Review

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Bladder Cancer Medication Bacillus Calmette-Guérin-Cell Wall Skeleton Focusing on Alternatives and Developments to Limitations

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Bacillus Calmette-Guérin (BCG) serves as an anticancer drug for bladder cancer by enhancing the innate immune response and facilitating the expression of beta-defensin-2/-3. BCG is significantly more effective than other treatment modalities; however, it has limitations due to the nonspecific secretion of immune proteins such as interleukin-2 (IL-2) and IFN-γ, necessitating frequent injections that result in toxicity. The newly developed BCG-cell wall skeleton (BCG-CWS) is intended to address the non-specificity and the requirement for repeated treatments associated with BCG. BCG-CWS stimulates antigen-presenting cells by secreting cytokines such as IL-12, using an adjuvant to enhance the immune response and synergize with it to provoke a potent immune reaction. Nevertheless, BCG-CWS encounters issues related to cellular uptake due to the substantial molecular weight of the drug. To meet this challenge, various strategies such as the introduction of R8 protein, the liposome evaporated via an emulsified lipid method, and nanoparticle formulation have been employed which can enhance targeted drug delivery, though issues related to particle size remain unresolved. This paper aims to discuss future perspectives by examining the mechanisms and challenges of BCG-CWS.

Key Words Bladder cancer, Bacillus Calmette-Guérin-cell wall skeleton, Vaccine, Nanoparticles, Programmed death ligand 1

INTRODUCTION

Bladder cancer, a malignant tumor originating in the bladder, ranks as the ninth most common cancer globally. In Korea, bladder cancer constituted 1.9% of all cancer cases in 2020, with the incidence rate in males being 2.9%, approximately three times higher than the rate in females, which was 0.8% [1]. Bacillus Calmette-Guérin (BCG), a live attenuated strain of *Mycobacterium bovis*, is employed as a therapeutic agent for some malignancies such as bladder cancer. Upon injection into the bladder, BCG enhances the innate immune system, preventing its recognition as a pathogen. This activation results in the expression of β-defensins 2 and 3, leading to the destruction of BCG [2]. Consequently, bladder cancer treatment with the BCG vaccine offers distinct benefits, diverging from other therapies.

Nevertheless, BCG anticancer medications are associated with numerous adverse effects, including severe pain and bladder inflammation during urination. Dissemination of BCG to other body parts via blood vessels can provoke various complications. Moreover, BCG can induce nonspecific secretion of immune proteins such as interleukin (IL)-2 and IFN-γ. Continuous administration of BCG can also engender toxicity [3]. Thus, BCG anticancer medications are known to precipitate multiple adverse effects. Owing to these side effects, complete removal of bladder cancer is often unachievable, leading to frequent recurrence of the disease in many patients [3,4]. Additionally, the use of BCG anticancer drugs complicates internalization within host cells. Chemotherapy serves as an alternate therapeutic strategy. Recent research has identified the chemoresistance-motility (CrM) signature in bladder cancer patients, demonstrating that the effectiveness of BCG treatment and immuno-oncology therapies can be predicted based on specific gene expression profiles. The findings indicate that patients with a high CrM signature display reduced responsiveness to BCG immunotherapy due to

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enhanced mobility of cancer cells and active interactions with cancer-associated fibroblasts [5].

The BCG-cell wall skeleton (BCG-CWS) is being researched as a potential alternative for treating bladder cancer, aiming to address BCG limitations. CWS, designed to augment BCG, features a nano-scale promotive structure enveloped in a lipid bilayer. Furthermore, nanoparticles have been designed utilizing modified emulsified lipids (liposome evaporated via an emulsified lipids [LEELs]) to encapsulate BCG-CWS. The employment of a modified ribosome from cell penetrating peptide-1 (Pep-1) for encapsulation enhances the selectivity and efficacy of CWS in targeted bladder cancer therapy [6]. These findings indicate that BCG-CWS could represent a promising therapeutic alternative for bladder cancer. Furthermore in this context, it would be worthehile utilizing the CrM signature for combination with chemotherapy to enhance the clinical utility of BCG-CWS [5].

