. Care must be taken to avoid contamination from dust in the air, powder on gloves or materials from the rat. The freshly voided urine is collected directly into a polystyrene test tube (glass must be avoided) and centrifuged for 30 minutes at 3,000 rpm (14,000 x g). The supernatant is removed for examination of the crystals, with the sediment being filtered onto a 0.22 μm Millipore filter using a low vacuum. The filter is coated with gold and examined by SEM and EDS (Fig. 7). In some instances, coating with carbon is more useful since the M-line of gold occasionally interferes with measurement of the K-line of silicon X-ray peak. We have been using a Philips 515 scanning electron microscope with attached Kevex energy dispersive X-ray spectrometer. Filters of urine from control rats have numerous magnesium ammonium phosphate crystals which are readily distinguishable from silicate crystals by SEM (Fig. 8). Also present are numerous small potassium chloride crystals, with a characteristic Y-shaped crack. These crystals form as part of the drying procedure secondary to the large amounts of potassium chloride present in the urine. The filters of urine from sodium saccharin-fed rats contain some magnesium ammonium phosphate and potassium chloride crystals, but also contain clumps of precipitate and jagged crystals, both of which contain varying amounts of silicate.

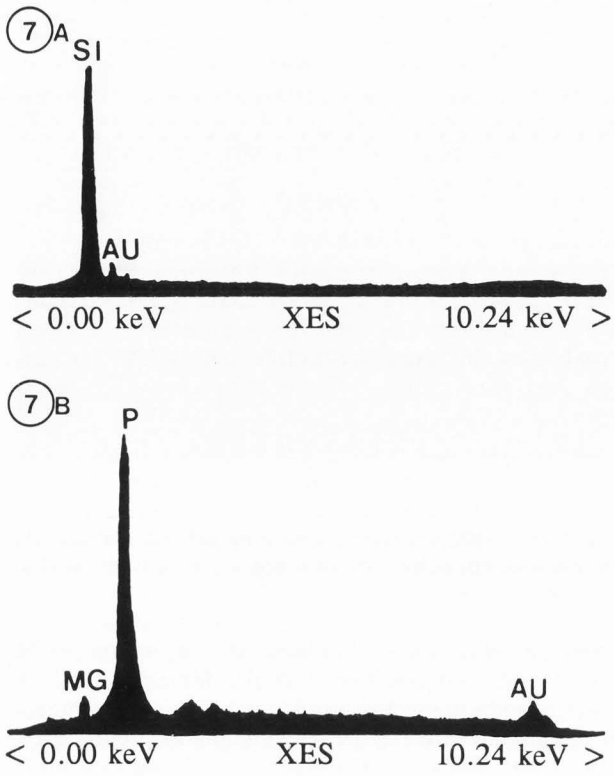


Figure 7. Energy dispersive X-ray spectra of (**A**) silicate-containing crystal from a sodium saccharin treated rat, and (**B**) typical $MgNH_4PO_4$ crystals present in urine of rats fed control diet.

The normal rat urine, like that of most other mammalian species, including humans, contain numerous magnesium ammonium phosphate crystals. There is little amorphous precipitated material in the normal rat urine. In rats fed high levels of sodium saccharin, the silicate-containing precipitate occurs with silicate-containing crystals, in addition to the presence of magnesium ammonium phosphate crystals. By light microscopy, the silicate-containing crystals are generally not distinguishable from the usual phosphate crystals. Female rats have considerably less of the silicate-containing precipitate and crystals, and male and female mice administered high doses of sodium saccharin, as high as 7.5% of the diet, do not contain the precipitate or the crystals, nor do rats administered acid saccharin.

