

## Antineoplastic Effect of Immunostimulatory DNA (CpG-ODN) in a Murine C57-BL6 / MB-49 Transitional Cell Carcinoma Model

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**Abstract.** *Background:* Intravesical BCG installation is the standard of care in the prophylaxis of recurrent intermediate and high-risk transitional cell carcinoma (TCC), but its mode of action has not yet been elucidated. However, a Th-1 biased immune response is postulated. Cell culture and animal models demonstrated the efficacy of synthetic CpG-oligodeoxynucleotides (ODN) as inducer and adjuvant for a strong Th1-response. The purpose of our study was to evaluate the antineoplastic effect of locally administered CpG ODN in a subcutaneous murine bladder cancer model. *Materials and Methods:* A subcutaneous murine TCC model was established in female C57/BL6 mice using the corresponding syngeneic MB49 TCC cell line. Three groups of 5 animals received a cell suspension, standardized for  $1 \times 10^6$  cells/50  $\mu$ l, injected s.c. into the right and left flank. Group I received 10 nmol of CpG-ODN only into the right cell depot. Group II received 10 nmol of GpC ODN. Group III served as untreated control and received only PBS. The animals were examined at various time points after injection until sacrifice on day 14. Tumor or scar tissue were excised, weighed and examined histopathologically (HE-stain). *Results:* Tumor sizes and weights showed no side differences. The average tumor weight on day 14 was 171 mg ( $SD \pm 8.9$ ), 110 mg ( $SD \pm 19.2$ ) and 18 mg ( $SD \pm 6.1$ ), respectively, in groups III, II and I ( $p < 0.05$ ). Histopathology revealed solid vital epithelial tumors in group III and reduced vital tumor mass with

central necrosis and moderate mononuclear infiltration in group II. Group I showed almost complete tumor necrosis and a considerable mononuclear inflammatory response. *Conclusion:* Immunostimulatory DNA has promising antineoplastic activity in a murine subcutaneous TCC-model. The histological findings suggest an immunologically-mediated mode of action. Further investigations are necessary to elucidate the immunological response.

The incidence of transitional cell carcinoma (TCC) is rising. The majority of patients initially present with superficial stages (75%), the minority with muscle-invasive (20%) or metastatic disease (5%). After primary treatment, TCC recurs in 70% of cases within 2 years (1). During their future clinical course, 10-20% of patients will progress to muscle-invasive or/and metastatic disease (2). To reduce recurrence rates and the risk of progression, adjuvant treatment regimens have been advocated for 30 years (3,4). For intermediate and high-risk superficial TCC, intravesical immunotherapy with bacillus Calmette-Guérin (BCG) is superior to intravesical chemoprophylaxis (5-7). It reduces recurrence rates significantly and is probably also beneficial in terms of progression, especially when applied as maintenance therapy.

The BCG-induced immune response is very complex and the mode of BCG action has not yet been elucidated. However, there is evidence for a predominantly unspecific, Th-1-biased, local immune response, including activation of phagocytic and dendritic cells and local production of Th-1 cytokines like  $TNF\alpha$  and  $IFN\gamma$  (8-11). BCG therapy has several potentially serious side-effects, associated with considerable discomfort for the patient. Hemorrhagic cystitis and severe dysuria occur, and often the intended therapy regimen with an optimal number of instillations has to be ceased or interrupted. Even BCG-sepsis with fatal outcome has been described in single cases (7).

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*Key Words:* Immunotherapy, bladder neoplasm, subcutaneous animal model, CpG-ODN.

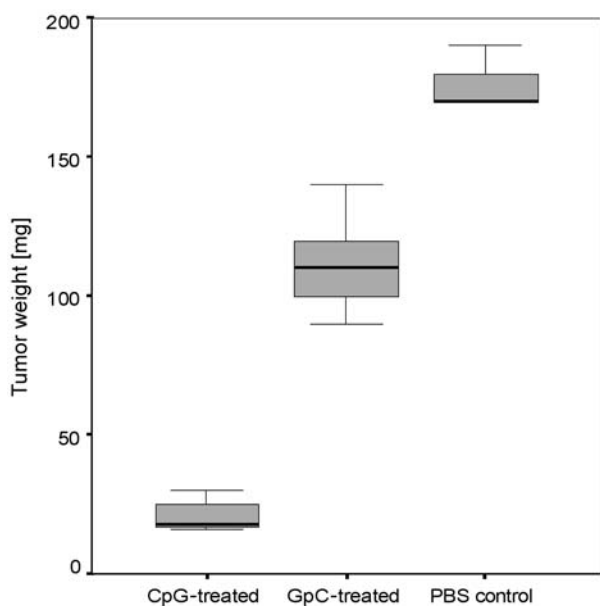


Figure 1. Tumor weight of all three groups 14 days after treatment showed a significant difference ( $p < 0.05$ , Kruskal Wallis ANOVA test).