MECHANISMS OF ACTION AND LIMITATIONS OF THE BCG VACCINE

Mechanisms of BCG vaccines

The BCG vaccine is utilized in immunotherapy to enable immune cells within the human body to recognize and eliminate cancer cells [3]. When administered intravenously, BCG identifies bladder cancer cells via fibronectin (FN) and $\alpha 5\ \beta 1$ integrin receptors, which serve as conduits on the cellular surface. Fibroblast activation protein and FN located on the surface of the BCG cell wall can bind together, facilitating BCG's adherence to the bladder cell surface. The bladder lumen features a hydrophilic peptidoglycan layer which shields the bladder from microorganisms [7]. The BCG cell wall, bearing a negative charge, and the peptidoglycan layer, a component of the bladder's normal lumen, are similarly negatively charged. Consequently, adverse effects such as BCG expression in normal cells are minimized.

BCG accumulates near the bladder wall and traverses the GAG layer, the innermost layer of the bladder wall. Subsequent internalization occurs through the immune cells, including B cells and T cells, which interact with glycoproteins within the components of the extracellular matrix of bladder cancer, including BCG, FN, and integrins. Following internalization, BCG antigens are presented to CD4+ T cells. BCG is then processed within the body. Moreover, when BCG is introduced into the bladder, macrophages are activated through BCG's interaction with Toll-like receptor (TLR) 2 and TLR4. Urinary epithelial cells can initiate the production of cytokines and chemokines. These include IL-6, which is involved in the proliferation and antibody secretion by the B cell lineage, IL-8, which recruits neutrophils, and TNF-α that enhances macrophage activity (Fig. 1). Through this mechanism, macrophages secrete TNF-α [8].

Neutrophils, recruited by IL-8, can secrete TNF-related apoptosis-induced ligand (TRAIL), thus inducing apoptosis in bladder cancer cells and facilitating the elimination of bladder cancer through secreted cytokines that attract effector cells [9,10]. TRAIL can induce apoptosis in various tumor cells and is non-toxic to normal cells. The release of macrophages and neutrophils can activate natural killer (NK) cells and dendritic cells. The expression of TRAIL, subsequent to IFN-α and IFN-γ stimulation, is induced by T cells, NK cells, dendritic cells, and macrophages, contributing to the immune-mediated elimination of bladder cancer cells [11,12].

Perforin is the primary cytolytic molecule in follicular lymphocytes. Typically, CTLs can lyse cells through perforin or via the Fas-FasL interaction. However, NK cells predominantly employ a perforin-dependent pathway for tumor elimination. It has been postulated that the BCG vaccine may selectively target and eliminate bladder cancer cells [13]. It is likely that the BCG vaccine facilitates the elimination of bladder cancer cells through the aforementioned lysis mechanism which is attributable to its enhancement of the initial immune response.

Limitations of BCG vaccines

Although BCG can reduce the recurrence and progression rate of bladder cancer, it has side effects that may restrict a patient's adaptability to external environmental changes [14]. The most common side effects include aseptic cystitis, diuretic-induced complications, hematuria, and hypothermia, typically manifesting within 48 hours. As BCG treatment progresses and side effects intensify, strategies such as prolonging treatment duration and reducing dosage may be implemented. Furthermore, side effects such as granulomatous prostatitis, arthritis, and skin rashes may occur, albeit with a low incidence of 1%. These conditions might necessitate ongoing BCG therapy along with additional treatments [15]. Besides these side effects, limitations in its operational mechanism also exist. It is challenging for BCG to be internalized by the immune responses of host cells. MEK inhibitors can enhance the growth inhibition of BCG-treated cancer cells potentially by suppressing the release of antimicrobial peptide, conferring an improved uptake by host cells [5].

