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Role of Neutrophils in BCG Immunotherapy for Bladder Cancer

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SUMMARY

Bladder cancer accounts for ~13,000 deaths annually, and >60,000 new cases will appear this year, making it the 4th and 10th most common cancer among men and women, respectively [1]. The majority of the newly diagnosed cases will be diagnosed prior to muscle invasion, thus potentially completely curable. Unfortunately, >20% of patients initially diagnosed with non-muscle-invasive bladder cancer will eventually die of their disease despite local endoscopic surgery [2].

Mycobacterium bovis bacillus Calmette-Guérin (BCG) has been used for the treatment of bladder cancer since 1976 [3], and continues to be at the forefront of therapeutic options for this malignancy. Despite its success and worldwide acceptance, the antitumor effector mechanisms remain elusive.

BCG therapy induces a massive local immune response characterized by cytokine expression of multiple cytokines in the urine and bladder tissue [4] and the influx of granulocytes and mononuclear cells into the bladder wall [5,6]. Findings from our laboratory have demonstrated that TNF-related apoptosis-inducing ligand (TRAIL) is induced by BCG treatment [7] and TRAIL was expressed on polymorphonuclear neutrophils (PMN) in the urine obtained from patients after intravesical BCG instillation. Subsequently, we have determined that BCG and components of the *Mycobacterial* cell wall can directly stimulate the release of soluble TRAIL from PMN through Toll- Like Receptor-2 (TLR2) recognition that is augmented by interferon (IFN) [8]. Based on our work and that of others implicating the need for T helper type 1 (Th-1) cytokine responses to BCG therapy for therapeutic results, we propose that TRAIL is released by PMN migrating to the bladder in response to BCG treatment. In addition, IFN acts to augment and prolong the amount of TRAIL released by PMN, resulting in an effective therapeutic outcome.

Keywords

TRAIL; neutrophil; PMN; BCG; *Mycobacterium*

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INTRODUCTION

Urothelial carcinoma of the bladder accounts for ~5% of all cancer deaths in humans. The majority (70–80%) of bladder tumors are non-muscle-invasive at diagnosis and, after local surgical therapy, have a high rate of local recurrence (70%) and progression (20%). Thus, patients require lifelong medical follow-up exams with inspections of their bladders and surgical resection, typically with additional prophylactic treatments in the event of recurrence. Current treatments extend time to recurrence, but do not alter disease survival. The resulting economic burden on the U.S. health care system is enormous, reaching over \$4 billion annually. As measured on the basis of cumulative per patient cost from diagnosis until death, the cost to treat bladder cancer exceeds all other forms of human cancer.

Mycobacterium bovis bacillus Calmette-Guerin (BCG) was isolated in 1921 [9], and has been given to billions of people as a vaccine against tuberculosis. Since its first use by Morales in 1976, BCG has become the treatment of choice for non-muscle-invasive bladder cancer. Despite nearly 30 years of clinical use, the anticancer mechanism of BCG in the treatment of bladder cancer has not been clearly defined, limiting rational improvements to this treatment strategy. Recent studies have demonstrated that polymorphonuclear neutrophils (PMN) migrating to the bladder after BCG instillation release large amounts of the apoptosis-inducing molecule TNF-related apoptosis-inducing ligand (TRAIL)[7], along with chemokines that recruit other immune cells, suggesting that PMN play a key role in the antitumor response to BCG therapy. This review discusses the impact of these recent findings on the understanding of the antitumor mechanisms underlying BCG-based immunotherapy for bladder cancer.

