Interactions of BCG with urothelial tumor cells in immunotherapy for superficial bladder cancer
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BCG internalization in human bladder cancer cell lines, especially with regard to cell surface expressed fibronectin


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Abstract

Purpose. Several lines of evidence suggest a functional role of urothelial (tumor) cells in the bacillus Calmette-Guérin (BCG) immunotherapy for superficial bladder cancer. In this study the interaction between BCG organisms and a series of human transitional bladder cancer (TCC) cell lines was addressed, focusing on the role of cell-associated fibronectin (FN) in BCG internalization.

Materials and Methods. FN expression, and BCG attachment and internalization were evaluated in a series of human TCC cell lines. Effects of anti-FN antibodies (αFN) on cell-associated BCG were studied in the human TCC cell line T24.
Results. In contrast to the well-differentiated cell lines RT-4, SBC-2 and SBC-7, fibronectin expression was high in the poorly differentiated cell lines T24, TCC-SUP and J82.

A correlation between FN expression and the capacity to internalize BCG was observed. The possibility of causal relationship between FN expression and BCG internalization was studied in detail with T24 cells. The results showed that pre-incubation of T24 cells with αFN did not prevent the internalization of BCG.

Conclusions. Cell membrane-expressed fibronectin does not seem to be crucially involved in the internalization of BCG by TCC cells. A correlation between cellular fibronectin expression and the ability of TCC cells to internalize BCG may be considered as a fortuitous coincidence.

Introduction

Local immunotherapy with BCG has been shown in numerous studies to be superior to surgery alone and is the most successfully applied modality in the superficial bladder carcinoma prophylaxis and treatment of carcinoma in situ [1-3]. Accumulating evidence indicates that BCG-mediated antitumor activity underlies an immune response against both BCG and the tumor [4] although the exact mechanism remains to be established [4,5]. For clinical efficacy several initial steps comprehending the first interaction of BCG with the bladder wall, prior to initiation of the local immune response, could be crucially involved. These primary, possibly initiating and/or modulating reactions, may be BCG internalization by bladder (carcinoma) cells followed by an upregulation of the synthesis of certain cytokines and BCG antigen presentation [4,6]. Attachment and internalization of BCG by bladder tumor cells have been reported [6-8]. Ingestion of mycobacteria both in vitro and in vivo by other non-professional phagocytic cells has also been reported [9]. Although an essential role of FN in the attachment and internalization of BCG by urothelial cells has been noted by Kuroda et al. [10], several studies suggest a FN-independent binding of BCG [6,8,11].

Increase of cytokines in the urine of patients during intravesical BCG treatment has been reported extensively [12-17], but the cellular source of several of these cytokines is not conclusively determined. Epithelial cells including urothelial cells possess the capacity to synthesize cytokines [6,18-21] and BCG organisms appear to upregulate cytokine synthesis of tumor urothelial cells grown in vitro, such as IL-6 and others [6,18,19]. In patients IL-6 (and other cytokine) levels in urine following BCG instillation appeared
to be related to the onset of the BCG response and/or the clinical BCG antitumor response [14,15].

In this study, using a series of TCC cell lines, the hypothesis was tested that cell surface-associated FN expression of bladder tumor cells is a causal factor for BCG internalization.

Material and method

_Urothelial tumor cells, culture conditions and BCG preparation_

Stock cultures of a series of cell lines derived from human bladder carcinomas with a different degree of differentiation, RT-4, SBC-2 and SBC-7 (grade 1), and T24, TCC-SUP and J82 (grade 3) were cultured as described previously [6]. For BCG attachment and internalization experiments the cells were incubated with BCG Connaught (Pasteur Mérieux Connaught, North York, Ontario, Canada). Prior to incubation, large clumps of bacteria were eliminated by reconstitution of freeze-dried BCG (3.2 x 10^8 CFU per vial) in 10 ml pre-warmed DMEM, followed by centrifugation (300 g; 3 min). The bacteria remaining in the top 5 ml were used for experiments [6].

