Urol Res (1994) 22:167–170

ORIGINAL PAPER

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Measurement of BBN-induced alterations in rat urothelium by electron microscopic X-ray microanalysis

Received: 18 June 1993/Accepted: 3 December 1993

Abstract The aim of this study was to analyse *N*-butyl-*n*-butanol-4-nitrosamine (BBN)-induced alterations of the urothelium in rats concerning its content of phosphorus, sulphur, chlorine, potassium and calcium using electron microscopic X-ray microanalysis (REM analysis). The following histopathological findings of the bladder mucosa were discovered after exposure to BBN: normal urothelium ($n = 36$); focal epithelial proliferations ($n = 12$) following 6–12 weeks' exposure; epithelial hyperplasia ($n = 8$) after urothelial carcinoma ($n = 4$) following 12 weeks' exposure. The observed phosphorus/sulphur and phosphorus/calcium ratios based on REM analysis did not show any statistical correlation with the morphological changes classified by light microscopy. Our data do not support the hypothesis raised by other investigators that an increase in phosphorus content or phosphorus/sulphur or phosphorus/calcium ratio could indicate early neoplastic transformations of urothelial cells as "tumor markers".

Key words Urothelial carcinoma · Animal experiment · *N*-butyl-*n*-butanol-4-nitrosamine · Chemical carcinogenesis · X-ray analytical measurement · Tumor markers

An analysis of chemical elements using the method of X-ray micro-analysis can be worked out at cell level by scanning electron microscopic micro-analysis (REM analysis) [11, 16–18]. This micro-analytical procedure was derived from metallurgy, where it has been used to detect the qualitative and quantitative formulation of metals. Applied to tissue specimens, it provides information about the morphology of the surface structure by electron microscopy as well as the distribution of chemical elements [20]. By REM analysis even traces of an element around 10^{-19} g could be detected [1, 12]. Recent studies by Friedrichs and Gupta [6, 8, 9, 10] analyzed biopsies of human neoplastic and normal bladder mucosa by means of the scanning electron microscope for their element composition. They detected phosphorus almost exclusively in carcinomatous tissue ($n = 14$), while weak phosphorus peaks were measured in only two specimens of normal bladder mucosa ($n = 14$).

Alterations of normal bladder mucosa were induced in this study by *N*-butyl-*n*-butanol-4-nitrosamine (BBN), a carcinogen that selectively produces bladder tumors in rats. Bladder specimens were examined histologically by light- and scanning electron microscopy to find whether differences in element composition in urothelial cells occur during BBN exposure and whether there is a significant correlation between histology and the phosphorus/sulphur or the phosphorus/calcium ratio.

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Materials and methods

Animals

Sixty female Wistar rats were used which initially weighed between 200 and 260 g. A standard diet (Herilan, Eggersmann, Freiburg, Germany) was fed ad libitum and drinking water, mixed with BBN, was constantly available in dark-painted, light-protected plastic bottles. Weight increase or loss, fluid intake and general state of health were assessed and recorded every 2 days. The average weight increase per week was 5–7 g per rat, and the mean consumption of drinking water was 29 ml per day per rat.

Experimental protocol

The 60 rats were marked with hair dye and split into five groups of 12 animals each. The drinking water of four groups was mixed with 0.05% BBN (German National Cancer Research Center, Heidelberg, Germany) and administered over periods of 4, 6, 8 and 12 weeks. The other 12 rats (control group) received pure drinking water without any additives throughout the experiment.