There are several features of silicate precipitation which are germane to the biological effects of sodium saccharin in the rat (9). To begin with, it has been observed by us and others that silicate precipitation does not occur if the urinary pH is below approximately 6.5 (this range is between 6.3 and 7.0 depending on the model system). As described above, procedures which produce acidification of the urine inhibit the proliferative and tumorigenic effects of saccharin and other sodium salts. In addition, a major requirement for precipitation of silicates is the presence of protein. The greater

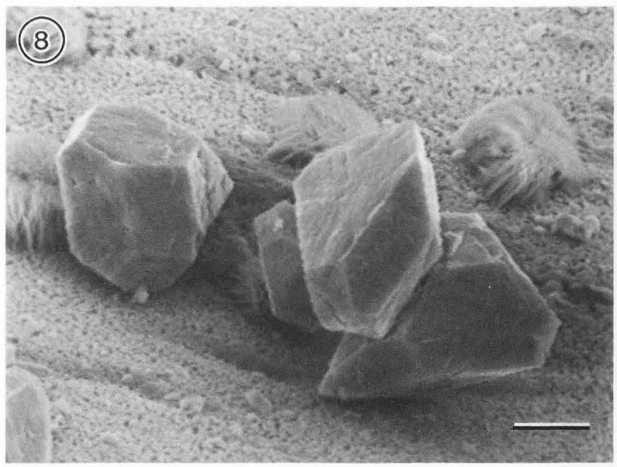


Figure 8. $MgNH_4PO_4$ crystals from the urine of control rats are not sharp and jagged as are silicate-containing crystals from the urine of sodium saccharin treated rats. Bar = 10 μm.

---

the amount of protein, the greater the likelihood for silicate precipitation. The rat has large amounts of protein, more than 100 times the levels observed in humans, and the level is greater in male rats than in female rats. In male rats, the increase occurs after puberty with the appearance of large amounts of α_{2u}-globulin being excreted in the urine (18). It appears that α_{2u}-globulin serves as a better vehicle for precipitation of the silicates than does albumin or other large molecular weight proteins (9). In addition, it is well known that increased concentrations of sodium increase the rate of precipitation of proteins, in general.

The concentration of soluble silicates actually decreases in the urine of rats fed high doses of sodium saccharin (9). This is due to a dilutional effect secondary to the nearly doubling of urinary volume in rats fed sodium saccharin in response to the increased consumption of water (9). Expressed in terms of the amount of silicate for unit of creatinine, the levels of soluble silicates are similar in control and sodium saccharin fed rats. We presently hypothesize that the precipitate occurs in the sodium saccharin fed rats rather than in the control rats because of interaction of the saccharin anion and sodium with the protein at sufficiently high pH and diluted urine (9). This modified protein is more susceptible to the precipitation of silicates. We have demonstrated that saccharin binds to urinary proteins, with greater binding per mole to α_{2u}-globulin than to albumin. Thus, before the appearance of α_{2u}-globulin in males at puberty, effects are postulated to be mild and similar in both sexes. With the addition of α_{2u}-globulin after puberty, the response is accentuated in the male.

If the hypothesis involving silicates is correct, it provides a rational basis for evaluating the risk of saccharin in humans. To begin with, it is unlikely that silicate precipitates will form in humans regardless of the

concentration of sodium saccharin. In the mouse, which can be administered levels of sodium saccharin as high as 7.5% of the diet, silicates do not form and there is no increased proliferation or tumorigenic effects with sodium saccharin. It is likely that the human response is similar to the mouse, rather than being similar to the rat. Our overall hypothesis at the present time is that the administration of high levels of sodium or analogous sodium salts leads to the generation of silicate-containing precipitate and crystals which leads to mild toxicity of the superficial urothelium, consequent regenerative hyperplasia, and ultimately tumor formation.

The mechanism for silicate toxicity is not completely known. Silicates are well known to cause toxicity in the respiratory tract if aspirated in relatively high amounts and in fiber form (20). In the respiratory tract, this is accompanied by an inflammatory reaction progressing to fibrosis and chronic regenerative proliferation of the pulmonary epithelium. There is evidence that the effect of the silicate is on cell membranes (19), and this has been studied in greater detail using red cell membranes. For red cells, exposure to silicates produces hemolysis.

Silicic acid has a high affinity for hydrogen exceptors in biological tissues which can react with secondary amide groups in protein and with phosphate lipids by hydrogen donation from its phenolyic hydroxyl groups. The greater the hydroxylation of the silica polymer, the more toxic the silicate becomes (14).

