Abstract. To date, the precise mechanism of intravesical immunomodulators remains unknown. In vitro, interferon alpha (IFN-α) acts directly on neoplastic cells and inhibits their proliferation while it induces their differentiation. Urothelium and transitional cell carcinoma (TCC) cells express IFN-α receptor, the density of which correlates with lesion grade. IFN-α reduces neo-microvascular density in the normal urothelium adjacent to the tumor after transurethral resection (TUR), possibly via inhibition of COX-1. Moreover, IFN-α induces the membrane expression of tumor-related antigens and MHC antigens, providing a basis for a cellular immune response. When given intravesically, IFN-α may result in local and systemic T cell and NK cell activation. By monitoring nitric oxide (NO) end-products in urine and evaluating inducible nitric oxide synthase (iNOS) expression immunohistochemically, we were able to show that IFN-α may induce urothelial iNOS expression with subsequent formation of peroxynitrite, which might contribute to the antineoplastic action of IFN-α. Bacillus Calmette-Guerin (BCG) is thought to bind to the bladder wall via interaction between the bacterial antigen 85 complex and fibronectin. Although systemic reactions (evolution of cellular immune response, systemic production of cytokines and oxygen free radicals) have been reported, a likely scenario is that exposure to BCG results in a massive local immune response, characterized by induced expression of cytokines in the urine and in the bladder wall, and by a marked infiltration of the bladder wall by granulocytes and mononuclear cells. BCG-induced changes in tumor cell phenotype render them able to act both as lymphokine-activated killer cell targets and antigen presenting cells. Although BCG may act directly on the proliferation of tumor cells, helper and cytotoxic T cells and, most probably, NK cells are absolutely necessary for any antitumor effects. Tumor cell killing is mediated through FasLigand, perforin and TNF-alpha. In a recent study, we found that BCG up-regulated iNOS expression in normal human urothelium in vivo, suggesting a role for NO in BCG-mediated antitumor activity.

Bladder carcinoma is the eleventh most common malignant neoplasm worldwide (1). An estimated 80% of these tumors present as superficial neoplasms which do not infiltrate the detrusor muscle (2). However, superficial bladder carcinomas are a heterogenous group of tumors with different biological potential and varied natural history. Despite the absence of any evident residual disease after the transurethral resection of the primary lesion(s), tumor recurrence is expected in 55-70% of patients, while in 10% of patients the disease will recur as a muscle-infiltrating tumor (3). Bladder transitional cell carcinomas may initiate functional alterations in peripheral T lymphocytes and NK cells (4-6), while the immune response is obvious, at least within the bladder wall, and has been recognized as an important prognostic factor (7, 8). Adjuvant treatment with intravesical instillations of several substances such as cytotoxic (epirubicin, mitomycin-C) and immunomodulating (Bacillus Calmette-Guerin, interferon-alpha) agents has been introduced for treating residual disease and for preventing tumor recurrence and/or progression (9, 10). To date, the precise mechanism of intravesical immunomodulators remains unknown.

Interferon instillations

Interferons are known as angiogenesis inhibitors, differentiation regulators, immune effector cell activators, cytokine production inducers and tumor-associated antigen...
expression enhancers, as well as for their antiviral and direct antiproliferative activity (11). Several studies have shown the efficacy of interferon-alpha2b (IFN-α2b) in reducing recurrence rates (12-16). In vitro, IFN-α acts directly and indirectly on bladder cancer cells (11, 17) and inhibits their proliferation, while it induces their differentiation (18-20). Normal urothelium and bladder cancer cells express IFN-α receptor and receptor densities correlate with the grade of the lesions (21). Receptor density might serve as a factor for selecting subgroups of patients who will benefit more from such therapy. In vivo, IFN-α2b reduces neo-microvascular density in the normal-appearing urothelium adjacent to the tumor after transurethral resection (22), and this can be attributed to its ability to inhibit cyclooxygenase-1 that stimulates angiogenesis (23). Ultrastructural studies have shown that it may also help in restoring the normal features of the non-involved urothelium (24-26). Moreover, IFN-α induces the membrane expression of tumor-related antigens and major histocompatibility complex antigens (11, 19, 27), providing a basis for a cellular immune response. In vitro, recombinant IFN-α increases lymphokine-activated killer (LAK) cell activity and lytic susceptibility of murine bladder transitional cell carcinoma to tumor infiltrating cytotoxic T lymphocytes (28, 29). When given intravesically, IFN-α may result in local (30, 31) and systemic (32, 33) T cell and NK cell activation. The enhanced immune cell activity persisted for 3 to 6 months after discontinuation of instillations and correlated with the response to treatment (31, 33). By monitoring nitric oxide end products in urine and evaluating inducible nitric oxide synthase (iNOS) expression immunohistochemically, we were able to show that IFN-α2b may induce urothelial iNOS expression which, subsequently, results in the production and formation of oxidative molecules in urine. The peroxynitrite formed by the combination of nitric oxide with superoxide provides important clues in its role as a causative factor in the antineoplastic action of IFN-α2b (34).

