Interactions of BCG with urothelial tumor cells in immunotherapy for superficial bladder cancer
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Introduction

In The Netherlands each year 2500 new patients are diagnosed for bladder cancer. Approximately 75% of these patients present themselves with a superficial form of bladder cancer [1]. Superficial bladder cancer can manifest itself in two forms. The majority are papillary tumors, but in 10% it consists of carcinoma in situ (CIS), which may be inconspicuous at cystoscopy. The initial treatment of these patients aims at complete transurethral resection (TUR) of papillary tumors. Because of the substantial (60-70%) chance of recurrence, and the inability to cure CIS by endoscopic surgery, (adjuvant) therapies have been introduced [2]. Adjuvant therapies encompass administration into the bladder of chemotherapeutic and immunomodifying substances, such as the clinically well-explored biological response modifier bacillus Calmette-Guérin (BCG) [3].

BCG is an attenuated strain of *Mycobacterium bovis* that was generated over the period 1908-1921 by Calmette and Guérin, aiming to develop a vaccine for human tuberculosis. Since 1976, it has been used with considerable success as an intravesical therapy for superficial bladder cancer, or for prophylaxis after TUR [4]. In comparative trials, it has been revealed that immunotherapy with BCG is superior to chemotherapy, especially in patients with high risk for progression or recurrence of bladder cancer [5,6]. Unfortunately, intravesical instillation with live BCG has several disadvantages, since it can cause infections, ranging from bothersome cystitis in the majority of patients to sepsis that have lead to death in rare cases [7].
More than a quarter of a century after its introduction, researchers are still trying to decipher the exact mechanism of the interaction between BCG and the host, and its association with anti-tumor activity. An improved understanding of the mechanism of BCG action, as an anti-tumor agent, should provide a solid base for optimization of BCG treatment schedules and/or dose, prediction of clinical response, and possibly the development of novel biological response modifiers. The latter goal intends to establish alternatives for BCG that further reduce the risk of recurrence, provide increased recurrence free periods, are easier applicable and exhibit less side effects [8].

Initiated by early histological observations and the experimental observations of Zbar and Ratliff and their associates, research efforts have largely focused on the BCG-induced local immune response [9,10]. Biopsies of the bladder wall, retrieved 24 hours after BCG instillation, reveal a strong inflammatory reaction. The submucosa and, to a lesser extend, the urothelium are infiltrated with mononuclear cells (predominantly T-lymphocytes) and polymorphonuclear granulocytes [11]. The local inflammatory reaction tends to increase during repeated BCG instillations. Histology shows infiltration with lymphocytes, plasma cells, histiocytes and polymorphonuclear cells. In a quarter of cases, characteristic granulomas are observed [12]. The importance of local immunostimulation induced by BCG is generally accepted, but no definite conclusion has been reached about the nature of the immune cells that actually contribute to (selective) tumor cell destruction. A growing body of evidence indicates, however, that a cascade of complex and multifactorial processes leads to antigen-specific activity of cytotoxic T lymphocytes, and/or the innate immune response of natural killer (NK) cells [13]. The abundance of inflammatory cells may well explain the characteristic, rapid and transient increase of an impressive series of urinary cytokines, such as interleukin (IL)-1, IL-2, IL-6, IL-8, IL-12, IL-18, tumor necrosis factor (TNF)-α and interferon (IFN)-γ during BCG instillations [8, 14-19].

It has not been fully established whether the BCG-induced local reaction is accompanied by a systemic immune modulation by e.g. a cross reaction against similar antigen(s) on the bladder tumor cell and BCG [20, 21]. However, a major causal involvement of a systemic immune reaction contradicts the inability to cure upper urinary tract tumors by intravesical BCG instillation [9, 22, 23]. When the anti-tumor effect could be accomplished solely by a systemic immune reaction, intravesical instillation should cure urothelial tumors regardless of their position (bladder, upper urinary tract) in the urinary tract.
In addition to the induction of a “classical” immune response, several lines of evidence suggest that BCG might interact directly with bladder (tumor) cells and, that these cells might be of importance for the BCG-mediated anti-tumor effect. *In vitro* experiments with human TCC cell lines have revealed that BCG possesses cytolytic and anti-proliferative properties [24-26]. Inhibition of cell proliferation was most pronounced for poorly differentiated TCC cells. Although the direct effects of BCG bacteria may contribute to the efficacy of BCG therapy, they alone are insufficient to explain the working mechanism of BCG.

