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Development of *N*-butyl-*N*-(hydroxybutyl)-nitrosamine-induced tumors in the partially resected, proliferating rat urinary bladder in dependence upon the time of onset of stimulated DNA synthesis

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Summary. Tumor development was investigated in the partially resected, proliferating urinary bladder of rats in dependence upon the onset of stimulated de novo DNA synthesis related to carcinogen dosing. *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN) was used as carcinogen and administered by gavage in three fractionated doses (100 mg/kg body weight each) either during the phase of the most pronounced proliferation of the urothelium 30, 45 and 70 h after one-third resection of the bladder or 24 h and 1 week prior to partial cystectomy. When BBN was given during most increased DNA synthesis subsequent to one-third resection, the incidence of bladder tumors was reduced to 8.7% compared with 19.6% found in control animals with a non-resected, quiescent bladder. Tumor formation was neither inhibited nor enhanced when BBN was initially administered, followed by partial cystectomy 24 h or 1 week after the last carcinogen dose, yielding tumor incidences of 18.2% and 22.5%, respectively. Thus, the feeding of BBN during the period of maximum DNA synthesis inhibited tumor development in the partially resected bladder, while stimulation of cell replication subsequent to carcinogen administration did not influence the carcinogenic process initiated. The results obtained indicate that time of onset of stimulated DNA synthesis related to carcinogen dosing is the decisive factor in modifying urothelial carcinogenesis in the proliferating urinary bladder.

Key words: Rat – Urinary bladder – Partial cystectomy – Stimulation of urothelial proliferation – *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine – Tumor development

Previous investigations have shown an inhibition of tumor development in the partially resected, reparatively regenerating urinary bladder after administration of *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN) and *N*-methyl-*N*-nitrosourea (MNU) during the phase of most pronounced proliferation of the urothelium [8, 11]. Recent studies using the cystectomy model following synchronization of the stimulated urothelial proliferation by hydroxyurea have revealed that the inhibition of urothelial carcinogenesis is dependent on the cell cycle [13]. The incidence of MNU-induced bladder tumors was significantly reduced when the carcinogen was instilled intravesically in a single dose during the early DNA synthesis phase; tumor induction was also remarkably – though not significantly – inhibited when MNU was given during the late postmitotic-presynthetic phase and the late DNA synthesis phase. The objective of the present experiments was to test whether stimulation of urothelial proliferation subsequent to initial treatment with BBN also modifies tumor development in the partially resected bladder either in the sense of inhibition or enhancement due to promotion of the initiated carcinogenic process.

Materials and methods

Animals

Pathogen-free adult female Wistar rats with an initial weight of between 180 and 220 g (animal breeding farm Mus-Rattus, Brunnthal, FRG) were used. Groups of four rats were housed in plastic cages and kept under standardized conditions in an air-conditioned room at a temperature of 22°C and a relative humidity of between 50 and 60% and on a 12-h light-dark cycle. The animals had free access to tap water and were fed a standard commercial ground diet (Altromen Company, Lage, FRG).

Partial cystectomy

To induce proliferation of the urothelium that normally shows a very low rate of cell renewal (for a review of the literature see [5]), one-third of the urinary bladder was resected, employing conventional surgical techniques [10]. Before laparotomy, the bladder was emptied from urine by application of gentle pressure to the lower abdomen. The rats were then anesthetized with Ketanest (Park-Davies Company, Munich, FRG) at a dose of 50 mg/kg body weight and Rompun (Bayer AG, Leverkusen, FRG) at a dose of 4 mg/kg body weight injected intraperitoneally in a mixed solution. The peritoneal cavity was opened by a midline, longitudinal incision approximately 10 mm in length. Thereafter, the bladder was pulled out slightly with forceps and affixed with two opposing sutures using

Table 1. Incidence of *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN)-induced (three fractionated doses of 100 mg/kg body weight each by gavage) tumors in the non-resected, resting and partially resected (one-third resection), proliferating rat urinary bladder after an experimental period of 19 months