BCG instillations

As far as Bacillus Calmette-Guerin (BCG) is concerned, a plethora of events following its intravesical administration has been described. The initial crucial step in BCG immunotherapy seems to be the binding of mycobacteria to the urothelial lining (35, 36) and enhanced binding to fibronectin can augment the antitumor activity of BCG (35, 37). This most probably depends on the interaction of antigen 85, an immunodominant protein complex on the bacteria surface (38), with the carboxyl-terminal heparin-binding domain of fibronectin (39). Further studies showed the fibronectin/BCG interaction to be mediated by bacterial receptors, the fibronectin attachment protein (FAP) that binds to fibronectin in an essentially irreversible manner (40) and is necessary for in vivo BCG attachment (41). Furthermore, glycosaminoglycans could provide another binding site (42). Following attachment, BCG are ingested by inflammatory, urothelial and tumor cells (43). BCG attachment and internalization in the latter are mediated, at least in part, by the αβ₂ integrin receptor and both processes can be modulated by fibronectin (44). The presence of both CD4⁺ helper and CD8⁺ cytolytic T cells is also absolutely necessary to induce any antitumor effects; the administration of T cells to athymic animals has been shown to restore their ability to respond to BCG therapy (45, 46). However, a recent study using an optimized murine bladder cancer cell model, MB49 cells, beige mice and anti-NK1.1 antibody suggested that NK cell activity is of prime importance for the therapeutic effect of BCG (47).

Systemic immune response after BCG instillations. Antibody response to intravesical BCG has been characterized in the urine and peripheral blood (48-51). Zlotta et al. showed that patients with superficial bladder carcinoma demonstrated an increased lymphoproliferation against mycobacterial antigens before BCG as compared to control subjects, indeed suggesting the possible existence of bladder carcinoma antigens cross-reactive with mycobacterial antigens (52). By studying the humoral response to defined mycobacterial antigens or heat-shock proteins, they further showed not only a specific lymphoproliferative response in the majority of patients, but also an increase in antibodies against mycobacterial antigens (53-55). Assessment of peripheral blood lymphocyte subsets revealed a statistically significant difference in the CD4⁺/CD8⁺ ratio (56-58) and activation of antigen expression (58) between baseline values and that obtained following BCG administration. However, such differences were not found in other studies (59, 60). Peripheral blood mononuclear cell (PBMC) killing activity has been found to increase significantly after the third BCG instillation, suggesting a systemic immune response (61). Analogous increases were found in the serum levels of IFN-γ (61, 62) and TNF-p75-receptor (62), while IL-1β, IL-6, TNF-α and M-CSF were not detectable (61). Production of IL-2 mRNA, but not IFN-γ mRNA, in PBMCs was observed during BCG treatment (63), resulting in a significant increase of the circulating IL-2 (61, 64). Moreover, serum levels of monocyte chemotactic protein-1 (MCP-1) and the "regulated on activation normal T expressed and secreted" chemokine (RANTES), which are potent chemotactic molecules that attract monocytes and memory T cells, were significantly higher in patients with superficial bladder cancer treated with BCG than in untreated cancer patients and controls (65). Finally, a possible indicator of a systemic rather than a local immune reaction could be the BCG-induced systemic production of free radicals resulting in enhanced red blood cells oxidative stress (66).
Local immune response to BCG instillations. The intravesical instillation of BCG results in a massive local immune response, characterized by induced expression of cytokines in the urine and in bladder tissue, and by an influx of granulocytes as well as mononuclear cells into the bladder wall. Besides these cells, both normal and malignant urothelial cells can produce a limited array of cytokines in response to this mycobacterial stimulus and bladder cancer cells take on some of the features of a BCG-infected phagocyte.