Immunocompetence of the host is required for effective BCG therapy [9]. Differentiation of normal urothelial cells from tumor cells is essential for patrolling immunocompetent cells, such as cytotoxic T-lymphocytes (CTL) and natural killer cells (NK). BCG seems to sensitize bladder tumor cells for CD4⁺ T-helper lymphocytes and the cytotoxic effects of NK-cells and CD8⁺ T-lymphocytes, but has no influence on normal urothelial cells [27]. So, BCG augments the recognition of surface antigens through induction of the antigen-presenting systems, the major histocompatibility complex (MHC) class I and II, and CD1, and their accessory molecules, such as intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function associated antigen-3 (LFA-3) [28-31]. As a result, there are several possible pathways for cells of the immune system to attack BCG treated urothelial tumor cells. Patrolling NK-cells may recognize altered antigens presented by MHC class I receptors, e.g. BCG antigens and/or tumor-associated MAGE antigens or heat shock proteins [32-34]. Furthermore, the urothelial cell could cause activation of the immune system by presenting antigens of internalized BCG [35]. Either in a MHC class I-restricted, or in a MHC independent CD1-restricted fashion, cell-mediated cytotoxicity may be accomplished by cytotoxic CD8⁺ T-cells [31]. The relative importance of each pathway remains to be established.

Activation of immunopotent cells has been considered an exclusive characteristic of antigen-presenting cells of the immune system itself, like dendritic cells. Interestingly, and in accordance with other nonprofessional phagocytes, it has been suggested that, in the presence of BCG, bladder tumor cells gain the characteristics and functions of antigen-presenting cells [29]. These observations challenge the traditional view of the epithelium as a relatively passive physical barrier to the internal environment and support the concept that epithelial cells play an important role in the regulation of an inflammatory response [36, 37]. An encounter of airway epithelial or urothelial cells with micro-organisms, like *E. coli* or *Citrobacter*, is accompanied by the production of a broad range of cytokines and chemokines that may initiate and modulate the inflammatory cascades [38,
Regarding BCG immunotherapy, it has been shown that BCG upregulates, in in vitro cultured urothelial tumor cells, the synthesis of various cytokines, additional to a constitutive synthesis [41, 42]. Recent studies suggest that BCG internalization and the induced upregulation of cytokine depend on the degree of dedifferentiation of TCC cells [43]. From these observations it is tempting to speculate that urothelial (tumor) cells play a role in a cytokine-mediated initiation and/or regulation of the early immune response. Interestingly, the efficacy of BCG as an immunotherapeutic agent, seems to correlate with the relative high rate of BCG-induced cytokine synthesis in urothelial tumor cells, compared to E. coli, S. faecalis or Nocardia rubra cell preparation [41].

Internalization of BCG in (urothelial) cells is probably a key process in the mechanism of action of BCG. Urothelial (tumor) cells are capable of internalization of BCG [44, 45, 46, 43, 47, 48]. BCG internalization in TCC cell lines is time- and dose-dependent. In vitro studies have revealed that normal urothelial cells are not capable of BCG internalization [48], whereas internalization is most pronounced in poorly differentiated tumor cells [46]. These observations are in accord with the absence of BCG adherence and internalization in the urothelium of guinea pigs [49]. These data may be related to the observed, better clinical response to BCG treatment of high-grade compared to low-grade tumors [50]. Profound knowledge about the process of BCG internalization is, however, lacking. An intermediate role of fibronectin (FN) has been suggested, since BCG bacteria possess a receptor that binds to the collagen domain of FN, the fibronectin attachment protein (FAP), while uroepithelial cells express an integrin (α5β1 receptor) with a special affinity for FN [51, 52]. In accordance with the proposed mechanism, some investigators found interference of anti-FN antibodies with BCG adherence and internalization in human bladder cancer cell lines, but others were not able to reproduce these inhibitory effects [52, 46, 53]. Although these conflicting results may be due to different experimental conditions or applied techniques, the existence of other nonfibronectin-mediated bacterial attachment and internalization mechanisms can not be excluded at present [54, 45, 44, 46].

