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Influence of pertussis toxin on superficial bladder carcinoma in rats

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Abstract The proliferation and migration of cells is a fundamental process for the metastasis of malignant tumour cells. In several in vitro studies, pertussis toxin (PTX) inhibited cell proliferation and cell motility in the human transitional cell carcinoma cell line J82. The present study investigated the effect of the intravesical application of PTX on the development of superficial bladder cancer in rats. We used the model of N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN, 0.05% via drinking water ×10 weeks) to induce superficial bladder carcinomas in 40 female rats. After 16 weeks the rats were treated in two groups with 0.4 ml PTX (1 μg/ml) or 0.4 ml phosphate buffered saline (PBS) by intravesical application (once a week for 10 weeks). In the 25th week urine cytology was determined and all rats were killed at week 26 followed by histological evaluation. In the control group, the urine cytology was positive for G2/G3 cells in ten of 17 rats. In the PTX group G2/G3 cells were determined in five of 20 rats (P two tailed < 0.05). Histopathologically 12 rats (71%) of the control group and 11 rats (55%) of the PTX group developed T1-T2 transitional-cell carcinomas. No local or systemic side effects were disclosed. PTX treatment reduces the development of G3 transitional cell carcinomas in rats and may represent a new approach for local therapy in superficial bladder cancer.

Keywords Transitional cell carcinoma · Pertussis toxin · G protein coupled receptors · N-butyl-N-(4-hydroxybutyl)nitrosamine

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Introduction

Superficial bladder cancer, which represents 70–75% of all bladder malignancies, has a variable potential for recurrence, progression to muscle invasion and subsequent metastasis. At least 50% of patients will have a recurrence or develop a new tumour, with a variable propensity for invasive disease despite complete resection of the initial tumour [23]. One of the major unsolved problems in clinical oncology is to prevent tumour progression in patients or to make progression reversible. We have demonstrated that tumour cell migration correlates with the malignant potential of tumour cells and the clinical course of patients with bladder carcinoma [17, 18]. Tumour cells migrate from the local tumour site into the circulatory system to invade adjacent organs and form distant metastases. The organisation of the cytoskeleton has been shown to play an important role in cell motility [29] and over the past years the signal transduction pathways that triggers different cytoskeletal patterns have been studied in great detail. In human transitional cell carcinoma cells J82, stimulation of serpentine-type receptors that are linked to heterotrimeric guanine-nucleotide-binding proteins (G proteins) induce rearrangements of the actin cytoskeleton resulting in stress fibers (coupled to attachment plaques) [14]. The extracellular ligands lysophosphatidic acid (LPA), thrombin and sphingosin-1-phosphate (SPP) induced stress fibres. We defined pertussis toxin (PTX) as a factor potentially influencing tumour cell motility as verified with in-vitro assays [27].

For the first time, antimotility factors were applied in a model of chemically induced bladder carcinoma in rats and adverse reactions as well as therapy effectiveness were evaluated.

Materials and methods

Animals

Forty female Wistar Unilever rats with a median body weight of 194 g and a median age of 8 weeks were selected for the topical

application of PTX. All animals were housed in plastic cages (four animals per cage) in an air-conditioned room at 22°C and a humidity of 55% under a 12 h-light/dark cycle with free access to water and commercial stock diet (10H10, Eggersmann, Germany).

Materials and chemicals

N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) is a yellow oily liquid, hardly volatile and soluble due to terminal hydroxylation. BBN was synthesised and provided by the German Cancer Research Centre, Heidelberg. It was added to drinking water at a concentration of 0.05% in light impermeable bottles and given ad libitum for 10 weeks. Given an average uptake of 6 ml water/day and animal each rat ingested approximately 2.0 ml BBN.

PTX was purchased from List Biological Laboratories (Campbell, Calif., USA) and dissolved in NaCl 0.9%.

Experimental design

After BBN treatment, 40 rats were randomly divided into two groups of 20. From 16 weeks onward topical therapy was carried out and the control group received 0.4 ml phosphate buffered saline (PBS) and the PTX-group 0.4 ml of 1 μg/ml PTX. The intravesical instillations were carried out once weekly over a period of 10 weeks by catheterisation (outside diameter 0.96 mm) under Rompun 2% and Ketamin 10% anaesthesia. In the 25th week, the urine from each rat was collected by catheterisation prior to therapy. This was then fixed in 3 ml thiomersal (30%, v:v) and placed on ice. Slides were prepared from the specimen via direct smears of centrifuged sediment. Prepared slides were stained by the Papanicolaou method. The slides were examined by light microscopy (100 and 400 magnification, Zeiss microscope) and the tumour grade was determined using the WHO grading classification of transitional cell carcinoma. The examiner of the urine cytology did not know the treatment groups. Before and during the treatment, the rats were monitored for side effects such as weight-loss, apathy, and change of behaviour. Red and white blood cell counts were taken at autopsy to test for haematological side effects of PTX.