The precise nature of the cytotoxic effects of silicate particles is of biological importance due to its universal presence in food and water as well as in the air we breathe. In small amounts, the ingestion of amorphous silica powder appears to be completely harmless since it dissolves to give only a monomer. Monomeric $Si(OH)_4$ permeates all body liquids at concentrations less than its maximum solubility (approximately 0.01%) and is readily excreted in the urine (14). Silicates can be absorbed from the gastrointestinal tract and passed in soluble form into the blood and then excreted in the urine. Numerous silicates are considered safe additives in foods as anti-caking agents and are present in numerous pharmaceuticals and in the ceramic industry. The respiratory toxicity occurs primarily in association with exposure to high levels of relatively large fibers.

Urinary tract effects in humans of silicates are primarily related to silicate-containing antacids when consumed at extremely high levels (23). At these extreme levels, the silicate concentration in the urine becomes quite high, and there is precipitation and ultimately formation of calculi. A few cases of such silicate-containing calculi have been reported in human urine. They have always been associated with exposures to extremely large amounts of silicate, not normally seen in human exposures.

Conclusions

SEM analysis of urine and bladder tissue and cells are useful in various experimental settings as well as in human cytology, both for the detection of abnormal cells as well as factors which contribute to the mechanism of action of various urinary bladder carcinogens. The proposed role of silicates in sodium saccharin carcinogenesis in the rat provides a rational basis for extrapolation from the rat model to human exposures, and suggests that humans are without a response of their urothelium to sodium saccharin exposure. This is supported by extensive epidemiological studies which indicate no effect of the human urothelium to exposure to sodium saccharin, whether utilizing urothelial proliferation or bladder cancer as the basis for evaluation. SEM and EDS analyses have proven essential in this investigation.

Acknowledgements

This research was supported in part by grants from the National Cancer Institute (CA32513 and CA36727) and from the International Life Sciences Institute-North America. We would like to thank Emily M. Garland and Linda S. Johnson for their technical assistance and we are grateful to Ginni Philbrick for assistance in the preparation of this manuscript.

References

1. Ayres PH, Shiohara Y, Frith CH (1985) Morphological observations on the epithelium of the developing urinary bladder of the mouse and rat. J. Urol. **133**, 506-512.

2. Cano M, Johansson SL, Wilson RB, Ellwein LB, Sakata T, Cohen SM. (1986) Preparation methods for light microscopic and ultrastructural studies of fetal rat bladder. Scanning Electron Microsc. **1986**; IV, 1357-1362.

3. Cano M, Wilson RB, Cohen SM (1985) Quantitation of scanning electron microscopic urinary cytology. Scanning Electron Microsc. **1985**; III, 1273-1278.

4. Cohen SM (1983) Pathology of experimental bladder cancer in rodents. In: The Pathology of Bladder Cancer. Bryan GT, Cohen SM (Eds.), Vol. II. CRC Press, Boca Raton, FL, pp. 1-40.

5. Cohen SM, Cano M, Sakata T. Johansson SL (1988) Ultrastructural characteristics of the fetal and neonatal rat urinary bladder. Scanning Microsc. **2**, 2091-2104.

6. Cohen SM, Ellwein LB (1990) Cell proliferation in carcinogenesis. Science **249**, 1007-1011.

7. Cohen SM, Fisher MJ, Sakata T, Cano M, Schoenig GP, Chappel CI, Garland EM (1990) Comparative analysis of the proliferative response of the rat urinary bladder to sodium saccharin by light and scanning electron microscopy and autoradiography. Scanning Microsc. **4**, 135-142.

8. Cohen SM, Ellwein LB, Okamura T, Masui T, Johansson SL, Smith RA, Wehner JM, Khachab M, Chappel CI, Schoenig GP, Emerson JL (1991) Comparative bladder tumor promoting activity of sodium saccha-

rin, sodium ascorbate, related acids and calcium salts in rats. Cancer Res. **51**, 1766-1777.