Within 24 hours of BCG instillation, significant urinary secretion of IL-1, IL-2, TNF, IFN-γ and other cytokines (IL-5, IL-6, IL-8, IL-10, IL-12, IL-18, GM-CSF) can be detected, with a maximum titer after 2-8 hours and a decrease to normal values within 24 hours. IL-1, TNF and IL-2 are formed in the course of an immunological reaction which, in contrast to non-specific inflammation, is associated with antigen recognition. The sharp increase in the urine level of these cytokines is, therefore, an indication of the immunological character of a BCG-induced reaction.

Detection of IL-2 in urine during BCG instillations was first reported in 1986 (67, 68) and subsequent studies confirmed the results (61, 62, 69-82). While IL-1 can be present in the urine of healthy and febrile subjects, the strong secretion of IL-2 and TNF following BCG treatment represents a qualitatively and quantitatively very different reaction compared with non-specific cystitis. Neither IL-2 nor TNF has been found in the urine of healthy individuals, while IL-2 has been detected in a few exceptional cases in the urine of patients with non-specific cystitis. IL-2 is mainly produced by activated T helper cells and acts specifically in the proliferation and differentiation of T lymphocytes. Furthermore, an association between urinary IL-2 levels and response to BCG treatment has been observed. TNF is mainly formed by activated macrophages and both TNF-α and TNF-β and their soluble receptors have been identified in the urine of BCG-treated patients (75, 83). Special attention has been paid by some groups to IL-8, due to its rapid onset in the immune response to intravesical BCG (76, 82, 84-88), although urinary IL-8 levels are also elevated in subjects with transitional cell carcinoma (89). However, its predictive value for clinical response to BCG is questionable. Other cytokines that have attracted research interest are IL-5 (90), IL-6 (61, 73, 74, 76-78, 82, 91, 92), IL-10 (62, 76, 78, 80-82), IL-12 (78, 93), IL-18 (88), IFN-γ (61, 62, 76, 78, 80-82, 93, 94), M-CSF (61) and GM-CSF (78, 92). Intercellular adhesion molecule-1 (ICAM-1) is one of the three major ligands for the β2 integrin leukocyte function-associated antigen-1 (LFA-1) and its detection in the urine indicates a response of the tumor to immunotherapy (76, 78, 95, 96). CD14 is important in the initiation phase of immune responses and high levels of its soluble form can be detected in urine; its source is likely to be the resident and infiltrating macrophages in the bladder wall (78, 97). However, the source of urinary cytokines is debatable since the production of pro-inflammatory cytokines by bladder cancer cells can be induced by BCG (92). The number of cells in urine increases significantly after BCG administration; the predominant cell type present is polymorphonuclear granulocyte, probably representing a defense mechanism against mycobacteria. The main mononuclear leukocytes are monocytes/macrophages and T lymphocytes, while natural killer cells (CD16+ and/or CD56+) and B cells (CD19+) are less numerous (73, 98, 99). The pattern of cytokines detected in the urine suggests that BCG predominantly induces a T helper type (TH)1 response in the bladder of patients. Nevertheless, in addition, certain TH2 cytokines (IL-5 and IL-10) and cytokines not specifically belonging to a TH1 or TH2 profile (such as IL-8, GM-CSF and TNF-alpha) are induced. Interestingly, in most investigations the TH2 cytokine IL-4 could not be detected (76, 78, 83). The important role of the TH1/TH2 balance for effective immunotherapy with BCG has also been shown in animal models (100), as well as with in vitro model investigations (102-105). Chemokines are involved in the initiation or maintenance of inflammatory processes, while they also probably contribute to the antitumor effect of immunotherapy. Indeed, chemokines known to be involved in the latter, such as interferon-inducible protein 10 kD (IP-10), macrophage-inflammatory-protein-1alpha, monocyte chemotactic protein-1, macrophage-derived chemoattractant and even eotaxin, have been detected in the urine of treated patients (93, 106).