_Assay of cell surface-associated fibronectin expression_

Cells were harvested from culture flasks with cell dissociation solution (Sigma, St Louis, MO), washed and incubated with 1:100 dilution of rabbit polyclonal anti-human fibronectin antibodies (aFN) (Dako Corp., Santa Barbara, CA). Subsequently, cells were stained with phycoerythrin (PE) conjugated goat anti-rabbit antibodies (1:25, Sigma, St Louis, MO). FN expression was expressed as the median fluorescence intensity, analyzing 10^4 cells (FI/10^4 cells) by a fluorescence activated cell sorter (FACScan, Becton Dickinson Immunocytometry Systems, Mountain View, CA).

_BCG attachment / internalization assay_

BCG attachment and internalization in the TCC cell lines were determined by double fluorescent flow cytometry as described previously [22]. The method consisted of labeling BCG bacteria chemically with fluorescein isothiocyanate (FITC). Subsequent to incubation of TCC cells with fluoresceinolated BCG bacteria, cell surface attached BCG bacteria were marked by two-step labeling with polyclonal rabbit anti-BCG antibodies (aBCG) (1: 100; Dakopatts, Denmark) and phycoerythrin (PE) conjugated goat anti-rabbit antibodies (1: 25). Double fluorescent flow cytometry
(FACScan) differentiated between FITC "stained" cells (FITC⁺), due to extra- and/or intracellular FITC-labeled BCG organisms, as well as PE "stained" cells (PE⁺), due to extracellular PE-labeled organisms. FITC⁺/PE⁺ cells were considered as cells containing intracellular or internalized (but no extracellular) BCG organisms.

Inhibition studies of BCG internalization
Rabbit polyclonal anti-human fibronectin antibodies (αFN) (Dako Corp., Santa Barbara, CA) were added to the urothelial tumor cells 30 min prior to the BCG internalization assay. To exclude an effect of possible aspecific attachment of BCG to the Fc portion of αFN, inhibition experiments were also performed with goat anti-human FN F(ab)'2 fragments (Organon Teknika, West Chester, PA).

Results

BCG internalization and fibronectin expression of bladder cancer cell lines.
Previously it was shown that, for T24 cells, BCG internalization was both dose- and time-dependent [22]. Based on these data, experiments in the present study were performed under conditions of a 4-h incubation period with 10 CFU BCG/cell. As depicted in Fig. 1, the capability of BCG internalization in a series of human TCC cells appeared to be cell line- and grade dependent. In the well-differentiated cell lines RT-4, SBC-2 and SBC-7, the proportion of cells containing internalized BCG varied from 0 to 2 %, whereas in the poorly differentiated cells T24, TCC-SUP and J82, the percentage of cells containing internalized BCG ranged from 34 to 68 %.
Comparing the level of cell surface-expressed FN with BCG internalizing capability indicated a correlation between FN expression and internalization of BCG. A virtual absence of FN expression (less than 5 Fl/10⁴ cells) was found in the well differentiated, BCG non-internalizing cell lines, whereas the poorly differentiated, BCG internalizing cell lines expressed FN to a considerable extent (30 to 60 Fl/10⁴ cells) (Fig. 1).

Relationship between fibronectin expression and BCG internalization
In a subsequent series of experiments the possibility of a causal role of cell surface-expressed FN and BCG internalization was studied by incubating TCC cells in the presence of rabbit polyclonal anti-human fibronectin antibodies (αFN). Pre-incubation of T24 cells, which exhibit a high
Figure 1. Fibronectin, and BCG attachment and internalization by a series of well and poorly differentiated human bladder cancer cell lines. Cell surface-expressed FN was expressed as fluorescence per $10^4$ cells (FI/$10^4$ cells) (left panel). The percentage of BCG internalizing cells (right panel) was determined after a 4-h incubation with (FITC-labeled) BCG (10 CFU/cell) [22]. Each value represents the mean of duplicate cultures; the SD is less than 10%. RT-4, SBC-2 and SBC-7 are well differentiated, grade 1 cell lines, while T24, TCC-SUP and J82 are poorly differentiated, grade 3 cell lines.