9. Cohen SM, Cano M, Earl RA, Carson SD, Garland EM (1991) A proposed role for silicates and protein in the proliferative effects of saccharin on the male rat urothelium. Carcinogenesis **12**, 1551-1555.

10. Ellwein LB, Cohen SM (1988) A cellular dynamics model of experimental bladder cancer: analysis of sodium saccharin in the rat. Risk Analysis **8**, 215-221.

11. Ellwein LB, Cohen SM (1990) The health risks of saccharin revisited. Toxicology **20**, 311-326.

12. Hasegawa R, Cohen SM (1986) The effect of different salts of saccharin on the rat urinary bladder. Cancer Lett. **30**, 261-268.

13. Jacobs JB, Cohen SM, Farrow GM, Friedell GH (1981) Scanning electron microscopic features of human urinary bladder cancer. Cancer **48**, 1399-1409.

14. Iler RK (1979) The chemistry of silica. Wiley, New York; pages 375, 707, and 757.

15. Jacobs JB, Cohen SM, Friedell GH (1983) Scanning electron microscopy of the lower urinary tract. In: The Pathology of Bladder Cancer. Bryan GT, Cohen SM (Eds.), Vol II. CRC Press, Boca Raton, FL, pp 141-181.

16. Okamura T, Garland EM, Masui T, Sakata T, St. John M, Cohen SM (1991) Lack of bladder tumor promoting activity in rats fed sodium saccharin in AIN-76A diet. Cancer Res. **51**, 1778-1782.

17. Okamura T, Garland EM, Johnson LS, Cano M, Johansson SL, Cohen SM (1992) Acute urinary tract toxicity of tetraethylorthosilicate in rats. Fund. Appl. Toxicol. **18**, 425-441.

18. Olson MJ, Johnson JT, Reidy CA (1990) A comparison of male rat and human urinary proteins: implications for human resistance to hyaline droplet nephropathy. Toxicol. Appl. Pharm. **102**, 524-536.

19. Razzaboni B, Bolsaitis P (1990) Evidence of an oxidative mechanism for the hemolytic activity of silica particles. Environ. Health Perspec. **87**, 337-341.

20. Saffiotti U, Stinson SF (1988) Lung cancer induction by crystalline silica: relationships to granulomatous reactions and host factors. J. Environ. Sci. Health **6**, 197-222.

21. Suzuki T, St. John M, Cohen SM, Friedell GH (1983) Benign squamous cells with pleomorphic microvilli in urine or bladder washings. Acta Cytol. **27**, 497-499.

22. Williamson DS, Nagel DL, Markin RS, Cohen SM (1987) Effect of pH and ions on the chemical structure of saccharin. Food Chem. Toxicol. **25**, 211-218.

23. Yamamoto N, Maeda S (1990) A case of silicate urolithiasis. Acta Urol. Jpn. **36**, 147-150.

Discussion with Reviewers

E. de Harven: Your analysis of the ultrastructural morphology of the transformed rat urothelium is based essentially on SEM in the secondary electron mode. Our own studies have benefitted considerably from the addition of immuno-SEM studies, with the colloidal gold marker visualized in the backscattered electron imaging mode of the SEM (Scanning Microscopy, **4** (2), 467-477, 1990). Was the phenotype of the bladder urothelium explored by immuno-SEM methods in attempts to demonstrate either the deletion of pre-existing antigens or the expression of tumor associated antigens?
Authors: We have not performed any immuno-SEM studies on our material.

G.M. Hodges: What is thought to be the nature of the cytotoxic effect of silicate precipitates on bladder urothelium? Since silicates in microcrystalline form may be relatively sharp and abrasive, could such microcrystals be capable of causing repeated local injury and, also possible anchorage and thus stimulate growth.
Authors: We do not yet have specific information concerning the cytotoxic effect of the precipitates and crystalline material.

G.M. Roomans: Do you have any quantitative data on the occurrence of various crystal types in rat (male, female) urine?
Authors: Unfortunately, we do not have quantitative date yet on the occurrence of various crystal types in urine.