Bacterial DNA is a potent stimulator of a Th-1-biased immune response with antineoplastic potential (12). In eukaryotic organisms, toll-like receptor 9 (TLR-9) detects bacterial DNA and probably mediates its immunostimulatory effects (13,14). Unmethylated CG dinucleotides within a defined base context (CG motifs) seem to be the critical component of bacterial DNA to trigger immune responses. In mice the CG motif is usually an unmethylated CG dinucleotide flanked by two 5' purines and two 3' pyrimidines. The 5' and 3' flanking regions have a modulating character, however, the mechanism behind these modulating effects is still unclear. Cell culture and animal models proved the efficacy of synthetic CpG-ODN as inducer and adjuvant for a strong Th1-response and there is evidence for a direct and/or adjuvant antineoplastic effect (15-18). It is now possible to develop synthetic, immunostimulatory CpG-ODN of high purity for use in experimental and clinical research. Protection against degradation *in vivo* and less toxicity make these molecules superior in comparison to complete bacterial DNA (19,20).

The parallels of the CpG-ODN and BCG effects in TCC formed the rationale for our studies. The purpose of our present study was to evaluate the antineoplastic effect of locally administered CpG-ODN in a subcutaneous murine bladder cancer model and to evaluate the perspectives of this innovative immuno-modulator for future TCC therapy.

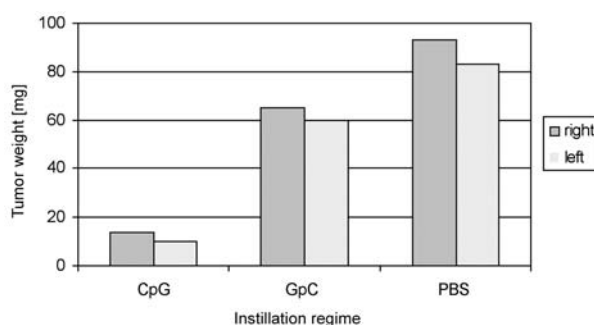


Figure 2. Comparison of tumor weight between the three groups separated after left and right (side of injection) flank.

### Materials and Methods

**Animals and tumor cells.** All animal experiments were approved by the local animal research committee. Female C57/BL6 mice (15-20g, Harlan, Borchon, Germany) were kept under standard conditions with food and water *ad libitum*. No antibiotics were given. All interventions were performed under general intraperitoneal anesthesia (Ketamin-HCl 100mg/kg body weight, Xylazin 10mg/kg body weight).

The corresponding syngeneic murine TCC line MB-49, originally described by Summerhaynes (21), was kindly provided by Prof. Totterman (Institute of Clinical Immunology, University of Uppsala, Sweden) and was maintained at our laboratory under standardized conditions (DMEM supplemented with 10% FCS, 1% glutamax and 1% penicillin/streptomycin) at 37°C in the presence of 5% CO<sub>2</sub>. Viability was determined by the trypan blue exclusion method. Only tumor cell suspensions with >95% viable cells were used for implantation.

**Reagents.** Lyophilized, completely phosphorothioate-(PTO) modified ODN (TIB-MolBiol, Berlin/Germany) were used for all experiments.

CpG, immunostimulative sequence: 5'-TCCATGACGTTTCCTGATGCT-3'(1668)

GpC, non-stimulative, control sequence: 5'-TCCATGAGCTTCCTGATGCT-3'(1668)

A 250 μM stem solution, storable at -20°C, was prepared from lyophilized ODN. For instillation, 40-μl aliquots were prepared, containing 10 nmol of the respective ODN.

