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Inhibition of Tumor Development in the Regenerating Rat Urinary Bladder Stimulated to Proliferate by Cyclophosphamide*

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Abstract—*The present study deals with the effect of stimulation of urothelial proliferation on experimental bladder carcinogenesis. To induce proliferative activity of the bladder mucosa cyclophosphamide (cp) was intraperitoneally administered to rats in a single dose* (100 mg/kg). N-*Butyl*-N-(4-*hydroxybutyl*)-*nitrosamine (BBN) was used as carcinogen and administered by gavage in* 3 *fractionated doses when proliferation of the urothelium was highest at* 28 *and* 40 hr *as well as* 7 days *following the injection of cp. Contrary to our original working hypothesis, tumor development proved to be inhibited in the bladder following initial stimulation of urothelial proliferation by cp. After administration of a low total dose of BBN* (300 mg/kg) *and an experimental period of* 6 *and* 12 months *none of the rats pretreated with cp developed a tumor in the regenerating bladder, whereas solitary transitional cell papillomas were observed in* 6.7% *of the control animals with a quiescent bladder. Following administration of BBN at a high total dose* (1.300 mg/kg) *and an induction time of* 4, 6 *and* 12 months *papillomas and non-invasive papillary transitional cell carcinomas occurred in only* 21.6% *of the rats initially receiving cp but in* 48.1% *of the control animals without stimulation of urothelial proliferation by cp. After treatment with BBN alone there was a far larger number of rats with multiple tumors in the quiescent bladder. The reduction in the incidence of tumors following administration of cp is not attributable to a prolongation of the latency period or induction time. It is an open question which mechanisms are responsible for the observed inhibition of experimental bladder carcinogenesis. An increased DNA repair induced synchronously with the stimulated replicative de-novo DNA synthesis or a decreased activity of urothelial enzymes metabolizing BBN to its ultimate carcinogen are proposed as the most likely explanations.*

INTRODUCTION

EXPERIMENTS on urinary bladder carcinogenesis have been mainly confined to the initially resting, physiologically slowly regenerating urothelium (for a review of the literature see [1–3]). It was therefore the object of the present study to test the influence of stimulated proliferation on urothelial carcinogenesis. This approach appeared promising, since it is well known that the DNA synthesis phase is important for the initiation and promotion of a carcinogenic process (for a review of the literature see [4–11]). Our original working hypothesis was that cells stimulated to proliferate are generally more susceptible to carcinogens than during their resting period, as could be demonstrated particularly in the proliferating liver following partial hepatectomy (for a review of the literature see [5, 9]). Thus we expected an enhancement of tumor development in the regenerating urinary bladder. However, we recently reported an inhibition of bladder carcinogenesis following stimulation of its proliferative activity by partial cystectomy [12–14].

In the present experiments cyclophosphamide was administered in order to stimulate proliferation of the bladder mucosa. Previous auto-

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radiographic studies [15] yielded a prompt increase of the [^{3}H]-thymidine labeling index 22 hr after administration of a single dose of cyclophosphamide, reaching a maximum of 17.8% at 30 hr. A second peak of DNA synthesis was observed at 7 days. Therefore this experimental model seemed well suited for studying a possible relationship between stimulated proliferation and carcinogenesis in the bladder. Because the cells were not proliferating enough in synchrony it was impossible to test the susceptibility of the different cell cycle phases to malignant transformation. *N*-Butyl-*N*-(4-hydroxybutyl)-nitrosamine was used as carcinogen and was administered in fractionated doses when proliferation of the bladder mucosa was at its peak.

MATERIALS AND METHODS

Animals

Pathogen-free female adult Wistar rats with an initial weight of 180–200 g (purchased from Winkelmann breeding farm, Borchen, F.R.G.) were used as experimental animals. The rats were housed in groups of five in plastic cages and maintained under standardized conditions (temperature: 22–33°C; atmospheric humidity: 50–60%; artificial light in a 12-hr light–dark cycle). They had free access to tap drinking water and were fed a standard ground commercial diet (Altromin, Lage, F.R.G.).

Chemicals

Proliferative activity of the bladder urothelium was stimulated by a single i.p. injection of cyclophosphamide (cp; Asta-Werke AG, Degussa Pharma Group, Bielefeld, F.R.G.). *N*-Butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN; special manufacture) dissolved in 1,2-propandiole was administered by gavage in a low (300 mg/kg body wt) and a high total dose (1300 mg/kg body wt) in order to evaluate a possible dose-dependence of tumor development. To avoid toxic–lethal effects on the urothelial cells BBN was given in several fractionated doses. The amount of propandiole mixed with BBN was 1 ml/gavage feeding.