MECHANISM OF BCG IMMUNOTHERAPY

Urinary tract infections (UTI) occur when uropathogens gain entry into the urinary tract and proliferate on the epithelial surface of the bladder. Unlike other mucosal surfaces, which are normally colonized with bacteria, the bladder mucosa is intended to line a sterile reservoir. Instillation of BCG into the bladder establishes a localized infection that involves both attachment and internalization into urothelial cells (normal and malignant) via a fibronectin dependent process mediated by integrins [10–12]. Thus, BCG therapy can be viewed as a method of inducing a chronic mycobacterial infection in the bladder [3]. BCG induces IL-1, -6, -8, and GM-CSF secretion from the infected urothelial cells [13]. High levels of IL-8 production early in the treatment cycle is associated with better clinical responses to BCG [14,15], and is responsible for PMN recruitment to the bladder. The net effect of these chemokine signals is an escalating recruitment of monocytes and granulocytes into the bladder with each successive weekly BCG instillation [16]. Within 4–6 h after a late cycle clinical BCG instillation, it is common to find massive pyuria with over 10^7 WBC/ml of urine (M. O'Donnell, unpublished data) associated with a typical constellation of irritative bladder symptoms including frequency, urgency, and dysuria. Over 75% of these cells are PMN, with 5–10% as macrophages (Mφ), and only 1–3% of the cells as T cells or natural killer (NK) cells [17]. In addition to the massive inflammation and cellular influx that occurs after BCG instillation, findings from our laboratory have demonstrated that TNF-related apoptosis-inducing ligand (TRAIL), a protein with tumoricidal activity, is present at high levels in urine samples from BCG patients that had responded well to therapy [7]. Furthermore, urinary TRAIL effectively killed bladder tumor cells *in vitro*. However, TRAIL levels and tumoricidal activity were greatly reduced in the urine from patients that did not respond to BCG therapy. Together, the establishment of a productive inflammatory response in the bladder that results in the accumulation of cytokines and TRAIL seems to be essential for an effective antitumor response during BCG therapy for bladder cancer.

Role of PMN in the BCG antitumor response

PMN recruitment to the bladder begins when BCG stimulates bladder epithelial cells to secrete chemokines prompting PMN to leave the circulation in response to the chemotactic gradient, traversing the mucosa to the epithelial barrier [18]. Much study into the antitumor mechanism of BCG has focused on the mononuclear infiltrate, whereas the role of the early granulocyte infiltrate has been largely ignored. From our analysis of leukocytes in the urine from patients after instillation of BCG, TRAIL is expressed on CD15⁺ PMN [19]. Our group is one of several to show that human PMN are a rich source of TRAIL [7,19–22], providing evidence that PMN have the potential to play an important role in the antitumor outcome of BCG therapy. In support of this, recent work by Suttman *et al.* suggests that PMN are essential for a positive outcome to BCG therapy in a mouse bladder tumor model [23]. They found that depletion of PMN eliminated the effect of BCG therapy, resulting in a reduction in survival compared to non-depleted controls. When stimulated with BCG *in vitro* PMN release IL-8, GRO- α , MIP-1 α , and MIF. Furthermore, using transwell assays it was determined that the BCG-induced chemokine release by PMN is sufficient to recruit M ϕ , which subsequently recruit T cells. Based on these findings, the authors suggest that BCG instillation results in the influx of PMN that orchestrate the subsequent M ϕ and T cell recruitment through the release of chemokines [23]. In their model, Suttman *et al.* propose that the BCG-induced antitumor response is mediated by activated T cells, whereas PMN act indirectly through recruitment of other immune cells. Although we agree that PMN likely release chemokines that induce the influx of other immune cells, our data suggests that PMN may also have a direct antitumor effect through the release of TRAIL into the bladder environment.

The idea of an antitumor role for PMN in BCG therapy is supported by recent evidence that 1) TRAIL is found in resting PMN, 2) functional surface bound and soluble TRAIL expression increases following IFN- α and - γ stimulation, and 3) high early IL-8 production is associated with a better clinical response to BCG [14,15,20,21,24]. In an effort to determine if *in vivo* TRAIL production by recruited PMN after BCG instillation in the bladder is mediated by exposure to complex cytokine milieu and/or due to direct stimulation by BCG, we examined PMN that were directly stimulated *in vitro* with BCG and/or IFN- α . Flow cytometry analysis revealed only a slight increase in surface-bound TRAIL was detectable on PMN after stimulation with BCG [19]. However, there was nearly a 4-fold increase in soluble TRAIL released into culture supernatants after PMN were stimulated with BCG compared to unstimulated PMN. The amount of soluble TRAIL released into supernatants increases over time, resulting in maximal accumulation after 24 h [19]. Interestingly, although IFN induced TRAIL mRNA synthesis in PMN, it did not stimulate TRAIL to be released from PMN in the absence of BCG stimulation [19]. These results demonstrate that BCG directly stimulates PMN to release TRAIL, further supporting a role of PMN in the BCG-induced antitumor response.