capability to internalize BCG, with αFN for 30 min prior to BCG addition did not reveal a decrease of the proportion of cells associated with BCG, attached and/or internalized, FITC$^+$ cells (Fig. 2). Binding of αFN to T24 cells under this condition was confirmed by FACS analysis (data not shown). Also, no inhibitory effect on the proportion of BCG-associated cells was found after pre-incubation (30 min) of T24 cells with αFN F(ab)$_2$ fragments prior to BCG addition. The latter observation indicated that the absence of an effect of the polyclonal αFN was not due to aspecific binding of the Fc portions of αFN. In contrast, addition of BCG, pre-incubated with rabbit polyclonal αBCG antibodies for 30 min, resulted in a significant
Figure 2. Effect of rabbit polyclonal anti-human fibronectin antibodies (αFN) and goat anti-human FN F(ab)$_2$ fragments on BCG internalization and/or attachment by T24 cells. Prior to incubation of T24 cells with BCG-FITC (10 CFU/cell) for 4 h, the epithelial cells were pre-incubated with αFN or αFN F(ab)$_2$ fragments at two concentrations. Subsequently, the proportion of T24 cells with cell-associated BCG (FITC$^+$) was determined with FACS analysis. It should be noted that the BCG attachment/internalization assay and the particularly applied αFN do not allow differentiation between cells with internalized BCG (FITC$^+$/PE$^-$) and cells associated with BCG (attached and/or internalized) (FITC$^+$/PE$^+$).

Inhibition of the percentage of T24 cells with attached and/or internalized BCG (1:100, 78%, $p=0.045$, 1:25, 30%, $p=0.003$). These results indicate that fibronectin expressed on the cell membrane of urothelial tumor cells does not attribute to BCG internalization.

Discussion

Current insights suggest the response of epithelial cells, such as upregulation of cytokine production, to a microbial challenge as an integral property of the immune reaction [20,21]. Recently we showed that upregulation of
cytokine (IL-6) synthesis by TCC cells grown in vitro in the presence of BCG depends on the capability to internalize BCG [6]. It is tempting to speculate that internalization of BCG, followed by an upregulation of the synthesis of certain cytokines by TCC cells, may play an initiating or modulating role in the therapeutic and prophylactic action of BCG [5, 6, 18]. Accepting such a hypothesis implies that studies addressing the mechanism of BCG attachment and internalization could be of clinical relevance.

The present study provides evidence that BCG internalization is specifically associated with high grade or poorly differentiated TCC cell lines grown in vitro. This observation agrees with the results reported by Schneider et al. [8], although these investigators did not clearly discriminate between cell surface-attached BCG and internalized BCG. Along with the previously reported BCG-induced upregulation of cytokine production of cells grown in vitro [6], these data support the hypothesis that (tumor) urothelial cells may be a significant source of (BCG-induced) urinary cytokines, additional to major cellular sources, such as macrophages and lymphocytes infiltrating the bladder wall. Furthermore, clinically these data may be related to observations showing a better response to BCG treatment of high-grade tumors compared to lower grade tumors [23].

The correlation between the level of cell surface-expressed FN and BCG internalization, as observed by comparing a series of TCC cell lines suggests an involvement of FN in the internalization of BCG and seems to be in agreement with observations described by Kuroda et al. [10]. These investigators suggested that BCG internalization depends on fibronectin and can be inhibited by αFN. However, in contrast to Kuroda et al., using a different BCG internalization assay, but identical polyclonal FN antibodies, we were not able to detect an inhibitory effect of αFN on BCG internalization by T24 cells. An absence of an inhibiting effect of αFN on BCG attachment/ internalization by TCC cells has been reported by other investigators too [8, 11]. All together, the current available data suggest no mandatory role of FN in BCG attachment and subsequent internalization by bladder carcinoma cells grown in vitro. It can be speculated that BCG internalization depends on yet unknown molecules co-expressed with fibronectin.

In conclusion, the data reported herein show an enhanced, not fibronectin-mediated internalization of BCG by poorly differentiated, high-grade tumor cells in vitro. BCG internalization by (tumor) urothelial cells may be part of the mode of action of intravesical BCG immunotherapy for superficial
bladder cancer, although the role of this phenomenon in vivo remains to be established.

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