Experimental groups

The rats were divided into 4 groups (see Tables 1 and 2).

Group 1. BBN was administered by gavage to 95 rats in three single doses of 100 mg/kg body wt each when proliferative activity of the bladder mucosa was highest, at 28 and 40 hr, and again 7 days after a single i.p. injection of cp in a dose of 100 mg/kg body wt. The animals were killed after 6 and 12 months.

Group 2. Ninety-five rats served as controls to those in group 1 and received BBN alone in the same doses and time intervals as in group 1. The experiment was terminated after 6 and 12 months.

Group 3. Ninety rats were fed by gavage in three fractionated doses of 300 mg/kg body wt each at 28 and 40 hr and 7 days after a single i.p. administration of cp (100 mg/kg body wt). Additionally, the carcinogen was given in the drinking water for 40 subsequent days in a dose of 10 mg/kg body wt. The total dose of BBN administered thus amounted to 1300 mg/kg body wt. The rats were killed in groups after 4, 6 and 12 months.

Group 4. Ninety rats, used as controls to group 3, received exclusively BBN in the same manner as in group 3. The animals were killed after an experimental period of 4, 6 and 12 months.

Histological examination

The rats were induced to empty their bladders by pinching their tails. Subsequently they were anesthestized with ether and then killed by decapitation. The bladders were removed, fixed in 4% formalin for 24 hr and embedded in paraffin. From each bladder at least 80 longitudinal step sections of 5 μm thickness were prepared and stained exclusively with hematoxylin.

The sections were examined for urothelial tumors, classified according to the WHO classification for human neoplasms [16]. Urothelial hyperplasias were not included in this study because it was frequently impossible to clearly distinguish between true preneoplastic and only persisting reactive proliferative lesions due to nonspecific effects of the carcinogen or cp. Statistical assessment was performed according to the 2×2 contingency table [17] with the correction of Ku [18] for zero frequencies. As a global test the number of surviving rats with and without a bladder tumor in the different groups were added and compared with the χ^2 distribution involving two and three degrees of freedom.

RESULTS

Findings after administration of a low total dose of BBN

Administration of cp and BBN (group 1). The fatality of the animals in this group was high. Thus 46 of the 95 rats originally used died prematurately. The cause of death was pulmonary infections at the beginning of the experiment, evidently due to the known immunosuppressive and bone-marrow-depressing effects of cp. After an experimental period of 6 and 12 months none of the 49 surviving rats had developed a tumor in the bladder (Table 1). Nearly all rats showed a moderate-to-severe diffuse, focally papillary

hyperplasia of the bladder urothelium which was much more pronounced than after administration of BBN alone (see group 2).

Administration of BBN alone (group 2). The mortality of the rats in this control group was considerably lower than after administration of cp and BBN (group 1). Only five of 95 rats died during the experiment. After an induction time of 6 months none of the 20 surviving rats showed a tumor in the bladder (Table 1). After 1 yr six of 70 (8.6%) rats had developed a solitary small transitional cell papilloma. The tumor incidence in all rats together (number of animals with tumors) was 6.7%. The difference between the tumor incidences in the rats of group 1 with a regenerating- and in group 2 with a quiescent bladder is not statistically significant (global P value for both experimental periods of 6 and 12 months: 0.19; P value for the induction time of 12 months alone: 0.08). However, it is difficult to establish a definite proof of statistical significance, since in three of the four subgroups (see Table 1) no animals with bladder tumors were noted. The majority of animals exhibited a slight-to-moderate diffuse hyperplasia of the bladder mucosa, which can be attributed to nonspecific proliferation-stimulating effects of BBN.

Findings after administration of a high total dose of BBN

Administration of cp and BBN (group 3). Twenty-five of the 90 originally used rats died of severe pneumonia during the first month of the experiment. The tumor incidence in each of the three subgroups was approximately one-half of that of the control animals (group 4) with a quiescent bladder (Table 2). After an experimental period of 4 months, in only three of 27 (11%) rats was a solitary transitional cell papilloma induced in the bladder. After 6 months four of 23 (17%) had developed a papilloma. After 1 yr seven of 15 (47%) rats showed urothelial tumors; only two animals had two and three discrete neoplasms, respectively, in their bladder. Histologically, seven transitional cell papillomas and three noninvasive papillary transitional cell carcinomas grade 1 were diagnosed. The overall tumor incidence (number of animals with tumors) in all three subgroups together was 21.6%. Moderate-to-