Since local side effects are common after intravesical BCG instillation, where approximately 5% of patients develop severe infections [25], future therapies that reduce the risk of serious infection would be desirable. However, there have been no significant improvements in the 30 years BCG has been used to treat bladder cancer. Based on our findings that BCG directly stimulates TRAIL release from PMN, we became interested in the mechanisms involved in the recognition of BCG by PMN. We compared the amount of TRAIL release by PMN when stimulated *in vitro* with either live or heat-killed BCG, demonstrating that the amount of TRAIL released from PMN was identical between live and heat-killed BCG [19]. This suggested that the surface cell wall components are a potential source of the TRAIL inducing activity, which was confirmed in later experiments that demonstrated the mycobacterial cell wall fraction is in fact a strong stimulus of TRAIL release from PMN [19]. These results are exciting because they suggest that non-viable BCG has equipotent activity as the viable BCG currently being used in the clinic. Furthermore, the identification of the stimulatory cell wall components may

have potential for improved future therapies for bladder cancer, by eliminating the risk of serious infections along with potentially increasing the potency of the treatment. Although previous studies have reported that viable BCG is necessary for effective therapy [12,25], presumably due to the requirement for fibronectin-mediated attachment and invasion that leads to an established infection sufficient for leukocytes recruitment, the use of specific non-viable preparations or cell wall components may have potential as a strategy for the development of future therapies. Indeed, various reports have highlighted the use of purified components of BCG as a potential noninfectious therapeutic alternative to live BCG therapy. The idea of using BCG cell wall extracts, instead of viable BCG, as a cancer immunotherapeutic has been known for over 30 years [26]. This concept has been advanced in recent years through the use of mycobacterial cell wall extracts that also contains short oligonucleotides derived from the mycobacterial DNA, which have demonstrated antitumor activity against a range of cancer cells. It is believed that the antitumor activity of these mycobacterial cell wall/DNA complexes is due to a direct apoptotic effect on the tumor cell and an indirect effect via the induction of immunostimulatory cytokines [27]. One can easily speculate that multiple TLR are being engaged by the cell wall extracts, which synergistically stimulate a multifaceted antitumor response.

Based on the potent activity of the cell wall fraction in our studies, we assessed the ability of individual cell wall components to stimulate TRAIL release from PMN. Our findings demonstrate that the cell wall proteins had the greatest potential for therapeutic use, inducing the highest amounts of TRAIL release from PMN activity as compared to other mycobacterial cell wall components [28]. Our results did not exclude the potential contribution of the individual lipids, but suggest that the strongest TRAIL-inducing stimuli present in the cell wall are proteins. In addition, we identified two candidates, alpha-crystallin and the Antigen85 complex, that each have significant TRAIL-inducing activity [28]. Furthermore, agonists of TLR2 and TLR4 induce TRAIL release from PMN [19], and cell wall proteins stimulate TLR2 expressing cell lines [28], suggesting that recognition of the cell wall components by TLR2 and TLR4 initiate signal cascades leading to TRAIL release by PMN.

In summary, recent findings suggest that PMN may play a key role in the antitumor mechanisms of BCG therapy. Findings from our laboratory demonstrate that PMN are a major source of TRAIL that is released through direct interactions with BCG, contributing to high levels of TRAIL in the bladder after BCG immunotherapy. Finally, our findings that the cell wall proteins of BCG possess potent TRAIL-inducing activity are exciting and provide potential candidates for improvements in the future treatment of bladder cancer.

Mechanisms of TRAIL release by PMN

The rapid release of TRAIL from BCG-stimulated PMN raises questions regarding underlying mechanisms involved in TRAIL secretion. Evidence from our laboratory has demonstrated that PMN possess intracellular stores of TRAIL that are released following BCG stimulation [19]. This intracellular source of TRAIL appears to be preformed, because PMN pretreated with either actinomycin D or cycloheximide prior to stimulation with BCG to inhibit new protein synthesis, release equal amounts of TRAIL into culture supernatants as unstimulated PMN [19].