BCG-treated patients typically have characteristic granulomatous cellular infiltrates surrounded by dense areas of lymphocytes and eosinophilic granulocytes (107-124), which differ significantly from non-specific cystitis or even from cystitis induced by cytostatic drugs in terms of duration as well as in qualitative terms. The early accumulation of granulocytes is followed by an influx of macrophages and lymphocytes that coincides with increased expression of activation markers (HLA-DR, CD25 and ICAM-1) on infiltrating cells and also on the urothelium, which strongly expressed major histocompatibility complex class II molecules at the end of the therapy (96, 109, 111, 112, 114, 116, 121). The infiltrate of mononuclear cells in the bladder wall consisted mainly of T cells, with a distinct predominance of CD4+ cells compared with CD8+ cells. The quotient of CD4+/CD8+ T cells detectable in the submucosa was approximately 2:1, which represents a reversal of the original ratio found before treatment (108-112, 116-118, 120-124). Honda et al. also found γδ+ T cells significantly increased after treatment compared with numbers before treatment (122). Immunohistology has been helpful for the localization of cytokines and the long-lasting mucosal inflammatory response (91, 96, 125). Urine
cytology and immunocytophagy have further helped study the local immune response to BCG and confirmed the immunohistochimical data (73, 98, 99, 126-128). The addition of BCG to bladder-wash-derived lymphocytes expanded in vitro enhanced their proliferation, suggesting that this population was sensitized against BCG. This was further confirmed by analysis of T cell receptor restriction patterns, showing that bladder lymphocytes from patients under BCG were oligoclonal (127). The number of bladder-wash derived T cells expressing the γ/δ receptor has been found to be significantly higher in patients receiving standard dose BCG than in those receiving low dose BCG (128).

Antigen presentation. Antigen presentation is performed via MHC class I and MHC class II molecules for endogenous and exogenous antigens, respectively. While MHC class I molecules are expressed by almost all cell types, MHC class II molecules are expressed by antigen-presenting cells (APCs: macrophages, B lymphocytes, dendritic cells and Langerhans cells) only. MHC class I molecules are recognized by CD8+ cytotoxic T lymphocytes, while MHC class II molecules are recognized by CD4+ helper T lymphocytes. Dendritic cell activation by BCG involves homotypic aggregation, up-regulation of surface antigens, down-modulation of endocytic activity, and release of TNF-α and IL-8 (129, 130). Besides APCs, activated bladder carcinoma cells can process and present antigens to CD4+ T lymphocytes. Using a murine bladder tumor model, Lattime et al. (131) demonstrated that bladder carcinoma cells were able to present BCG antigens to CD4+ T cells in an MHC class II restricted fashion. The inflammatory response to the intravesical instillations of BCG is associated with change in the phenotype of bladder tumor cells. Before BCG therapy, bladder tumor cells express the class I MHC antigen (HLA-ABC), but the class II MHC molecule (HLA-DR) is expressed weakly or not at all. ICAM-1 and ICAM-2 are not expressed. After BCG therapy, the bladder cancer cells, as well as normal-appearing urothelial cells, express HLA-ABC, HLA-DR, ICAM-1 and costimulatory B7-1 molecules (95, 96, 109, 112, 114, 124). In vitro studies have shown that BCG can also produce the same results directly rather than via the host immune mechanisms (132). Nevertheless, the key cytokine for altering tumor cell phenotype in vivo seems to be IFN-γ that is produced by the T cells in response to BCG. By adding IFN-γ alone or, to a lesser extent, TNF-α or IL-1α to the culture medium, researchers were able to induce or enhance the expression of both HLA-DR and ICAM-1, but not ICAM-2 on the cell lines in a dose-dependent fashion (133, 134). These effects were also seen if urine from BCG-treated patients was added to the cell culture medium and could be largely abolished by preincubation of the urine with anti-IFN-γ antibody (135).

The change in phenotype has a significant impact on the function of tumor cells, rendering them capable of acting as both lymphokine-activated killer cell-sensitive targets and antigen-presenting cells for BCG.

BCG antitumor activity. Initial in vitro studies showed a lack of specificity of lymphocyte-mediated cytotoxicity against bladder cancer cells (136), that was attributed to a low incidence of CD56+ and CD57+ mononuclear blood cells (137). Subsequent studies have shown that IL-2- and TNF-α-stimulated human PBMCs are mediators of the antitumor response that kill tumor cells in an MHC-unrestricted fashion and are probably effector cells in the BCG-induced antitumor response (28). Binding to the target was shown to be a requirement, and was achieved through the interaction between the leukocyte function-associated antigen-1 (LFA-1) on the LAK cells and ICAMs on the tumor cells (138, 139). LAK cells were shown to mediate apoptosis of human bladder cancer cells, involving a pH-dependent endonuclease system in the cancer cell and the fibronectin molecule present on the LAK cell membrane (140).