Treatment	Exclusively BBN (group 1)	Initially PC; BBN 30, 45 and 70 h postoperatively (group 2)	Initially BBN; PC 24 h after the last carcinogen dosing (group 3)	Initially BBN; PC 1 week after the last carcinogen dose (group 4)
Effective no. of rats	46	46	55	49
No. of rats with tumors	9	4	10	11
Tumor incidence (%)	19.6	8.7	18.2	22.5
Histological tumor types	4 papillomas[a] 4 pap. TCC (1) 2 pap. TCC (2)	3 papillomas 1 pap. TCC (1)	1 papilloma 8 pap. TCC (1) 1 pap. TCC (2)	7 papillomas 3 pap. TCC (1) 1 pap. TCC (2)

[a] One of the animals had two independent tumors in the bladder
PC = Partial cystectomy (one-third resection of the bladder); pap. TCC = papillary transitional cell carcinoma; histopathological grades of the transitional cell carcinomas indicated in parentheses

atraumatic, resorbable material (Dexon; Braun-Dexon GmbH, Melsungen, FRG) at the transition of the middle to the upper third. Subsequently, the upper third of the bladder was resected using a pair of scissors. The stump was closed by two sutures through the outer muscular layer and serosa using resorbable material (Dexon). Care was taken to avoid damage of the mucosa by the sutures and, hence, the risk of stone formation in the bladder. The abdominal incision was closed in three layers, fascia, muscle and skin, using catgut. The operative and postoperative mortality was lower than 5%. The rats were kept warm under a lamp to prevent hypothermia during the operative procedure and postoperatively. Catherization was not needed since the rats spontaneously urinated.

As thoroughly analyzed in our previous autoradiographical studies, the ^{3}H-TdR index of the urothelium begins to increase dramatically following a short preproliferative phase of 15 h at the site of operation and reaches a peak of 24.7% 45 h after one-third resection of the bladder [10]. This represents a 193-fold increase in cell proliferation above normal levels (0.13%). Subsequently, the labelling index rapidly decreases and returns to low values 90 h postoperatively (0.6%). After 2 weeks a ^{3}H-TdR index of 0.02% was determined, which largely corresponds to that of the normal, physiologically regenerating urothelium. There is also an increase of the proliferative activity in the remainder of the bladder stump, away from the immediate operative region, but here the maximum ^{3}H-TdR index amounts to only 10.1% 25 h postoperatively [10].

N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN)

In agreement with our previous studies [11, 12], BBN (prepared by D. Zelesny, Deutsches Krebsforschungszentrum, Heidelberg, FRG) was dissolved in 1.2 propandiol as a vehicle and administered by gavage in three fractionated doses. For each gavage feeding the animals received 1 ml of the propandiol-carcinogen solution. BBN was given in fractionated low doses rather than in a single high dose to avoid toxic effects on the experimental animals in general and, in particular, on the urothelial cells, possibly causing cell death. As ascertained earlier [11], the low doses used induce urothelial bladder tumors at an incidence of 21% after an experimental period of 18 months.

Experimental groups

The experimental animals were randomly divided into four groups and received BBN in three fractioned doses of 100 mg/kg body weight each (total dose: 300 mg/kg). In all rats – with the exception of the controls – one-third of the urinary bladder was resected either prior to or subsequent to carcinogen administration. After an experimental period of 19 months the animals were killed by cervical dislocation.

Group 1. The rats of this group had an intact, non-resected urinary bladder and received BBN at the beginning of the experiment in three fractionated doses at 24-h intervals. This control group consisted of 60 animals.

Group 2. Following one-third resection of the bladder, 70 rats were administered BBN in three single doses during the phase of most pronounced proliferation of the stimulated urothelium 30, 45 and 70 h postoperatively.

Group 3. In this group, 70 rats were initially given BBN at three fractionated doses at 24-h intervals. Twenty-four hours following the last carcinogen dose the animals were subjected to one-third resection of the bladder.

Group 4. At the start of the experiment, a total of 70 rats received BBN at three single doses at 24-h intervals. One week after the last carcinogen feeding the bladders were partially resected.