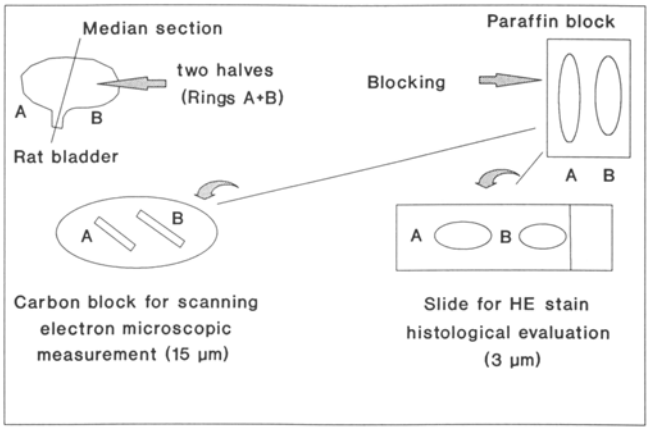

Fig. 1 Fixation and embedding of rat bladder

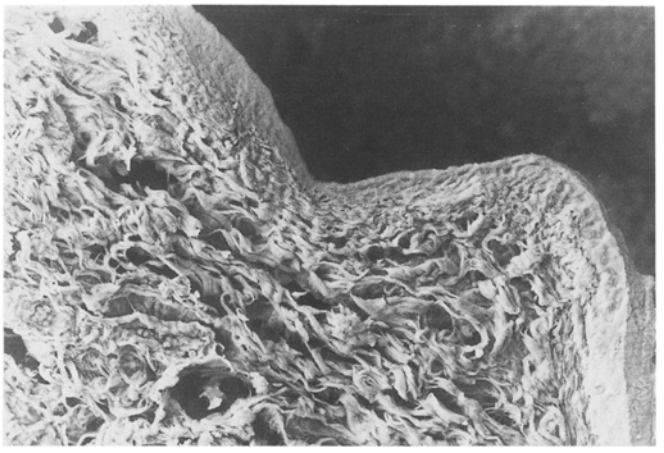

Fig. 3 REM-image of bladder specimen from rat 41, group 1. (×300)

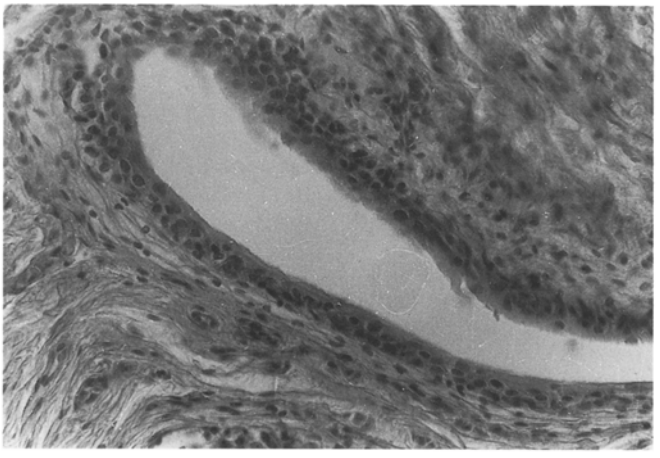

Fig. 2 Light microscopy analysis of bladder specimen from rat 41, group 1. (HE)

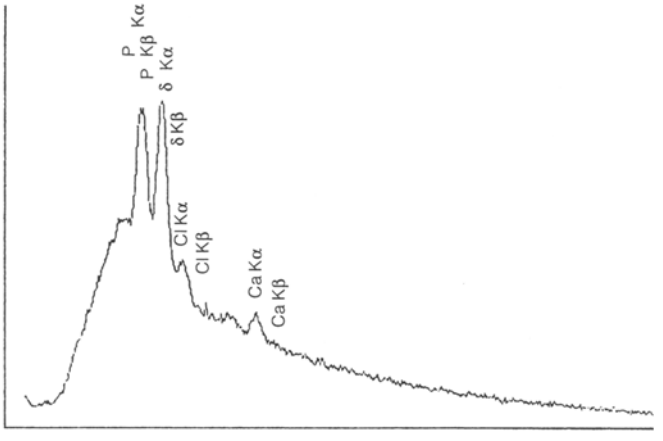

Fig. 4 X-ray analytical REM measurements of elements in rat urothelium from rat 41, group 1

Group 1: 12 weeks with pure drinking water (control group)
Group 2: 4 weeks with drinking water containing BBN
Group 3: 6 weeks with drinking water containing BBN
Group 4: 8 weeks with drinking water containing BBN
Group 5: 12 weeks with drinking water containing BBN

BBN-treated and control (group 1) animals received carcinogen-free water for 5 days before being killed by a lethal dose of a pentobarbital derivative (Nembutal)™.