At the end of the 26th week all rats were killed. To assess the clinical tumour stage, the thoracic and abdominal contents were examined macroscopically. Advanced, bladder wall-penetrating carcinomas as well as tumour related obstruction of the upper urinary tract were macroscopically verified and classified as locally advanced tumour (≥pT2b). The bladder was filled with 0.05 ml saline to facilitate fixation and staining.

After fixation of the 5 μm frozen sections in ethanol, the slides were stained with hematoxylin-eosin and evaluated at 100 and 400 magnification (Zeiss microscope). The tumour progression was determined according the UICC-criteria and the tumours were classified as superficial and early invasive carcinomas (pTa, pT1, pT2) in contrast to extravesical tumour growth (pT3, pT4), though this not expected in this animal model. Histopathological examination of the removed organs (bladder, kidney, liver, lung, spleen, lymph nodes) was performed to disclose therapy related side effects and the presence of distant metastases (liver, lung, lymph nodes).

Statistical methods

Either Fisher's exact test, the χ^2-test or the Mantel-Haenszel test were applied to compare the frequencies of tumour development in the animal model. The level of statistical significance was set at $P < 5\%$ unless otherwise indicated.

Results

During the experimental period, three rats died due to overdoses of the anaesthetic used during instillation of PTX or PBS. The other rats grew normally until the end of the experiment.

Influence on body weight

Initially, the adult rats had a median body weight of 194 g (range 163 g–250 g). At the end of the experiment, at 26 weeks, the mean body weight (±SD) of the PTX group was 261 ± 35 g which is comparable to the median body weight in the control group with 260 ± 27 g.

In summery, rats treated with PTX have the same median body weight during the treatment period as the control group (Fig. 1).

Side effects

Severe side effects did not occur in either treatment group nor was PTX related toxicity detectable (weight-loss, apathy, behavioural changes). At the end of the experiment, seven rats in the PTX group and six in the control group developed gross hematuria. The histopathological examination of different organs, i.e. kidney, liver, lung, spleen and lymph nodes revealed normal findings. Furthermore, we found no pathological changes in the differential blood count compared to control rats, though Howell-Jolly bodies were found in 25% of the blood smears after the application of PTX. Holly-Jolly bodies were normally observed in patients with hyposplenism or after splenectomy. Circulating erythrocytes reflect the loss of the splenic filtration function and are found to contain mitochondrial remnants and inclusions of nuclear fragments [4].

Influence on tumour development

In the control group, the urine cytology was positive for G2/G3 cells in ten out of 17 rats (Fig. 2). In three rats,

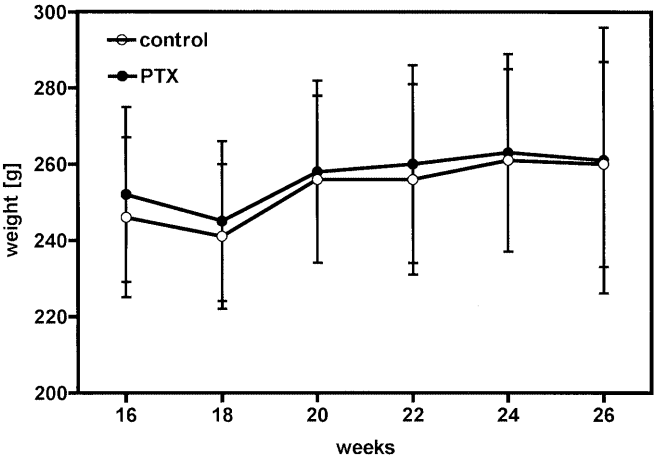

Fig. 1. Changes in body weight in rats treated with PTX or PBS (weeks 16–26, median body weight)