Histological examination

The animals that died before the scheduled time of sacrifice were autopsied and examined macroscopically and histologically, but excluded from the effective number. For histological examination, the urinary bladder, both ureters and both kidneys were removed and fixed in 4% buffered formalin for 24 h. After embedding in paraffin, 80–100 step sections of 5-μm thickness were prepared from each bladder and stained with hematoxylin. At least ten longitudinal sections from each kidney, including the renal pelvis, and approximately 15 sections from the proximal, middle, and distal portions of both ureters were stained with hematoxylin and eosin. The sections were examined for the presence of tumors and urothelial hyperplasias. Histological typing of the epithelial tumors induced was based on the classification systems proposed for the rat [1, 4, 7, 9]. Staging was performed according to the UICC guidelines. For statistical analysis of the tumor incidences obtained in the different experimental groups, the two-tailed Fisher exact probability test was used.

Results

Treatment with BBN alone: group 1; control group

Of the initially 60 rats used, 14 died before the scheduled experimental period mainly of pneumonia. Nine of the

surviving animals had developed a tumor in the urinary bladder, which corresponds to an incidence of 19.6% (Table 1). One of the tumor-bearing rats exhibited two independent neoplasms. The histological types and grades of the tumors induced are listed in Table 1. All papillary transitional cell carcinomas were non-invasive. Six of the 37 rats (= 16.2%) without a bladder tumor revealed focal simple and papillary urothelial hyperplasias. Nearly all animals showed moderate, diffuse hyperplasia of the urothelium, probably due to non-specific, proliferation-stimulating effects of BBN. This phenomenon was also seen in most rats in the other experimental groups. No tumor development was observed in the renal pelvis or and ureters.

Initial partial cystectomy and subsequent administration of BBN: group 2

In this group 47 rats survived; 1 showed bladder concretions that caused severe, diffuse, tumorlike papillary proliferation of the urothelium. This "papillomatosis" is interpreted as being regenerative and not truly neoplastic lesion [6]. Since nevertheless urolithiasis may play a role as a promotor in the pathogenesis of bladder tumors, the stone-bearing animal was discarded from the effective number. Of the remaining 46 rats only 4 developed a bladder neoplasm (Table 1). The tumor incidence of 8.7% proved not to be significantly different ($p < 0.23$) from that found in the control animals (19.6%) using the two-sided Fisher exact probability test, but the reduction of the tumor yield by more than one-half suggests a real biological difference between the two groups that is not realized by the statistical method applied. Histologically, three transitional cell papillomas and one non-invasive papillary transitional cell carcinoma, grade 1, were diagnosed (Table 1). In 2 of the 42 rats (4.8%) without a tumor, focal simple urothelial hyperplasia of the bladder was found. In none of the animals was a pelvic or ureteric tumor induced.

Initial administration of BBN and subsequent partial cystectomy after 24 h: group 3

In 3 of the surviving 58 rats concretions were present in the bladder associated with a severe reactive papillomatous proliferation of the urothelium. These animals were eliminated from the final evaluation. Ten of the remaining 55 animals showed a solitary bladder neoplasm corresponding to a tumor incidence of 18.2% (Table 1), which was as high as in the control group ($p < 1.00$). The histology and grades of the urothelial neoplasms induced are given in Table 1. One of the papillary transitional cell carcinomas infiltrated the lamina propria (pT1-stage). Three of the 45 rats (6.7%) lacking tumor growth in the bladder developed simple urothelial hyperplasia. No tumor formation was observed in the renal pelvis or ureters.

Initial administration of BBN and subsequent partial cystectomy after 1 week: group 4

Of the 54 survivors 5 were excluded from the effective number, since they had concretions in their bladders associated with tumorlike regenerative papillomatosis. In 11 of the remaining 49 rats a solitary bladder neoplasm was produced, yielding a tumor incidence of 22.5% (Table 1), which was not significantly different from that found in the controls ($p < 0.80$). The histological types of tumors are summarized in Table 1. None of the papillary transitional cell carcinomas was invasive. Eight of the 38 animals (21.5%) without tumor formation in the bladder exhibited focal simple and papillary urothelial hyperplasias. There was no tumor development in the renal pelvis or ureters.