Fixation and embedding

Removed rat bladders were fixed in 2.5% glutaric dialdehyde (5–7 h) and afterwards stored in cacodylate buffer (pH 7.9). Specimens were dehydrated in an ascending alcohol series, followed by treatment with xylene (3× 6 h). The bladders were then transferred to paraffin (3× 6 h; 60°C) and poured out in Paraplast™.

The bladders were bisected sagitally and positioned in such a way that the resulting slides would exactly represent the median plane; one half was used for histology and the other, for REM element analysis. This procedure made direct comparison of histology and element analysis at corresponding regions possible (Fig. 1).

Staining

Paraffin blocks were cut into cross sections (3 µm), rehydrated and stained with hematoxylin-eosin following the protocol of Harrison et al. [13] (Fig. 2).

Preparative techniques for REM analysis

Paraffin blocks were cut into cross sections of 15 µm and then melted for 1 h in a water bath at 60°C. Deparaffinization of the sections was carried out with xylene and 100% alcohol. The cross sections were afterwards carefully fixed on acetone-cleaned carbon blocks, using a small drop of glue (Uhu Hart, Henkel, Düsseldorf, Germany), and dried for 30 min.

X-ray analytical measurement

Two sagittal sections (rings A and B, Fig. 1, 15 µm) from each rat bladder were prepared on a carbon block as described above. Two measurements at two different locations were taken per ring. At each location one measurement was done in the urothelial mucosa and one measurement in the connective tissue underneath at a depth of

Table 1 Histological findings

Group	Number of rats reflected in histological results by treatment group	Total number of rats
A	Normal urothelium (3 layers in general) Group 1, $n=12$; 2, $n=12$; 3, $n=7$; 4, $n=3$; 5, $n=2$	36
B	Focally epithelial hyperplasia (3–5 layers), with normal cell differentiation (G0) Group 3, $n=5$; 4, $n=3$; 5, $n=3$	11
	Papillary protrusion Group 5, $n=1$	1
C	Epithelial hyperplasia (> 25–50% of bladder mucosa) Group 4, $n=6$; 5, $n=1$	7
	Epithelial proliferation Group 5, $n=1$	1
D	Occurrence of "bud protrusions," some infiltrating into lamina propria ($T_{a/l}$) Group 5, $n=2$	2
	Squamous cell carcinoma (T_1, G_x) Group 5, $n=1$	1
	Exophytic urothelial carcinoma with squamous cell metaplasia (T_1, G_2) Group 5, $n=1$	1

Table 2 Phosphorus/sulphur ratio (derived by REM analysis) concerning histopathological groups

Group	Urothelium			Connective tissue		
	Mean value	SD	r	Mean value	SD	r
A	1.16	0.71	0.13	0.54	0.44	0.11
B	1.64	0.69	–	0.63	0.21	–
C	1.18	0.74	–	0.47	0.2	–
D	0.97	0.2	–	0.44	0.32	–

Table 3 Phosphorus/calcium ratio concerning histopathological groups

Group	Urothelium			Connective tissue		
	Mean value	SD	r	Mean value	SD	r
A	14.38	14.11	0.28	7.7	6.2	0.13
B	19.59	9.17	–	6.87	3.04	–
C	14.48	8.4	–	10.24	6.74	–
D	27.33	21.61	–	14.04	0.4	–

40 µm. In total, there were eight measurements per rat bladder, distributed randomly over the complete bladder surface. Analytical measurements were carried out with an SEM, brad JSM-35-CF, equipped with a back scatter electron detector (silicon semiconductor) and an X-ray system ORTEC 5000 EGuG (Einrichtung der Gesellschaft für Elektronenmikroskopie der RWTH Aachen, Germany).

Operations were run using the following standardized parameters: acceleration voltage 15 kV; scanned surface ratio 0.0030756 mm^2; sample current $10^{-8} \times 0.32$ A (measured on the carbon mount); amplification 1000-fold; measuring period 150 s. The data obtained were calculated by a computer system and expressed (Einrichtung der Gesellschaft für Elektronenmikroskopie der RWTH Aachen) as graphs with pertinent peaks for the concentration of phosphorus (P) chlorine (Cl) and calcium (Ca) (Figs. 3, 4).

