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Research Paper

Preparation and in-vitro bioactivity of a novel superantigen conjugate targeting bladder carcinoma

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Abstract

Objectives Superantigens have shown potent effects against bladder tumours by inducing Vβ-specific T-lymphocyte proliferation and massive cytokine release but therapeutic benefit is compromised by cytotoxicity towards non-malignant cells and hypotoxicity to major histocompability complex (MHC) II-negative tumour cells. We are therefore interested in a conjugate preparation of a monoclonal antibody (MAb)–superantigens conjugate for which these drawbacks would be resolved.

Methods The Fab fragment of the anti-bladder carcinoma MAb BDI-1 was conjugated to one member of the staphylococcal enterotoxin A (SEA) superantigen using the chemical conjugating reagent, *N*-succinimidyl 3-(2-pyridyldithio) propionate.

Results After HPLC purification through a Superdex-200 gel column, another peak with a molecular mass of 250 KDa was observed before Fab and SEA were eluted. Indirect immunocytochemical analysis and immunofluorescence tests showed that the cell membranes of most human bladder cancer cells were positively stained only by the conjugate, confirming the ability of the conjugate to target human bladder carcinoma. Peripheral blood mononuclear cell proliferation and cytokine release were similar with the conjugate and SEA. Cytotoxicity targeting in MHC II-negative bladder cancer cell lines, evaluated by flow cytometry, showed significant differences between the conjugate and SEA, whereas there was no difference in the Lovo colon cancer cell line.

Conclusions These findings indicate the conjugate of SEA protein and BDI-1 Fab fragment was prepared successfully and targeted bladder carcinoma *in vitro*.

Keywords bladder carcinoma; immunotherapy; monoclonal antibodies; staphylococcal enterotoxin A; superantigens

Introduction

Bladder cancer is one of the most common genitourinary cancers, 70–80% of which are non-muscle invasive bladder cancer (NMIBC), characterised by repeated recurrences and/or progression. Standard treatment is transurethral resection (TUR) followed by adjuvant intravesical instillation of chemotherapeutic or immunotherapeutic agents, including mitomycin C, adriamycin and bacille Calmette-Guérin (BCG). However, recurrences still occur with current adjuvant treatments, and they have minimum influence on progression.[1–3] These treatments also induce local or systemic side-effects such as bladder disorders, fever and single cases of miliary tuberculosis, as well as deaths.[4–6] Thus, the current treatments either result in an unsatisfactory high local failure rate or adversely affect patients' quality of life. A novel approach to the intravesical therapy of NMIBC is therefore needed.

As with BCG, superantigens (SAgs) are potent T-cell stimulators but have a unique ability to interact simultaneously with major histocompatibility complex (MHC) class II molecules and the T-cell receptor (TCR) Vβ domain, forming a trimolecular complex that induces profound proliferation of T-cells which express TCR Vβ and massive cytokine release. This in turn directly suppresses tumour growth (resulting from released cytokines) and MHC-II-restricted cytotoxicity (resulting from activated T-cells) *in vivo* and *in vitro*.[7–9]

Recently, SAgs such as staphylococcal enterotoxin A (SEA) have been shown to be effective against bladder carcinoma *in vitro*[10,11], *in vivo*[10] and in intravesical studies.[12]

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However, only a minority of human bladder cancer cells express MHC II,[13,14] and activated T-cells, as well as produced cytokines, are also expected to cause severe toxicity to non-malignant cells, particularly those that express MHC II. Thus, a single application of SAgs did not produce a satisfactory therapeutic effect in the treatment of bladder carcinoma.

Recent studies in lymphatic leukaemia and colon cancer suggest that monoclonal antibody (MAb)-targeted SAgs may not exhibit these limitations.[15,16] We therefore believe that a MAb–SEA-based conjugate may represent a more powerful and acceptable approach to intravesical therapy of bladder carcinoma that may have significant advantages compared with single SAg-based strategies.

Materials and Methods

Reagents

SEA protein was purchased from the Academy of Military Medical Sciences (Beijing, China). BDI-1, a MAb reacting with human bladder carcinoma, was obtained from Jiuyuan Gene Engineering Co. (Hangzhou, China). Papain, dithiothreitol (DTT), *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), rabbit MAb against SEA, and 7-aminoactinomycin D (7-AAD) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Protein standards were purchased from Fermentas Co. (Glen Burnie, MD, USA). The Cell Counting Kit-8 (CCK-8) and ELISA kit were obtained from Dojindo Co. (Kumamoto, Japan) and ADL Co. (San Diego, CA, USA), respectively. Mouse MAb against MHC II (HLA-DR) was purchased from BD Biosciences Co. (San Jose, CA, USA). 5- and 6-Carboxy fluorescein diacetate succinimidyl ester (CFSE) was purchased from Molecular Probes (Eugene, OR, USA).

Cell lines

The human colorectal cancer cell line Lovo, and the human bladder carcinomas BIU-87, T-24 and E-J were obtained from the cell bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco).