Discussion

The present experiments confirm our previous findings documenting an inhibition of chemically induced tumor development in the partially resected, regenerating bladder following administration of carcinogens during the phase of most pronounced urothelial proliferation. Thus, the tumor incidence was reduced from 12.6% in the intact, resting bladder to 2.6% in the partially resected, proliferating bladder after administration of three fractionated low doses (100 mg/kg body weight) and from 48.1% to 27.4% after three high doses (300 mg/kg body weight) of BBN fed by gavage during the period of most increased proliferation of the stimulated urothelium 30, 34 and 70 h postoperatively [11]. An inhibition of tumor induction was also obtained using the direct-acting urothelial carcinogen MNU. After a single intravesical dose given during maximal DNA synthesis 45 h after a one-third resection of the bladder, the tumor incidence was 17.9% compared with 32.6% in the intact, resting bladder [8]. Recently we were able to demonstrate the inhibition of MNU-induced urothelial carcinogenesis in the proliferating bladder to be cell-cycle specific [13]. Following synchronization of the stimulated urothelial proliferation by hydroxyurea, the tumor incidence was significantly reduced (3.5-fold) when MNU was instilled during the early DNA synthesis phase. A remarkable – although statistically nonsignificant – decrease in the tumor yield was also observed following application of the carcinogen during the late G_1- and late S-phase. When the carcinogen was given during the G_2+M-phase and the early postmitotic phase, the tumor incidences increased and reached the control value after instillation of MNU during the late postmitotic phase. These results are quite compelling, as they suggest a direct and causal interrelationship between stimulated de novo DNA synthesis and malignant cell transformation in the regenerating urinary bladder.

The current data documenting a reduction of the incidence of BBN-induced tumors after initial partial cystectomy and subsequent carcinogen feeding during the phase of most increased urothelial proliferation to approximately one-half (8.7%) the incidence of the controls (19.6%) are in close agreement with our previous results, showing a tumor yield of 21% in the resting bladder and

8% in the proliferating bladder after an induction time of 18 months [11]. Likewise, the frequency of focal urothelial hyperplasia proved to be decreased from 16.2% in the control group to 4.8% in the animals with an initially proliferating bladder. Subsequent stimulation of urothelial proliferation 24 h or 1 week following feeding of BBN had no influence on tumor induction. The fact that initial proliferative stimulus resulted in a reduction of the tumor incidence, while subsequent stimulation of proliferative activity did not alter the tumor formation, clearly indicates that the time of onset of de novo DNA synthesis related to carcinogen dosing is evidently the decisive factor in modifying tumor development in the regenerating urinary bladder. Another conclusion is that subsequent proliferative stimulus cannot promote the initiated carcinogenic process. This was an unexpected observation since one of the effects ascribed to most promoters is to induce cell replication, thus ensuring transmission of carcinogen-caused DNA damages to subsequent cell generations to finally produce overt tumor growth. The results presented here agree reasonable well with those of Shirai [21], who also found a reduction in the incidence of BBN-induced bladder tumors to approximately one-half following stimulation of urothelial proliferation by freeze-ulceration prior to carcinogen administration, whilst freezing 24 h or 8 weeks after feeding of BBN did not change the tumor yield.

The mechanisms underlying the inhibition of tumor development in the regenerating bladder following initial stimulation of urothelial proliferation and subsequent carcinogen administration are not yet understood; this aspect is discussed in detail elsewhere [8, 11, 13]. It is possible that an increased capacity of proliferating urothelial cells to repair carcinogen-induced DNA injuries may play a role. In favor of this concept are findings in stimulated cell propulations showing an increase in DNA repair synthesis, as well as the activity of repair enzymes concomitant with the induced de novo DNA synthesis [2, 3, 14, 20, 22] and the increased elimination of DNA adducts in the partially resected, regenerating liver [15–17] in particular when the carcinogen is given during the early S-phase [18, 19]. Further studies must be carried out to ascertain whether increased repair capacity is also responsible for the inhibition of tumor development observed in the partially resected, proliferating urinary bladder.

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