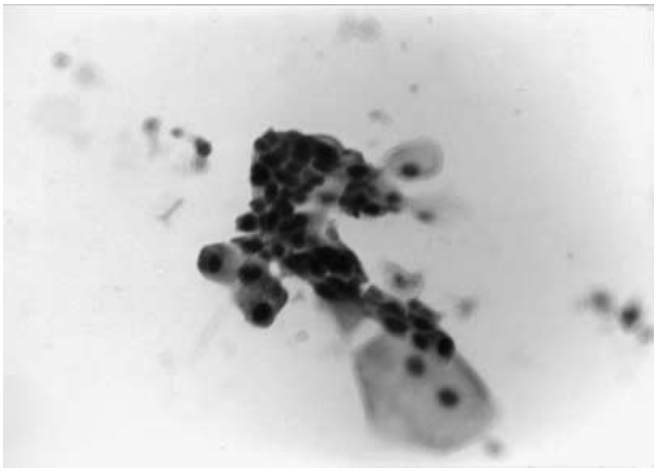

Fig. 2. Loosely clustered high-grade malignant urothelial cells show an irregular and hyperchromatic nucleus, anisocytosis and increased N/C (nuclear to cytoplasmic) ratios (stained using the Papanicolaou method, magnification ×400)

the urine cytology was normal and four rats showed G1 differentiated cells. In contrast to the control group, only five out of 20 rats of the PTX group developed G2/G3 differentiated cells (P two tailed < 0.05). Normal urine cytology was detected in 12 rats and three rats showed G1 differentiated cells (Table 1).

Histopathologically confirmed carcinomas developed in the control group as well as in the PTX treated group. The incidence of carcinoma tended to be higher in the control group than in the PTX group. In the control group, 12 out of 17 rats (71%) with urine cytology developed a T1-T2 transitional cell carcinoma and four rats developed a papillary or nodular hyperplasia. In the PTX group, carcinomas were detected in 11 out of 20 rats (55%) (P two tailed $= 0.5$) and six rats developed a papillary or nodular hyperplasia (Table 2). The grading of the carcinomas is given in Table 3. Only one G1-tumour in the PTX-group was classified as a G2-tumour

Table 1. Effect of treatment on urine cytology (25 weeks)

	n	≥G2	P
Control	17	10 (59%)	
PTX	20	5 (25%)	< 0.05

Table 2. Histological findings in rat urinary bladder at 26 weeks

	Control ($n = 17$)	PTX ($n = 20$)
Normal	1	3
Papillary or nodulary hyperplasia	4	6
Cancer	12	11

Table 3. Grading of the carcinomas (26 weeks)

	n	G1	G2	G3
Control	12	2 (17%)	6 (50%)	4 (33%)
PTX	11	7 (64%)	3 (27%)	1 (9%)

using urine cytology. Histopathologically, no difference could be found in the bladder tumours of the control group and treatment group with respect to a possible effect of PTX on the morphological appearance using light microscopy.

Discussion

The migration of cells is a fundamental process for the life of multi-cellular organisms and is involved in a wide variety of physiological and pathological processes. In metastasis, tumour cells migrate from the initial tumour site into the circulatory system, they then invade a new site and disrupt normal tissue architecture, which accounts for as much or more of the lethality of cancer than does uncontrolled growth [12, 31]. The initiation of cell migration is caused by both substrate-bound and soluble extracellular molecules, interacting with specific cell surface receptors [28, 29]. We recently demonstrated that the cell motility and migration of human transitional carcinoma cells is potently stimulated by G protein coupled receptors correlated to calcium mobilisation [14, 27]. Out of the many different agonists studied, lysophosphatidic acid (LPA) strongly stimulated migration of the transitional cell carcinoma cell line J82. PTX appeared to inhibit the migratory response of J82 cells to LPA by inhibiting the G_i type G protein. J82 cells exhibited a very high sensitivity to PTX, with maximal inhibition being observed after PTX treatment with 0.1–1 ng/ml PTX [14]. Roos and van de Pavert showed that PTX-treatment inhibits lymphoma invasion and liver metastasis formation without affecting cell proliferation [25]. In an animal model of orthotopic implantation of human prostate cancer cell lines into the dorsal prostate of athymic nude mice, systemic PTX caused significantly less local tumour growth and loco-regional lymph node metastases [2]. From these data we conclude that LPA related cell motility is a common process in cancer cells and that it is potentially inhibited by PTX-treatment.

Animal models of carcinogenesis in the bladder have been reported for rats, mice and dogs. Mice treated by the oral administration of BBN develop highly malignant invasive bladder carcinoma [16]. To investigate the influence of PTX on the development of advanced bladder cancer we used the model of BBN-induced bladder carcinoma in female mice. We demonstrated that the intraperitoneal application of PTX significantly reduced the development of locally advanced transitional cell carcinoma compared to the control group (7% vs 41%; $P < 0.015$). Neither local nor systemic side effects were disclosed after application of PTX [20].