Table 1. Incidence of urinary bladder tumors induced by a low total dose of BBN (300 mg/kg; three fractionated doses of 100 mg/kg by gavage) in rats without initial administration of cp and in those pretreated with a single i.p. dose (100 mg/kg) of cp to stimulate urothelial proliferation (statistical analysis according to the contingency table with correction for zero frequencies)

Experimental period (months)	BBN alone		BBN and cp		
	No. of surviving rats	No. of rats with tumors	No. of surviving rats	No. of rats with tumors	χ^2
6	20	0	20	0	0
12	70	6 = 8.6%	29	0	3.32 $P = 0.08$
Total	90	6 = 6.7%	49	0	3.32 $P = 0.19$

cp = cyclophosphamide; BBN = *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine.

Table 2. Incidence of urinary bladder tumors induced by a high total dose of BBN (1300 mg/kg; three fractionated doses of 100 mg/kg by gavage and additional administration in the drinking water for 40 subsequent days in a daily dose of 10 mg/kg) in rats without initial administration of cp and in those pretreated with a single i.p. dose (100 mg/kg) of cp to stimulate urothelial proliferation (statistical analysis according to the contingency table)

Experimental period (months)	BBN alone		BBN and cp		
	No. of surviving rats	No. of rats with tumors	No. of surviving rats	No. of rats with tumors	χ^2
4	30	8 = 27%	27	3 = 11%	2.29
6	26	10 = 38%	23	4 = 17%	2.73
12	23	20 = 87%	15	7 = 47%	7.19
Summary	79	38 = 48.1%	65	14 = 21.6%	12.21 $P = 0.007$

cp = cyclophosphamide; BBN = *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine.

severe diffuse hyperplasia of the urothelium was also present in these rats.

Administration of BBN alone (group 4). The death rate in these animals was considerably lower than in those which had received additional cp (group 3). Only 11 of 90 rats died prematurely. The tumor incidence was substantially greater than that observed in the animals receiving the low dose of BBN (group 2). Increasing induction time yielded an increased frequency of tumor-bearing animals. After an experimental period of 4 months eight of 30 (27%) rats had developed a solitary transitional cell papilloma in the urinary bladder (Table 2). After 6 months a bladder tumor was found in ten of 26 (38%) animals (eight transitional cell papillomas and two noninvasive transitional cell carcinomas of grade 1). After 1 yr in 20 of 23 (87%) rats tumors were induced in the urinary bladder, with 2–3 discrete neoplasms in seven animals (19 transitional cell papillomas, nine noninvasive papillary transitional cell carcinomas of grades 1 and 2 and one invasive squamous cell carcinoma). In summary, 48.1% of the rats in all three subgroups had developed bladder tumors. The difference between the total tumor incidences in the rats with a proliferating bladder (group 3) and those with a quiescent bladder (group 4) proved to be highly statistically significant (global P values: 0.007).

DISCUSSION

The present investigation showed considerable inhibition of experimental urinary bladder carcinogenesis following initial stimulation of urothelial proliferation by cp. Because the difference in the tumor incidences in rats with a proliferating or quiescent bladder persisted with increasing induction time, it can be concluded that following administration of cp bladder carcinogenesis is primarily inhibited rather than merely delayed due to prolongation of the latency period and/or induction time. A further argument in favor of a true inhibition consists in the observation of a lower percentage of animals with multiple bladder tumors when proliferative activity of the urothelium had been stimulated by cp.

The observed inhibition of tumor development in the urinary bladder by initial administration of cp is not contradictory to the suspicion that the cytostatic drug may act as a urothelial carcinogen in man [19–21], since in the present experiment cp was given only in a single dose whereas in patients it is usually applied over a long period of time, resulting in a high total dose. Our findings were also not comparable with animal experiments revealing development of bladder tumors after lifetime administration of cp in the drinking water [22]. Furthermore, it can be assumed that the known immunosuppressive effects of cp do not influence urothelial carcinogenesis in the present experiment, since BBN was not given until a long time after treatment with cp, when its metabolites would have long been eliminated.