PMN contain many intracellular granules: azurophilic granules, specific granules, gelatinase granules, and secretory vesicles. Our findings that PMN contain preformed intracellular stores of TRAIL suggest that TRAIL may be stored within one or more of these granule subtypes. Initial observations from our laboratory support this idea demonstrating that each of the granule populations isolated from Percoll gradients is positive for TRAIL [19]. In addition, Cassatella and coworkers have found high amounts of TRAIL in the plasma membrane and secretory vesicle fraction isolated from PMN primed with IFN [22]. IFN induces transcription of the

TRAIL gene, enhancing the amount of TRAIL protein stored by PMN, and augments TRAIL release from PMN stimulated with BCG [19,28]. Although the study by Cassatella *et al.* did not report significant amounts of TRAIL in the azurophilic, specific, and gelatinase granule fractions, it is possible that the amount of TRAIL in these fractions was underestimated due to the high amount of newly synthesized TRAIL present in the secretory vesicle and plasma membrane fraction. We have since repeated our initial fractionation studies using both resting and IFN-primed PMN (manuscript in revision), demonstrating that TRAIL was present in all granule populations as we previously reported [19]. In addition, we also found that IFN priming resulted in high amounts of TRAIL in the secretory vesicle and plasma membrane fraction, confirming the study by Cassatella *et al.*, with significant amounts of TRAIL still found in the other granule fractions. PMN-derived TRAIL is the soluble truncated form of the protein [19], similar to the type of TRAIL found in the urine of bladder cancer patients undergoing BCG therapy [7]. The presence of soluble TRAIL in the granule fraction is consistent with its rapid release into culture supernatants following stimulation with BCG and also the inability of actinomycin D and cycloheximide to inhibit TRAIL secretion. However, the findings that IFN treatment induces *de novo* synthesis of TRAIL [19] that accumulates in secretory vesicles [22], suggest that IFN present in the urine either during BCG therapy [29,30] or supplied exogenously [31] may augment the total amount of TRAIL released by PMN and contribute to the sustained secretion of TRAIL after preformed intracellular stores are expended.

The broad distribution of TRAIL in PMN is unexpected. Packaging of PMN proteins into granules is tightly regulated during maturation in the bone marrow, leading to targeted localization of proteins into distinct granule subtypes [32]. Our recent findings that TRAIL may be found in all the granule populations suggests that TRAIL may be uniquely expressed throughout PMN development, resulting in the broad granular distribution. We are currently investigating TRAIL expression during PMN maturation using myeloid cell lines as well as hematopoietic stem cells. In addition, the presence of soluble TRAIL in PMN is also an interesting observation and has stimulated studies examining the biosynthesis and packaging of TRAIL during PMN development and in response to IFN priming. These studies will provide interesting insights that may provide additional clinical strategies to enhance the response of patients to BCG therapy.

CONCLUDING REMARKS

Recent evidence suggests that PMN can act both directly and indirectly in the BCG-induced antitumor response through the release of TRAIL and chemokines. Based on these findings we propose the following model as a mechanism for the antitumor response that occurs during BCG therapy: 1) BCG instillation results in local infection of bladder epithelial cells inducing the release IL-8 and other inflammatory cytokines, 2) high levels of IL-8 recruit PMN into the bladder, 3) PMN become activated by BCG to release their intracellular stores of soluble TRAIL and release chemokines to recruit other immune cells to the inflamed bladder, 4) Activated monocytes produce more chemokines, including those produced by the PMN, which promote T cell migration into the bladder, 5) IFN produced by activated monocytes induces TRAIL expression on the surface of T cells and augments the amount of TRAIL released by PMN, 6) Accumulating levels of soluble TRAIL in the urine and TRAIL expression on lymphocytes induce apoptosis in bladder cancer cells. Furthermore, each subsequent cycle of BCG therapy results in a more rapid and sustained inflammatory response, leading to an overall effective clinical response to BCG immunotherapy.

Many questions regarding the antitumor mechanisms underlying BCG therapy remain, such as the kinetics of chemokine release, cell recruitment, and accumulation of TRAIL; the differences in responses to BCG therapy between patients who respond well to therapy and those that do not; and potential improvements to BCG therapy that result in more favorable

responses with less risk for serious complications. Recent contributions from several laboratories have provided initial insight into each of these issues, but ongoing studies are needed to further resolve these and other areas that need attention. In addition, the evidence that PMN may provide defense against cancer cells is compelling and suggests a role that is not conventionally assigned to these innate immune cells.

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Abbreviations

TRAIL	TNF-related apoptosis-inducing ligand
TNF	tumor necrosis factor
TLR	Toll-Like Receptor
IFN	Interferon
PMN	Polymorphonuclear leukocyte (neutrophil)
BCG	<i>Mycobacterium bovis</i> bacillus Calmette-Guérin
MPO	myeloperoxidase
LF	lactoferrin, G-CSF, granulocyte-colony stimulating factor

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