Therapeutic effect of BCG in bladder cancer

BCG immunotherapy is recognized as a crucial treatment for the prevention of progression and recurrence of bladder cancer. According to a meta-analysis by research team from European Organisation for Research and Treatment of Cancer in Belgium, the recurrence rate in non-muscle invasive bladder cancer patients receiving BCG treatment post-transurethral resection of bladder tumor was reduced by 32%, particularly a significant benefit noted in patients who maintained BCG treatment for over a year [16]. On the other hand, another study focused on enhancing the efficacy of BCG treatment [5]. According to this study, antimicrobial peptides,, secreted during the innate immune response, could attenuate the anticancer effects of BCG. The use of MEK inhibitors

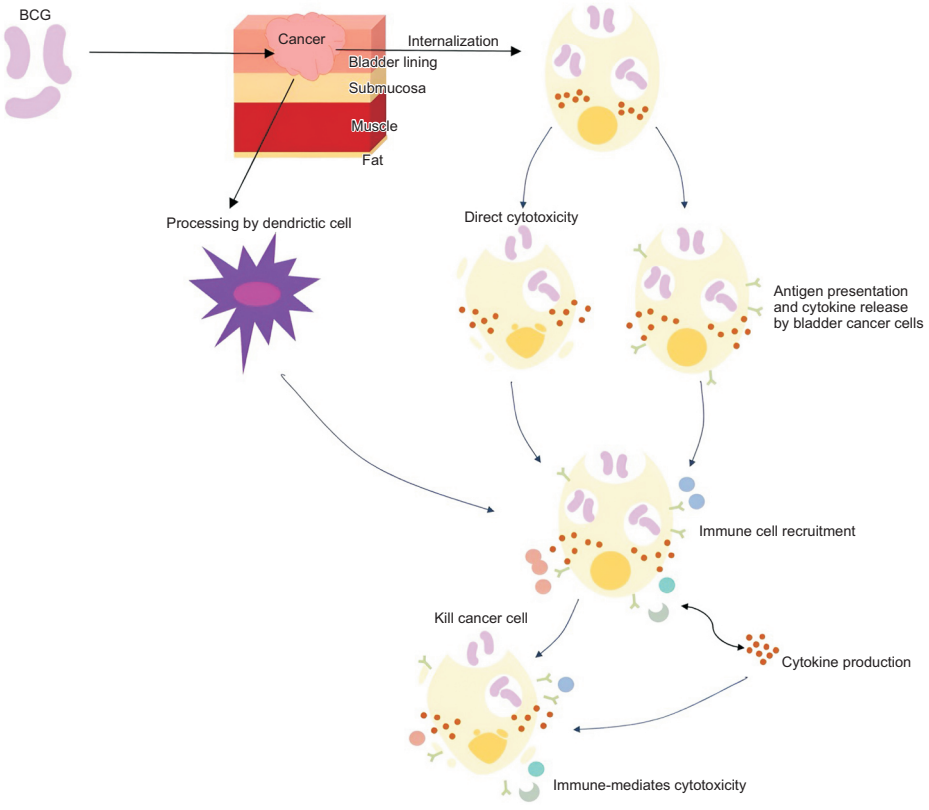

Figure 1. Action mechanism of BCG against bladder cancer. When BCG reaches the tumor-producing environment, it triggers two processes: 1) It attaches to the cell surface and immediately goes through the process of being endogenized by macrophages, which immediately leads to the death of cancer cells through phagocytosis. 2) Immune cells are recruited by bladder cancer cells. Then, it produces cytokines, which induce the proliferation of macrophages, and through sensitization of T cells, an immune response with antibodies takes place. This leads to the death of cancer cells. BCG, Bacillus Calmette-Guérin. Reproduced from the article of Redelman-Sidi et al. (Nat Rev Urol 2014;11:153-62) with original copyright holder's permission [8].

has proven to address this challenge [5]. These findings on BCG's efficacy in treating bladder cancer highlight the necessity for additional research to refine the therapy.

