9 Summary and conclusions

Chapter 1: Introduction

Each year the diagnosis bladder cancer is made in approximately 2500 people in The Netherlands. Mostly, it concerns superficial bladder cancer treated by trans urethral resection alone. Because of the substantial risk for recurrence (60-70%) adjuvant treatment is given, consisting of intravesical bladder instillations with chemo- or immunotherapeutics. The majority of studies indicates that immunotherapy with life bacillus Calmette-Guérin (BCG) is more effective than other adjuvant treatment modalities. BCG is an attenuated strain of *Mycobacterium bovis,* originally developed as a vaccine against tuberculosis. However, in clinical practice the potential risks of the treatment of superficial bladder cancer, associated with the introduction of a life microorganism, cannot be ignored. Since its first therapeutic application in 1976, major research efforts have been directed to decipher the exact mechanism of action of the BCG-associated anti-tumor effect. BCG causes an extensive local inflammatory reaction in the bladder wall. Of this reaction, the massive appearance of cytokines in the urine of BCG-treated patients stands out. Activated lymphocytes and macrophages are the most likely sources of these cytokines, but at present other cellular sources, such as urothelial tumor cells cannot be ruled out.

This thesis is devoted to the role of the urothelial cell in the mechanism of BCG action. In the chapters 2 to 7, questions concerning the interaction between BGC, bladder wall and urothelial cells are dealt with. In chapter 8, a review is presented treating the contemporary knowledge of the mechanism of action of BCG.

Chapter 2

Urothelial cells are covered with a protective layer that mainly consists of proteoglycans or proteins with associated glycosaminoglycans (GAG). Intending to reduce side effects of BCG treatment, addition of pentosan polysulphate (PPS), a substance resembling GAG and applied to treat interstitial cystitis, was hypothesized. However, the effects of PPS on the BCG induced (immune) reactions were unknown. Interference of PPS, having a strong affinity for the bladder wall, with the binding of BCG to the bladder wall could result in a reduced immune reaction.

The effects of PPS were studied in a guinea pig model. Contrary to expectations, a PPS concentration dependent enhancement of BCG effects was found in guinea pigs, reflected by the number of bladder wall infiltrates, weight of iliac lymph nodes and skin reaction to PPD (a protein derived of *M. tuberculosis).* Moreover, PPS possessed a high affinity for BCG, but not for other tested microorganisms (*Klebsiella pneumonia, S. faecalis, E.Coli en Proteus*).

It was concluded that PPS might provide an anchoring function between BCG and the bladder wall that results in an enhanced stimulation of the immune response towards BCG.

Chapter 3

The anti-tumor effect of BCG appears to be founded on an immune system-mediated reaction induced after intravesical BCG instillation. A series of cytokines are found in the urine of patients treated with BCG, such as interleukin (IL)-1, IL-2, IL-6, IL-8, tumor necrosis factor (TNF)-α and interferon (IFN)-γ. Presumably the major cellular sources of these cytokines are lymphocytes as well as macrophages infiltrating the bladder wall, but other cellular sources cannot be excluded yet.

In this study cytokine synthesis of an urothelial cancer cell line (T24), grown *in vitro*, was determined. T24 cells possess a constitutive synthesis of TNF-α and IL-6 that can be induced by BCG. Upregulation of cytokine synthesis requires a minimum time period of 0.5-1 hour. This period of time equals more or less the clinical practice of a 1 to 2-hour intravesical instillation. Interestingly the induction of cytokine synthesis seems to be a unique property of (live) BCG, since neither BCG-conditioned culture medium, other bacteria (*E.coli* en *S. faecalis*) nor a cell wall preparation of *N. rubra* did induce the IL-6 or TNF-α synthesis of T24 cells.

In conclusion, the BCG-induced upregulation of cytokine synthesis of bladder tumor cells may contribute to the BCG-induced immune response in bladder cancer patients.

Chapter 4

It has been assumed that alteration of gene expression of urothelial cells requires internalization of BCG into these cells. Several techniques are available to study internalization of bacteria in eukaryotic cells. However, in order to study BCG internalization in detail and in large quantities of (urothelial) cells a new method is needed.

This study reports a new, reliable, rapid and relatively easy technique to differentiate between adhered and internalized microorganism in (non-professional) phagocytosing cells. Cultured eukaryotic cells are incubated with fluorescein isothiocyanate (FITC)-labeled bacteria, followed by washing of the cultured cells, leaving only adhered and/or internalized bacteria. Using a two-step technique, non-phagocytosed bacteria are stained with a specific antibody followed by a phycoerythrin (PE)-conjugated, second antibody. Double fluorescence FACS analysis distinguishes adhered bacteria from internalized bacteria. This method was used to study internalization of BCG bacteria in a series of human bladder cancer cell lines. Conditions that inhibit (4 °C, cytochalasin B) or block (anti-BCG antibodies) phagocytosis were used as controls. It was observed that, contrary to well differentiated cells, poorly differentiated cell lines are capable of internalization of BCG.

The newly developed method is highly suitable for studying internalization in large quantities of eukaryotic cells *in vitro*.