**Tumor implant and treatment schedule.** For tumor induction tumor cells were washed, centrifuged (1500 rpm, 5 min) and resuspended in PBS. The cell suspension was normalized for 1x10<sup>6</sup> cells/50 μl. One cell depot was injected subcutaneously both into the right and left mouse flank. Simultaneously, the ODN were injected only into the right-sided cell depot. The mice were divided into 3 groups of 5 animals. Group I received 10 nmol of CpG-ODN. Group II received 10 nmol of GpC-ODN as non-stimulative control and to control PTO-backbone effects. Group III served as untreated control and received only PBS. Tumor volume (length x width<sup>2</sup> x 0.52 in mm<sup>3</sup>) and body weight were measured every 3 days. Mice were sacrificed 14 days after tumor implantation. Tumor or scar tissue at both injection sites were excised, weighed and examined histopathologically.

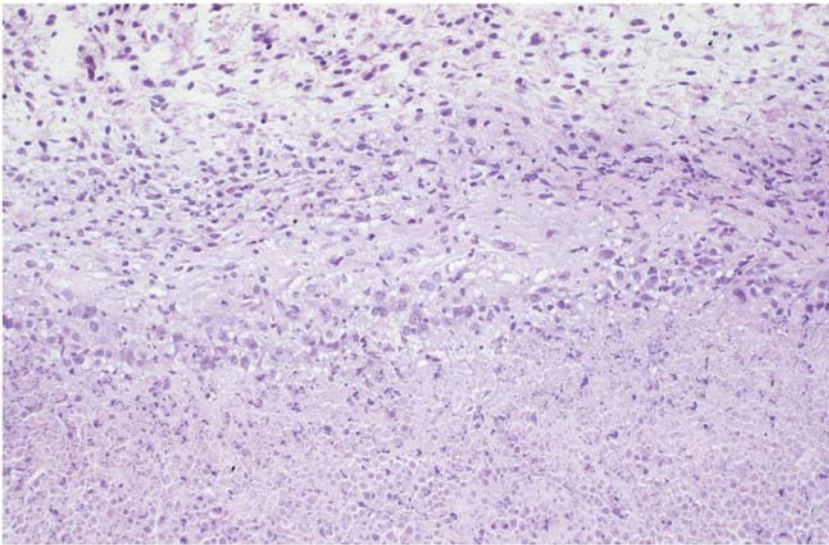


Figure 3. After CpG-ODN application, small lace-like complexes of carcinoma cells with extensive central tumor necrosis and massive inflammatory infiltrate, mainly consisting of macrophages, were found in group I.

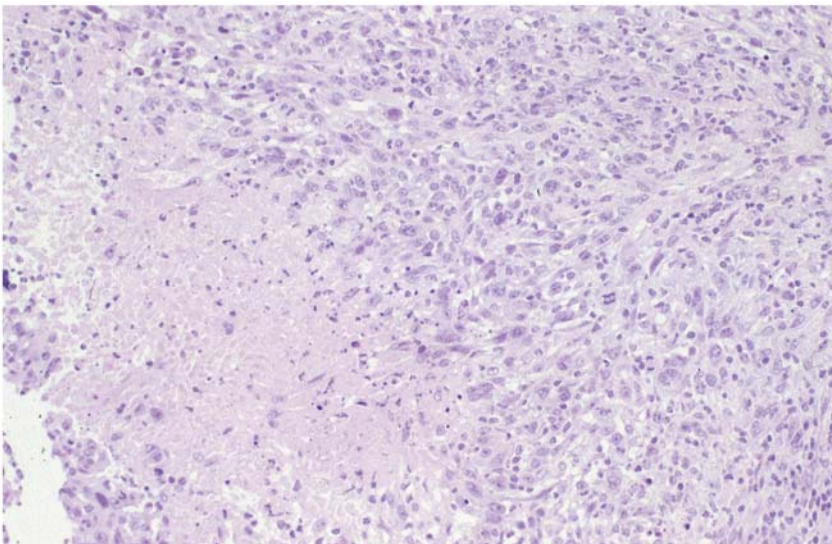


Figure 4. After application of non-stimulative CpC-ODN (group II), the amount of vital tumor cells was higher and the inflammatory infiltrate was less extensive in comparison to group I.