Clinical as well as experimental observations have been interpreted as evidence for an absolute necessity to treat with live organisms in order to provoke a local immune and clinical response [9, 55, 56, 57]. Depending on the state (live or dead) and nature of the antigen (protein or lipid) mycobacterial antigens are presented through different pathways, either MHC class I-, class II-, or CD1-restricted [30, 31]. In this light the clinical application of isoniazid (INH), a tuberculostatic drug, to diminish the side
effects of BCG treatment is of interest. INH interferes with bacterial cell wall formation, resulting in BCG cell death during the process of cell division. Non-dividing BCG microorganisms remain metabolically active. A different immune response towards live or dead mycobacteria implies that the effect of INH on clinical efficacy of BCG should be considered. Accepting urothelial tumor cells as (non-professional) antigen presenting cells and their importance in the BCG-provoked immune response, Bevers et al. studied the effects of INH on various characteristics of TCC cells [58]. INH did not interfere with the BCG-induced upregulation of IL-6 and IL-8, and proliferation of the human bladder cancer cell line T24. These observations are in accord with clinical data, indicating an equal BCG-induced immune response and clinical efficacy of BCG without and with INH [59,60].

Conclusive differentiation between the respective roles of cells of the immune system on one hand and of the urothelial cell on the other hand, in triggering the BCG induced anti-tumor effect will be difficult if not impossible: in the end, both are needed. A thorough knowledge however of the underlying mechanism is crucial for improving the adjuvant treatment of superficial bladder cancer.

It is well known that a subpopulation of patients fails to develop an immune response to BCG [61]. Although a considerable number of possible explanations can be put forward, suboptimal clinically effective binding and a limited passage of BCG through the protective glycosaminoglycan (GAG)-layer of the bladder have been raised [62-64]. Observations of Zbar et al. suggest a direct or close contact between BCG and the target organ to be treated as crucial for its anti-tumor effect, but profound knowledge about binding of and passage into the bladder wall is lacking [9]. Taking into account that both the highly sulphated GAG-layer and BCG cell wall are highly negatively charged, a physiochemical model of the interaction between BCG and the bladder wall has been presented [64]. This model describes an accumulation, without adherence, of BCG organisms at a close (70 - 100 Å) distance, the so-called docking distance, of the bladder wall, the (low) probability of actual adherence and the possible clinical relevant manipulation of BCG adherence by polyelectrolytes. Several aspects of this theoretical model seems to be in accord with experimental, in vivo findings in animals, including (1) the optimal BCG diluent (pH, saline) [65], (2) the apparently low abundance of BCG bacteria adherence to the uninjured bladder wall [49,62,63], and (3) the increased BCG-induced immune response after pre-instillation with the polyvalent polysaccharide pentosanpolysulphate (PPS) at an certain, predicted concentration [66].
should be stressed, however, that the adherence of BCG to the bladder wall is not adequately understood and that other, more specific mechanisms are involved. So, a crucial involvement of the interaction of BCG to fibronectin in the bladder mucosa has been postulated, but this is still controversial [63,67].

The subject of this thesis comprehends several, experimental aspects of the early events of the BCG-associated anti-tumor effect, studied in both a *in vitro*, human cell line model and *in vivo*, animal model: the encounter of BCG with the GAG-layer of the bladder wall and factors that influence this interaction, and the relation between BCG internalization into uroepithelial cells and cytokine production of uroepithelial cells in the presence of BCG and other microorganisms.
References