DEVELOPMENT OF BCG-CWS AS AN ALTERNATIVE OF BCG FOR TREATING BLADDER CANCER

Mechanism of action

BCG-CWS is a ligand of TLR2 and TLR4, which can activate monocytes to differentiate into macrophages and immature dendritic cells to mature antigen-presenting cells, thereby inducing potent innate immunity [17]. Additionally, BCG-CWS can produce proteins, such as TNF-α, IL-6, and IL-12, which are capable of inducing cellular immune responses and the cytokines that mediate them [18]. TNF-α production is partially inhibited in mouse macrophages lacking either TLR2 or TLR4, and completely inhibited in macrophages deficient in both TLR2 and TLR4. It is evident from this that BCG-CWS can induce the secretion of TNF-α from dendritic cells by activating TLR2 and TLR4. Secreted TNF-α can also promote the maturation of dendritic cells [19]. Furthermore, BCG-CWS exerts immunological effects by inducing apoptosis of cancer cells through activation of TLR2, enhancing antibody production with adjuvants, and boosting the proliferation of CTLs [3]. In the context of BCG, the addition of adjuvants can enhance the immune response compared to using the BCG vaccine alone. Adjuvants can stimulate cytokine secretion, such as IL-12, to activate antigen-presenting cells. Thus, BCG can be used in conjunction with adjuvants to elicit a more effective immune response [3].

However, recent studies have demonstrated that cancer-associated fibroblasts are more likely to be activated in the CrM-high group of patients, potentially inhibiting the immune effects of BCG-CWS. In particular, it has been shown that focal adhesion kinase, phosphoinositide 3-kinase-AKT, mitogen-activated protein kinase, and TGF-β signaling pathways are active in patients with a high CrM signature, and this heightened interaction with cancer-associated fibroblasts diminishes the efficacy of BCG therapy. Consequently, it is challenging to assert that treatment with BCG-CWS alone will be universally effective, necessitating the analysis of CrM signatures for patient selection and the development of combined therapeutic strategies [5]. Furthermore, recent studies propose that immune checkpoint proteins like programmed death ligand 1 (PD-L1) may facilitate immune evasion following BCG therapy [20]. PD-L1 interacts with CD8+ T cells to suppress their cytotoxic function, thereby diminishing the antitumor efficacy of the immune response [20]. Although BCG-CWS triggers a robust T-cell-mediated immune reaction, the potential upregulation of PD-L1 post-treatment could curtail its long-term efficacy. This indicates that targeting PD-L1-mediated immune suppression may be pivotal in optimizing BCG-CWS therapy.

Limitations of BCG-CWS

BCG-CWS, the primary immunoactive component of BCG, holds potential as a substitute for live BCG [21]. However, its clinical application is limited by several factors. Initially, BCG-CWS has a high molecular weight and is insoluble in both water-soluble and fat-soluble solvents [22]. BCG is administered via the Foley catheter during intracranial instillation procedures in bladder cancer patients. BCG-CWS exhibits significant insolubility. When constituents that are fat-soluble accumulate, they may obstruct the catheter during procedures. Consequently, a solvent to dissolve and a carrier solution to deliver it to bladder cells are necessary [3]. Secondly, formulating a suitable water-soluble drug with BCG-CWS is extremely challenging without causing aggregation. The capacity of cancer cells to uptake BCG-CWS is notably low. Forced dispersion of BCG-CWS in animal and human studies typically employs oil-in-water (O/W) emulsions containing detergents [22]. However, emulsified BCG-CWS induces intense inflammation upon administration in animals and humans, leading to severe local toxic side effects. Therefore, its application is confined to intracutaneous injections [3,23]. The size of BCG-CWS is influenced by the polarity of the solvent. A formula for BCG-CWS, dissolved via O/W emulsification, poses significant application challenges. Consequently, reducing the size of BCG-CWS is essential [3].