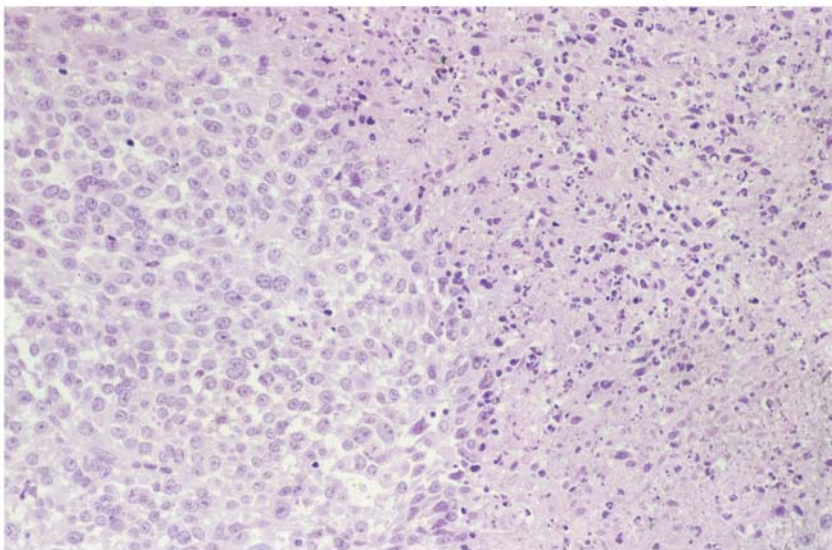


Figure 5. The PBS control group III showed large solid tumor with small necrotic areas. The inflammatory reaction was not as strong as in groups I and II, respectively.

**Histopathological examinations.** After sacrifice the tissue was excised *in toto* and fixed in a 10% unbuffered formalin solution, thereafter dehydrated in graded alcohols and embedded in paraffin, cut at a thickness of approximately 3  $\mu$ m and stained with Hematoxylin-Eosin (HE).

**Statistical analysis.** Statistical analysis for comparison of tumor weight was performed using the nonparametric Mann-Whitney *U*-test and the Kruskal-Wallis nonparametric ANOVA test. Results are given in box-plots. A *p*-value < 0.05 was accepted as statistically significant. Commercially available software (SPSS 10.0) was used for statistical calculations.

## Results

**Tumor implantation, tumor growth and sensitivity to intratumoral immunotherapy.** There was no procedure-related mortality and all animals survived until being sacrificed on day 14. All mice in groups II and III developed palpable tumors on either side and showed stable body weight. In group I there was no palpable tumor growth, either at the site of CpG-ODN injection or on the opposite side.

In group I, median tissue weight was 18 mg (SD  $\pm$  6.1 mg), in group II 110 mg (SD  $\pm$  19.2 mg) and in group III 171 mg (SD  $\pm$  8.9 mg). Statistical analysis showed a significant difference of tissue weight between all three groups (*p*<0.05, Kruskal Wallis ANOVA test, Figure 1) as well as between groups I and II, I and III and II and III, respectively, (*p*<0.01, Mann-Whitney *U*-Test). No significant differences in tumor weight and size between the left and right flank were detectable (Figure 2).

**Histopathology.** Histologically group I disclosed small lace-like complexes of carcinoma cell with extensive central tumor cell necrosis surrounded by densely packed myofibroblasts, and an inflammatory infiltrate mainly consisting of macrophages. Few neutrophil and eosinophil granulocytes were found adjacent to areas of tumor necrosis (Figure 3). In contrast, in group II the amount of vital tumor appeared to be higher. Necrosis and inflammatory infiltrate were less extensive (Figure 4). Group III disclosed large solid tumor with small centrally-located necroses devoid of myofibroblast proliferation or histiocytic infiltration. The inflammatory reaction consisted mainly of neutrophil granulocytes located within areas of tumor necrosis (Figure 5).

## Discussion

To reduce recurrence and the progression rates of intermediate and high-risk TCC, intravesical BCG therapy is the present standard of care. However, it may be associated with severe side-effects and considerable discomfort for the patient. The mode of action of intravesical BCG is still

unclear but a Th-1-biased immune response is postulated. Recent studies have shown the ability of immunostimulatory DNA to modulate the immune responses in a Th-1 direction (16). CpG-ODN has been described to activate an innate, humoral and cellular immune response and can be used as a potent adjuvant to vaccines with low toxicity (22,23). The goal of the present study was to evaluate the immune response and antineoplastic effects after locally administered CpG-ODN in a subcutaneous murine bladder cancer model.