Results

Histological findings

The specimens from the 60 rats initially distributes to five groups of 12 animals according to the administration of BBN were callocated according to histopathological morphology into four groups, A–D (Table 1).

Phosphorus/sulphur ratio

In group A, the P/S ratio was 1.16 (SD 0.71), i.e. the phosphorus intensities were larger than the sulphur intensities. In group B the P/S ratio was the highest (1.64; SD 0.69), because phosphorus concentrations were highest in this group. In group C the mean value was almost identical to that found in group A. In group D the mean value was lowest, with 0.97 (SD 0.2). When the nominal mean values are considered, a tendency for increasing urothelial changes in the mucosa becomes apparent to be accompanied by a declining relative phosphorus intensity (Table 2).

Measurements in the corresponding connective tissue yielded values half those for intensity in the mucosa. This can be explained by the lower phosphorus concentrations in the connective tissue. For group A the mean value was 0.54 (SD 0.44), for group B, 0.63 (SD 0.21), for group C, 0.47 (SD 0.2) and for group D, 0.44 (SD 0.32).

Phosphorus/calcium ratio

The calcium values reached only very low intensities so that P/Ca ratio increased generally and reached double figures (Table 3). Again, the values measured in connective tissue gave ratios approximately half those in the urothelium. Statistical analysis for the histopathological groups did not reveal any correlation with the element ratios except for P/Ca in one case. The correlation coefficient (r) was 0.28, with a possibility of error of 4.3%. The other results were not statistically significant.

Discussion

It is well known from cell culture experiments that cells of various origins only grow in the presence of calcium (available in the medium). Thus, fibroblasts require high Ca^{2+} concentrations, while human urothelial cells, prostate epithelial cells of the rat, and the majority of tumor

cells, require lower Ca^{2+} concentrations [19]. In addition, it is known from atom absorbtion spectrometry that a lower concentration of selenium and zinc is present in the mitochondria fraction of bladder carcinoma than in healthy bladder tissue. Furthermore, the zinc concentration is higher in tumor cells than in normal urothelium [4, 7].

In a study by Friedrichs and Gupta [8–10], human bladder mucosa biopsies of normal ($n = 14$) and tumor tissue ($n = 14$) were analyzed for element composition by SEM. Phosphorus, sulphur, chlorine, calcium and potassium were detected. In general, phosphorus was found almost exclusively in bladder carcinomas ($n = 12$). It is assumed that the increased concentration of phosphorus in carcinomatous tissue resulted from raised DNA content and increased metabolic activity [15].

Data derived from REM analysis are dependent on numerous factors. Problems can arise from the preparation of the samples, background radiation, the radiation damage, contamination with dust, etc. and, especially, from different qualitative and quantitative evaluation possibilities [1–3, 5, 14]. Therefore, in most surveys, semiquantitative evaluations are used that cannot give precise element concentrations but do allow evaluation of the relative concentration quotients of the elements within a sample and within the same experiment [12, 14]. The data presented in this study suggest the P/S ratio is the most reliable value in urothelial tissue, since the clearest intensity peaks were observed for P/S. The P/S ratio differed significantly between urothelium and connective tissue. This means a decline in phosphorus intensity from urothelium to connective tissue. In cases of very low concentrations, such as those of Ca^{2+}, the computer program used, which computed the pertinent background radiation and listed the net intensity of the elements, was unable to detect small differences. The minimum calcium peaks reached 20–300 counts/s, while normal values ranged between 10000 and 45000 counts/s. In addition, the number of samples in which the calcium peaks were analyzed was inadequate for statistical evaluation. This was similar to chlorine, that was only detected in a few specimens and thus was not included in the statistics.

Due to these "insensitivities", the results of the correlation analyses must be interpreted critically with regard to the P/Ca ratios. The statistically determined significances obtained for correlations between groups 1 and 2 and groups 1 and 5 are probably caused by the above-mentioned facts and are thus not reliable.

The aim of the study was to investigate whether early neoplastic transformations could be detected by REM analysis. The results, however, do not show any significant correlations between element intensities in urothelial hyperplasia and urothelial carcinomas (<T1,G2) of the bladder mucosa.

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