The primary purpose of employing a drug delivery system for anticancer agents is to target only the cancerous areas without harming the adjacent normal tissues or cells. For the live vaccine BCG, it is administered so that it undergoes intracellular internalization through bladder wall FN-mediated binding, owing to its antigen-targeting capability [24]. Nevertheless, BCG-CWS, which consists only of the cell wall, is limited by its inability teo penetrate the cell wall when injected into bladder tissues. Hence, the development of a drug delivery system that allows BCG-CWS to enter cells and achieve penetration into bladder tissue through cancer cell binding—ensuring uniform distribution of BCG-CWS in vivo and enhancing anti-cancer immune responsiveness—is necessary. In summary, BCG-CWS faces the challenge that an optimal drug delivery system needs to be engineered to internalize BCG-CWS into cancer cells by converting BCG-CWS into nanoparticles and using a suspension to augment solubility [3].

Another potential limitation of BCG-CWS is its vulnerability to the immune escape mechanism mediated by PD-L1. PD-L1 expression escalates in a subset of tumors that do not respond to BCG treatment (from 7% in BCG-naïve tumors to 14% in recurrences) [20], suggesting that BCG-CWS may also be susceptible to a similar mechanism of immune evasion over time, especially in recurrent cases. Elevated PD-L1 expression post-treatment may decrease the potency of cytotoxic T-cell responses, thereby restricting the sustainable therapeutic impact of BCG-CWS. Consequently, integrating BCG-CWS with immune checkpoint inhibitors (anti-PD-L1 therapy) could represent a viable approach to maintain antitumor immunity and enhance clinical outcomes [20].

IMPROVED FORMULATIONS OF BCG-CWS FOR BETTER BIOAVAILABILITY

BCG-CWS has a very large molecular weight, which hampers its efficacy in certain contexts. To address this issue, R8-liposomes, LEEL, and nanoparticles have been proposed. Initially, R8-liposomes were introduced and transformed into CWS to improve cellular transport in the bladder. Developed originally for conveying negatively charged DNA molecules via macropinocytosis into the cytoplasm, experiments with mice demonstrated the efficacy of R8-liposome-BCG-CWS in inhibiting bladder tumor growth. Mice injected with R8 into the bladder exhibited greater growth inhibition compared to control mice, indicating that R8-liposome-BCG-CWS can effectively suppress the development of bladder cancer [25]. Subsequently, Nakamura et al. [26] developed a nanoparticle technique through LEEL (Fig. 2) utilizing an organic solvent that enhances the formation of condensed nano-sized formulations at the core of lipid vesicles [27]. A modified LEEL method was used for the novel encapsulation of BCG-CWS in liposomes, and surface functionalization was achieved using FA and Pep-1. FA- and Pep-1-modified liposomes encapsulating BCG-CWS have shown high target selectivity and effectiveness in treating bladder cancer [6].

Recent studies have further validated the LEEL method for increasing the bioavailability of BCG-CWS. This method, which involves LEEL-based liposomal encapsulation, improves stability and dispersibility while significantly enhancing

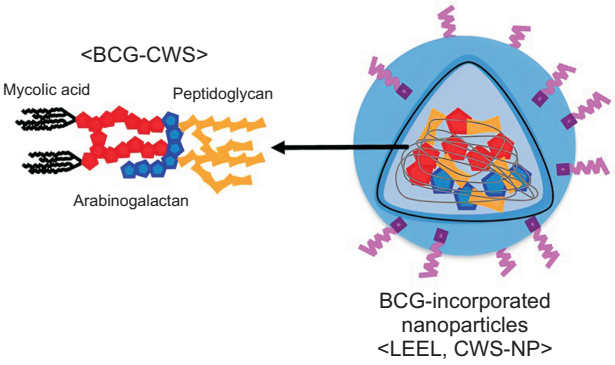

Figure 2. BCG-incorporated NP (CWS-NP/LEEL). For vascular injection, BCG-CWS undergoes nanoparticulation to form CWS-NP, wherein Mycolic acid, Arabinogalactan, and Peptidoglycan of BCG-CWS aggregate to create modified BCG-CWS, which then fuses with R8-modified ribosomes to form LEEL and CWS-NP. NP based on BCG-CWS, functionalized on their surface, exhibit enhanced intracellular delivery efficiency, enabling effective targeting in bladder cancer therapy. BCG, Bacillus Calmette-Guérin; CWS, cell wall skeleton; NP, nanoparticles; LEEL, liposome evaporated via an emulsified lipid. Reproduced from the article of Nakamura et al. (J Control Release 2014;176:44-53) with original copyright holder's permission [27].