Chapter 5

Although fibronectin (FN) is reported to play an important role in adherence and internalization of BCG in bladder cancer cells, the results of several research groups have been conflicting.

Here we present the results on FN expression and the capacity to internalize BCG, determined by a newly developed technique, in a series of human bladder carcinoma cell lines. Well-differentiated cell lines (RT-4, SBC-2, SBC-7) neither express cell membrane-associated FN nor do they internalize BCG. In contrast, poorly differentiated cell lines (T24, TCC-SUP, J82) express FN on their cell surface and internalize BCG. Blocking experiments with anti-FN antibodies or $F(ab)_2$ fragments of anti-FN antibodies are not able to prevent BCG internalization.

These *in vitro* experiments suggest that BCG internalization is a non-FN dependent process and is mediated by (additional) molecule(s), possibly co-expressed with FN.

Chapter 6

IL-6 is found in the urine of bladder carcinoma patients treated with BCG. It has been shown that BCG mediates an upregulation of IL-6 synthesis in *in vitro* cultured human bladder carcinoma (TCC) cell lines.

This report relates IL-6 synthesis of a series of TCC cell lines to their degree of differentiation and ability to internalize BCG. Well-differentiated cell lines (RT-4, SBC-2, SBC-7) do not internalize BCG, and BCG does not affect their Il-6 synthesis. Cell lines (T24, TCC-SUP, J82) with a high degree of dedifferentiation possess the ability to internalize BCG. In T24 and J82 cells BCG upregulates IL-6 synthesis, while in TCC-SUP cells no upregulation is seen, possible related to the high constitutive level of IL-6 synthesis. Detailed experiments with T24 cells show that BCG-induced IL-6 synthesis depends on the BCG incubation period and concentration. Cytochalasin B, a drug inhibiting phagocytosis, or anti-BCG antibodies have a negative affect on the BCG-induced upregulation of IL-6 synthesis. Contrary to the proposed role of fibronectin for internalization of BCG, anti-fibronectin antibodies do not reduce IL-6 upregulation.

Internalization of BCG, as shown for poorly differentiated bladder tumor cell lines, and BCG-induced upregulation of IL-6 synthesis may contribute to the action of BCG in the induction of the immune response and eradication of tumor cells. This hypothesis is in accord with clinical observations that BCG is more effective in patients with high-grade bladder tumors.

Chapter 7

BCG treatment can introduce bothersome to severe side effects and efforts to diminish these side effects have been subject of study. Isoniazid (INH) is a tuberculostatic drug that is investigated in clinical trials in order to achieve fewer side effects. It is assumed that INH does not interfere with the BCG-induced activity against tumor cells. However, conflicting results concerning this issue have been presented.

In this report we studied the effects of INH on the proliferation and the BCG-induced upregulation of IL-6 and IL-8 of a series of human bladder cancer cell lines. INH at increasing, physiological concentrations does not affect cell proliferation of the cell lines T24, TCC-SUP and BT-B. Also, both the constitutive and BCG-induced synthesis of IL-6 and IL-8 appear insensitive to the presence of INH.

Presuming that the interaction between BCG and urothelial tumor cells is causally involved in the BCG-induced immune response, this study indicates that, at this level, INH does not interfere with clinical efficacy.

Chapter 8

Several years of research have improved the knowledge about the interaction of BCG and host that results in an anti-tumor effect, although our understanding is not yet complete. In a detailed review the current insight in the complex working mechanism of BCG is presented.

Intravesical instillation of BCG in the human bladder initiates a sequence of processes. BCG bacteria accumulate near the bladder wall, followed by adherence and passage through the GAG-layer of the bladder wall. BCG is internalized and processed by urothelial tumor cells and professional antigen-presenting cells (APC). BCG antigens of peptide nature are presented via MHC class II molecules to $CD4^+$ T-cells and via MHC class I to $CD8^+$ T-cells. Lipid and glycolipid BCG antigens can be presented to $CD4^+$ and $CD8^+$ T-cells in a non MHC-restricted, CD1 restricted fashion. Internalized BCG initiates an alteration of gene expression and secretion of particular cytokines (also termed interleukins). Chemokines like IL-8, partly produced by BCG-internalized tumor cells, contribute to the local activation of the immune system and attract leukocytes and mononuclear cells into the bladder wall. A Th1 or cell-mediated immune response develops, characterized by a particular set of cytokines, such as IFN-γ, IL-2, IL-12 and TNF-β. The Th1 response promotes the delayed-type-hypersensitivity (DTH) reaction, and cytotoxic cell response. A Th2 response or humoral immune response, noted by among others IL-6 and IL-10, may occur to some degree. The Th1 and Th2 responses affect each other and the final balance depends on both constitutive and induced bacterial and host components and is not always predictable. As a result of the Th1 response, cytotoxic effector cells are recruited and matured. Natural killer cells are proposed as the actual effector cells of BCG therapy. However, a definite conclusion concerning the most important effector cell is not yet possible, as several other cell types are identified in *in vitro* research.

The anti-tumor effect of BCG largely results from activated effector cells killing BCG infected urothelial tumor cells, although to some extent some of the cytokines and BCG itself may directly contribute to the cytotoxic effect.