As early as 1984, Tokunaga and coworkers isolated a DNA-rich fraction of *Mycobacterium bovis* and characterized its antitumor potency (12). In further investigations the same group showed the alteration and activation of natural killer (NK) cells and the production of cytokines like IFN $\gamma$ , IL-12 and TNF $\alpha$  *in vitro* was caused by defined, palindromic CG-sequences (24,25).

Infiltration of the tumor by predominantly mononuclear cells and a corresponding cytokine secretion pattern might be the correlate for the immunogenic activity of CpG-ODN, and is probably responsible for the antitumor activity of simultaneous intratumoral CpG-ODN therapy in our murine TCC model. However, the nature of this intratumoral immune response will have to be evaluated in further studies.

Additionally, subcutaneously applied CpG-ODN induced a systemic antineoplastic response that prevented development of tumors at the injection site as well as on the opposite, tumor-implanted site. Comparable antineoplastic effects after intratumoral application of CpG-ODN have been described in other tumor entities, *e.g.* colon carcinoma, in animal models (18,26,27). The mode of CpG-ODN action after application has not yet been elucidated. However, CpG-ODN induced tumor-specific CD4(+) and CD8(+) T cell response of the type 1 effector class (18,26,28). In immuno-incompetent T cell/B cell deficient RAG-1 knockout mice, application of CpG-ODN did not result in a sufficient antitumoral immune response. However, enhanced expression of the major histocompatibility complex (MHC) I and II antigens by tumor cells demonstrated the antitumoral effects of CpG-ODN in animal models (27,29). The cellular response to prokaryotic DNA and to synthetic CpG-ODN is mediated by TLR-9. This pathway was originally described by Hemmi and coworkers using TLR 9 knockout mice (14). For use *in vivo*, CpG-ODN must be protected from the attack of nucleases to ensure sustained effectiveness. This is usually accomplished by phosphorothioate (PTO) modification of the ODN's backbone. This modification reduces the affinity of CpG-ODN to a putative CpG binding protein and seems to be responsible for activation of different immune competent cells and the induction of unwanted side-effects: after administration of PTO-modified CpG-ODN, prolonged local IFN $\gamma$ , IL-12 production and lymphadenopathy have been described (30-32).

Our results underline the ability of PTO modifications to mediate immunostimulatory effects themselves. We found significantly smaller tumors and more immunostimulative effects, possibly induced by the PTO modification in comparison to PBS controls when using non-stimulative PTO-modified GpC-ODN. However, histopathological examinations and clinical findings also revealed that the induced antineoplastic and immunostimulatory response is not as strong as the PTO CpG-ODN effect. To prevent the undesired immunostimulatory effects mediated by PTO modification, the first studies showed a strongly enhanced uptake and a improved immunostimulatory activity of phosphodiester (PO) CpG-ODN by poly guanosine runs added at the 3' end of the ODN *in vivo* and *in vitro*. No lymphadenopathy or prolonged cytokine production were detected and, in future studies, PO ODN might have to be recommended for use as adjuvant in vaccination protocols and for experimental research as well as to exclude the immunostimulatory side-effects of the PTO modification (33,34).

To evaluate, for the first time, the antineoplastic effect of CpG-ODN in TCC, we established a subcutaneous murine bladder cancer model using the MB49 cell line in C57/BL6 mice. Furthermore, we demonstrated that CpG-ODN, a defined agent mimicking bacterial DNA, induced a local and systemic antitumor response after injection into a subcutaneous murine TCC cell depot. Before using CpG-ODN in clinical settings, further investigations including the development of an intravesical murine TCC model and consecutive CpG-ODN treatment will be necessary. First clinical trials and studies in primates showed a broad therapeutic window and low systemic toxicity levels of CpG-ODN therapy used as adjuvants. However, CpG-ODN motifs used in murine models are not easily transferable into therapy regimens for stimulating the human immune system, and appropriate CpG-ODN have to be selected for therapy in human TCC (30,35-37). Moreover, the mode of CpG-ODN action in TCC and the details of influencing and activating the immune system to achieve antineoplastic effects will have to be elucidated.

CpG-ODN may complement immunotherapeutic approaches to reduce recurrence and progression of human TCC of the bladder with low toxicity rates and tolerable side-effects in comparison to intravesical BCG therapy. Furthermore, there might be a role for intratumorally-applied CpG-ODN in neoadjuvant settings to induce tumor regression and reduce tumor load.

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