We also observed an inhibition of experimental bladder carcinogenesis after initial stimulation of urothelial proliferation by partial cystectomy. Using this experimental model [23, 24], the incidence of bladder tumors was found to be five times less after a low and reduced by approximately one-half following a higher dose administration of BBN compared to controls with an intact bladder [13, 14]. Our findings in the regenerating bladder stimulated to proliferate by either partial cystectomy or initial administration of cp are in close agreement with those of Shirai [25], who induced regenerative proliferation of the bladder mucosa by ulceration using a new freezing technique. He found a significantly reduced incidence of BBN-initiated papillomas and carcinomas to approximately one-half of that in untreated rats. On the other hand, Cohen *et al.* [26] observed an enhancement of tumor development in the proliferating bladder following freeze ulceration or a single injection of cp and subsequent feeding of *N*-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) and sodium saccharin. However, FANFT was continuously fed for 2 weeks in a 'noncarcinogenic' dose, whereas in the present investigation BBN was administered in three single carcinogenic doses at the very time when proliferative activity of the stimulated bladder urothelium was at its peak. Furthermore, sodium saccharin fed over almost 2 yr acts as a strong promoting agent in multistage carcinogenesis [27]. By contrast, in our experiments no additional promotor was used.

At first glance our resuts appear difficult to reconcile with the findings in the regenerating liver, in which carcinogenesis following partial hepatectomy is enhanced when carcinogens are administered during the DNA synthesis phase (for a review of the literature see [5, 7, 9, 11]). The urinary bladder and liver, however, cannot really be compared in this regard, since the two organs play a different role in the activation of carcinogenic substances. The hepatocarcinogenic nitrosamines are completely metabolized in the hepatocytes by microsomal enzymes forming the ultimate carcinogen *in loco*, whereas this is not known to occur in urothelial cells. Thus BBN initially undergoes metabolic transformation in the liver, resulting in a proximate carcinogen which is then excreted into the urine. In the bladder these liver metabolites of BBN are converted to the ultimate carcinogenic agent

through α-hydroxylation by urothelial enzymes [28–33]. Thus in the liver the locally formed ultimate carcinogen is immediately available and can directly initiate malignant transformation of the proliferating hepatocytes, whereas bladder carcinogenesis can start only after a lag period due to the multiple steps of enzymatic activation of BBN in the liver and urothelium. This could be a decisive factor in the initiation of urothelial carcinogenesis, since the different phases of proliferation are differentially susceptible to carcinogenic agents (for a review of the literature see [9]).

At the present time it is a completely open question as to which mechanisms are responsible for the observed inhibition of experimental bladder carcinogenesis. The following possibilities of explanation should be considered:

1. The loss or damage of urothelial cells due to the cytotoxic action of cp leads to a decrease in the activity of urothelial hydroxylases which metabolize BBN, resulting in reduced concentration of the ultimate carcinogen and thus in lower tumor incidence. This possibility seems unlikely, since the second and third doses of BBN were administeed at times when the urothelium had already regenerated to a considerable extent.

2. The reduced tumor incidence is attributable to the fact that with the cytotoxic loss of urothelial cells a decreased number of cells is available for neoplastic transformation, and therefore fewer tumors had developed. However, as mentioned above, BBN was administered during the phase of intensive urothelial regeneration, when there are even more cells available for initiation of carcinogenesis than in the normal bladder.

3. The newly formed urothelial cells are immature and have a decreased ability to metabolize BBN to its ultimate carcinogenic agent [25]. This possibility is supported by the finding that rapidly regenerated transitional cells have a reduced number of fusiform vesicles and are covered with a symmetrical rather than the asymmetrical membrane found in normal urothelium [34]. This could be of importance in reducing the uptake of carcinogenic metabolites from the urine by the urothelial cells [35].

4. It is conceivable that inducible repair processes [36–39] eliminate BBN-caused damage to the DNA, thus preventing the initiation of carcinogenesis. Evidence in support of the concept that concomitant with a stimulation of replicative de-novo DNA synthesis DNA repair synthesis is also increased can be derived from several studies in different experimental models. Thus, following damage of the DNA of human peripheral blood lymphocytes by alkylating agents and ionizing radiation, DNA repair synthesis and the activity of the base-excision repair enzyme uracil-DNA glycosylase showed a many-fold increase synchronously with a stimulation of proliferative activity [40–43]. Furthermore, the activity of uracil-DNA glycosylase [44] and the elimination of the putative promutagen O^6-methylguanine from dimethylnitrosamine-damaged DNA [45, 46] was also considerably increased in the regenerating liver following partial hepatectomy when DNA synthesis was highest. There may also be a close association between DNA replication and DNA repair in the urothelium, resulting in increased activity of inducible repair enzymes.

In considering different possible mechanisms underlying the observed inhibition of carcinogenesis in the proliferating bladder it must be remembered that during the phase of DNA synthesis initiation and promotion on the one hand compete with repair mechanisms on the other, and that whether or not malignant transformation will ensue is dependent on many factors. We hope that our findings will stimulate subsequent studies to eludicate the role of induced proliferation for urothelial carcinogenesis.

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