A total to 70% of bladder tumours are superficial at initial presentation, i.e. confined to the mucosa (Ta, Tis) or submucosa (T1). Depending on the characteristics of the population included and the length of follow-up, 80% of the superficial bladder tumours will recur with 2–50% showing stage progression [26]. As a consequence,

major clinical and basic research efforts are dedicated to preventing the recurrence of and, even more importantly, to preventing the progression to muscle-invasive disease (≥T2). A large number of studies have been devoted to determining the efficacy of adjuvant application of chemo- and immunotherapeutic agents (i.e. doxorubicin, mitomycin C, BCG). These studies have provided conclusive evidence that adjuvant chemo- and immunotherapy is superior to transurethral resection alone with regard to the time of first recurrence and the recurrence rate per year [10, 21]. Nevertheless, the benefit from adjuvant chemo- and immunotherapy with regard to the prevention of local progression is still under discussion [10, 13, 32]. This disadvantage initiates new, experimental approaches and the exploration of novel or non-established biological response modifiers.

The positive in vitro and in vivo studies have encouraged us to investigate the antimotility effect of PTX in an in vivo model of superficial bladder cancer. Rats treated by the oral administration of BBN develop multiple papillary peduncular carcinomas, which are generally low grade and non-invasive and provide a useful experimental therapeutic model for the study of human superficial bladder tumours [8, 24]. It appears that the intravesical application of PTX has no influence on the formation of superficial bladder cancer in rats, although the incidence of cancer in the control group was somewhat higher than in the PTX group. Nevertheless, because of the great conformity of histological and cytological grading, the development of high-grade (G2/G3) superficial bladder cancer is significantly less in the PTX group compared to the control group. Several studies have shown that tumour grading has the greatest independent value in the prognosis of superficial bladder cancer becoming invasive [9, 26]. In the WHO grading system, 6% of G1 lesions are invasive in comparison with 52% of G2 and 82% of G3 lesions [11]. Intravesical application of PTX influences the development of high-grade superficial bladder cancer and in this way PTX prevents progression and metastasis. The mechanism by which PTX prevents the development of high grade tumours is not really clear. Beside cell motility, LPA and other agonists of G protein coupled receptors stimulates the growth of transitional cell carcinoma cells [21]. Several studies have shown that the α-subunits, as well as $\beta\gamma$-subunits of these G proteins, regulate several critical signalling pathways involved in cell proliferation, differentiation and apoptosis [3]. Recent studies have indicated that the asynchronous activation of these proteins can lead to the oncogenic transformation of different cell types [22]. PTX uncoupled $G\alpha_i$ type G proteins from the receptor and inhibited the signal transduction pathway of these receptors [14]. Mutations of $G\alpha_i$ have been observed in different forms of tumours and inactivation of these mutant forms of $G\alpha_i$ has been shown to inhibit cell growth and tumour formation [5, 6]. Since all rats were killed at 26 weeks, it remains unresolved as to whether the reduced development of the high-grade superficial bladder cancer which we observed after PTX-treatment is limited in time, nor is it clear how the transitional cells behave upon the withdrawal of PTX. There are only a few examples of other adjuvant drugs tested in this animal model. Othani et al. reported an intravesical instillation of Adriamycin and mitomycin, both well-known adjuvant drugs in superficial bladder cancer, once a week for 12 weeks into rats pre-treated with BBN for 4 weeks. They observed a markedly enhanced development of bladder cancers compared to a control group and concluded that the intravesical instillation of Adriamycin or mitomycin promotes two-stage bladder carcinogenesis in rats [15]. The systemic single application of the chemotherapeutics 5-fluorouracil, vincristine and cis-diamminedichloroplatinum was effective in inhibiting the incidence of bladder tumours induced by BBN in male Wistar strain rats [1]. Tanaka et al. performed a histological study on intravesical instillation of Bacillus Calmette Guerin (BCG) for BBN-induced bladder tumour in rats. They observed, after BCG-treatment, an enlargement of the intercellular spaces of superficial tumour cells and electron microscopic findings revealed a decrease or disappearance of the junctional complex. These results indicate that BCG may enlarge the intercellular spaces by changing the junctional complex, and lead to desquamation of the tumour cells from the superficial cell layer [30].

In a recently published clinical trial, we investigated the side effects of the local instillation of PTX. Intravesical treatment with PTX before radical cystectomy in patients with bladder cancer was safe and well tolerated without any significant local or systemic toxicity [19].

In conclusion, the intravesical application of PTX after the induction of superficial transitional cell carcinomas in rats by BBN leads to a significant reduction of high-grade superficial transitional cell carcinomas compared to the control group in the period of time observed. We therefore conclude that PTX acts as a factor which delays rather than prevents progression and metastasis. This supports the idea of successful application of PTX in an adjuvant setting after the transurethral resection of superficial bladder cancer.

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