tumor-suppressive effects via activation of the AMP-activated protein kinase (AMPK) pathway. This strategy has proven effective in orthotopic bladder cancer xenograft models, demonstrating its efficacy in tumor inhibition and targeted drug delivery. Nanoparticles developed through this approach exhibit improved dispersibility, stability, and size compatibility as functionalized liposomal delivery systems. Importantly, they also initiate various stress responses, which contribute to the inhibition of mTOR and activation of AMPK. These advancements underscore the potential of the BCG-CWS delivery system, utilizing the LEEL method, to efficiently internalize CWS through intravesical instillation in bladder cancer treatment, positioning it as a viable and clinically applicable therapeutic strategy [28]. In addition, nanoparticles designed for encapsulation were created to complement the large size and high molecular weight of CWS using liposomes processed through the LEEL method. As CWS-nanoparticles is readily dispersible, it boasts a wide application range and has demonstrated anticancer effects in bladder cancer treatment. For CWS-nanoparticles, employing a nanoparticles formulation containing BCG-CWS facilitates administration via an intravenous injection route to elicit antigen-specific CTL responses. Consequently, nanoparticles demonstrate significant potential as therapeutic agents for bladder cancer [22,29].

CONCLUSION

Immunization with BCG demonstrates a notable anticancer effect on bladder cancer compared to chemical anticancer agents, with few associated side effects. Nonetheless, BCG is limited in treating bladder cancer due to ineffective drug activity arising from its particle size. BCG-CWS could address these challenges, showing therapeutic effects on bladder cancer when combined with an adjuvant to induce an immune response [3]. However, BCG-CWS possesses hydrophobic properties, which can inhibit drug dispersion into cells by promoting the formation of large aggregates in a hydrophobic environment [25]. To overcome these limitations, R8-liposomes were employed. These liposomes attach to the surface of mouse bladder tumor line-2 cells and are effectively internalized within the cytoplasm [25,30]. Furthermore, the liposomes were encapsulated using the LEEL method, and BCG-CWS was converted into nano-sized particles through this approach [31]. Using an ultrasonic shredder, the particle sizes were reduced to less than 160 nm, allowing encapsulation and intravenous delivery of reduced BCG-CWS nanoparticles to facilitate antigen-specific actions [24]. Despite these advancements, the issue of particle size remains unresolved, and clinical studies are ongoing. The development of a replacement therapy for BCG-CWS is anticipated to be a protracted process. Overcoming the penetration barriers posed by particle size in bladder cancer tissue remains a significant challenge, but success could lead to safer treatment modalities.

To further optimize BCG-CWS therapy, future research should assess the role of PD-L1 expression in immune evasion post-treatment. Although PD-L1 is not an absolute biomarker for BCG responsiveness, its elevated expression in recurrent tumors implies a role in immune [28]. Combining PD-L1 inhibitors with BCG-CWS therapy could maintain prolonged immune and mitigate immune evasion. Moreover, integrating nanoparticle-based delivery systems (e.g., R8-liposomes, LEEL) with immune checkpoint blockade could forge an innovative path to enhance therapeutic efficacy. Thus, devising a comprehensive strategy that merges optimized drug delivery with immune checkpoint modulation could considerably enhance the clinical application of BCG-CWS as a bladder cancer immunotherapy. Additionally, the efficacy of BCG and BCG-CWS may be limited in patients with high CrM signatures, and activation of cancer-associated fibroblasts could facilitate immune avoidance. Hence, patient screening based on CrM signatures and evaluating the efficacy of combination therapies are crucial to maximize the clinical benefits of BCG-CWS. Future research will necessitate studies to predict the efficacy of BCG-CWS through CrM signature analysis and to optimize combinatory strategies with chemotherapy (doraxel, nafabucacin, etc.). It is anticipated that this will facilitate the development of effective bladder cancer treatments in the CrM-high group as well [28].

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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