Stimulation of PBMCs with BCG enhances their cytotoxic activity against tumor cells (141, 142), leading to the induction of MHC-unrestricted cytotoxicity mediated by the so-called BAK cells (103, 143-146). The cytotoxicity of BAK cells has been attributed to a small subpopulation of activated lymphocytes which belong to the CD8+/CD56+ phenotype and, unlike LAK cells, they require the accessory function of CD4+ T cells and monocytes, while they also need (like LAK cells) IL-2, IFN-γ and IL-12 during the stimulation period. Subsequent studies revealed that BAK cells correspond to a subset of NK cells with a CD3–/CD8+/CD56+/CD16 very dim /perforin + phenotype that accounts for roughly 10-20% of the total BCG-stimulated PBMCs (147). The contribution of CD8+ T cells, known to be up-regulated by mycobacterial heat-shock proteins (148), to the BAK cell phenomenon can be excluded, since these cells are known to be CD4+/CD8−. However, peripheral blood γ/δ + T lymphocytes, specifically activated by sonicated antigens of Mycobacterium tuberculosis, exhibit profound cytotoxicity against NK-resistant bladder tumor cells in an MHC non-restricted manner and their cytotoxicity is augmented by the addition of recombinant TNF (149). While LAK cell killing activity is mediated through the FasLigand, perforin and TNF-α (150), BAK cells kill tumor cells predominantly via perforin without significant contribution of the FasLigand pathway (151). Similarly to LAK cells (138, 139), BAK cell lysis of bladder tumor targets depends on the establishment of cell-cell contact and the expression of LFA-1 and its two subunits CD11a and CD18 (152).

Besides the direct cytotoxic activity of immune cells, another important effector mechanism could be the direct
antitumor activity of various cytokines. Indeed, anti-IFN-γ antibodies reduce the BCG antiproliferative activity against bladder carcinoma cells (153), while BCG-induced urinary cytokines (IFN-γ, TNF-α, IP-10) inhibit microvascular endothelial cell proliferation, thus affecting angiogenesis (154, 155). Urine-derived tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) that is induced in patients responding to BCG therapy, is capable of bladder tumor cell killing in vitro (156).

Exposure to BCG has an antiproliferative effect on certain tumor cell lines (157-160). Live BCG and, to a lesser but still significant extent, heat-inactivated BCG suppress tumor cell growth in a dose-dependent manner. Jackson et al. (158) reported that BCG is not directly cytoxic to tumor cells in vitro, while the supernatant from heat-killed BCG does not have an effect on tumor cells. However, another group of researchers has reported that BCG directly inhibited the growth of bladder carcinoma cells, through the induction of apoptosis and decreased telomerase activity (161, 162).

BCG interaction with the malignant urothelium is not a passive phenomenon; besides the activation of MHC class II molecules, BCG adherence induces the expression of cytokines by the tumor cells, such as IL-6 (91, 92, 160, 162-165), IL-8 (92), TNF-α (92, 160, 163) and GM-CSF (92). BCG stimulates tumor cell expression of IL-6 via an immediate early pathway requiring the signal transducers nuclear factor (NF)-κB and AP-1 (165). Initiation of the intracellular signaling is probably achieved following interaction of multiple fibronectin binding sites present on BCG with α5β1 integrin receptor bound fibronectin molecules to cross-link α5β1 integrin receptors on the tumor cell surface (166). In addition to facilitating the interaction of BCG with bladder carcinoma cells through the up-regulation of α5β1 integrin, IL-6 has shown potential to inhibit the growth of these cells conditionally.

In a recent study (167), we focused on the probable cytostatic role of nitric oxide (NO). Inducible NOS expression is enhanced in bladder cancer (168-173), while the endogenously produced NO modulates bladder cancer cell growth (174). BCG treatment has been found to increase both constitutive and inducible NOS activity in the bladder mucosa, as well as the mean NO concentration in the air aspirated from the bladder (175). In the rat, BCG up-regulates iNOS and eNOS gene and protein expression (176), while BCG treatment of cultured normal human urothelial cells and bladder cancer cells resulted in the induction of constitutive and inducible NOS activity and urine nitrite and luminal NO levels in the bladder (177). In our study, we showed that BCG instillations resulted in induction of iNOS expression of phenotypically normal urothelial cells in 66.6% of the cases studied. Interestingly, we found that 4/36 tumor specimens expressing iNOS before BCG instillations corresponded to the only early recurrences following BCG treatment. This could be an indicator of aggressive biological behavior or an endogenous resistance to BCG. Moreover, NO exposure inhibited induction of splenocyte-derived LAK cells by inducing apoptosis (178). The different effects of reactive oxygen species (NO, superoxide anion, hydroxyl radical) on bladder cancer cells has been recently reported (179). NO and superoxide anion enhanced the proliferation and activation and superoxide anion up-regulated the antitumor cytotoxicity of LAK cells. In contrast, the hydroxyl radical down-regulated LAK cell growth.

References


Mitropoulos: Intravesical Immunomodulators