Production and purification of the Fab fragment of MAb BDI-1

The MAb BDI-1 IgG was digested with papain (10 μg papain per mg MAb) in 0.02 M sodium phosphate buffer, pH 7.2, containing 20 μM β-mercaptoethanol at 37°C for 3 h. The papain was inactivated with iodoacetamide and the digestion mixture was extensively dialysed at 4°C versus 20 mM sodium phosphate buffer, pH 7.2. It was then applied to a Protein G affinity column (HiTrap Protein G 1 ml; Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer for purification. The Fab fragments were eluted earlier in the flow and concentrated by ultrafiltration through an UF centrifuge tube (Sartorius, Goettingen, Germany) while the Fc portion was retained and was eluted later in 0.1 M Tris-HC1, pH 2.7 buffer.[17]

Conjugation of MAb BDI-1Fab to SEA

Briefly, the principle used to conjugate MAb BDI-1 Fab to SEA involved activation of the amino group with SPDP, followed by disulfide exchange.[18] For activation, SEA protein (0.24 mg) and MAb BDI-1 Fab (1.6 mg), purified by HPLC (Agilent 1100, Santa Clara, CA, USA) through Superdex-200 (Pharmacia Biotech), were dissolved in 0.1 M Bicine buffer solution, pH 8.5 and reacted with SPDP for 30 min at a molar ratio of SEA protein : MAb BDI-1 Fab of 6 : 10. After dialysis in 0.02 M acetate buffer, pH 4.5, to remove excess SPDP, followed by concentration by ultrafiltration, the activated SEA was reduced by 20 μmol DTT for 30 min. The conjugation of reduced SEA with activated BDI-1 Fab, both of which were dialysed and ultrafiltered again, was done for 24 h under nitrogen using a 5 : 1 molar ratio. Finally, solutions were purified by HPLC through Superdex-200 and tested by the assays described below.

SDS-PAGE analysis

Fractions of products before and after purification were pooled and analysed by SDS-PAGE using a discontinuous buffer with 8% gel gradient and Coomassie brilliant blue staining.[19]

Protein assay

Total protein concentration of the collected product was determined using a bicinchoninic acid protein assay kit (KeyGen Biotech, Nanjing, China) and the final results were expressed as mg/ml.[20]

Targeting assay

The targeting selectivity of the conjugate to human bladder carcinoma was identified by immunocytochemical analysis and immunofluorescence.[21] Briefly, BIU-87, T-24 and E-J cells were cultured in RPMI 1640 medium at 37°C in Petri dishes containing some coverslips until the cells grew into log phase. After fixation in ice-cold acetone, the coverslips were sequentially stained with conjugate and rabbit MAb against SEA, followed by biotinylated or FITC goat anti-rabbit IgG. SEA and phosphate-buffered saline (PBS) were used as conjugate controls. Lovo cells were used as controls for bladder carcinoma cells.

For immunocytochemical analysis, colour was developed with a DAB kit (KeyGen Biotech) and counterstained with haematoxylin. The slides were photographed by standard light microscopy or by fluorescence microscopy.

Proliferation assay

Proliferation of lymphocytes was followed by CCK-8 assay, as described previously.[22] Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by density-gradient centrifugation using lymphocyte separation medium (TBD, Tianjing, China) and aliquoted into 96-well plates at 2×10^5 cells per well in RPMI 1640 supplemented with 10% FBS. A series of concentrations of SEA or conjugate were added to the wells (six wells for each agent); 10% FBS was used as a negative control. After 72 h' incubation at 37°C in 5% CO_2, 10 μl CCK-8 solution was added to each well and the incubation continued for 4 h. Absorbance was measured

at 450 nm using a microplate reader. The proliferation effect was reported as a proliferation index (PI), PI = absorbance value in experimental groups/absorbance value in control groups.

Cytokine assays

Culture supernatants were collected after stimulation with SEA or conjugate and centrifuged once to remove particulate material. Interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumour necrosis factor α (TNF-α) were assayed using commercial ELISA kits.

MHC II expression assay

Expression of MHC II (HLA-DR) on BIU-87, T-24, E-J and Lovo cells was examined using immunofluorescence flow cytometry. Cells (1×10^6 per line) collected from cultures were washed in PBS and resuspended in binding buffer. After labelling with optimal concentrations of a FITC-conjugated mouse anti-HLA-DR MAb at 4°C for 20 min, cells were analysed by flow cytometry using Cell Queste software (Becton Dickinson). An irrelevant isotype-matched antibody (FITC mouse IgG2a; BD Biosciences) was used as a negative control in each labelling experiment. Expression was quantified by comparison with control staining of matched samples.

Cytotoxicity assay

Cytotoxicity was measured at a 10 : 1 effector : target cell ratio using a flow cytometric CFSE/7-AAD cytotoxicity assay, and was expressed as % specific lysis = ([$CFSE^+7 - AAD^+$]/$CFSE^+$) $\times$ 100.[23,24] The effector PBMC at a density of 6×10^5 cells per well were pre-incubated with SEA or conjugate at various concentrations in six-well plates for 48 h and co-cultured with BIU-87, T-24, E-J or Lovo cells labelled with 1 μM CFSE. After 24 h, cells were harvested from cultures and washed in PBS. Cells were then resuspended in binding buffer containing 20 μg/ml 7-AAD and incubated in the dark at 4°C for 20 min. Acquisition was analysed immediately by flow cytometry. All experiments were performed in triplicate.

Statistical analysis

Data are presented as mean ± SD. The Kruskal–Wallis test or one-way analysis of variance (ANOVA) followed by Dunn's test were used to assess the significance of differences in stimulating bioactivity. Two-way ANOVA followed by Tukey's test was also used to determine the significance of differences in cytotoxicity.[25] Statistical comparisons were performed using R2.7.2 for Windows (www.r-project.org). A P value of less than 0.05 was considered statistically significant.

Results

Purification and identification of the conjugate

Using a protein G affinity column and SPDP, the Fab fragment was successfully purified and conjugated to SEA. During the HPLC purification with Superdex-200, another peak was observed before the Fab and SEA eluted. The fractions corresponding to these peaks were analysed by SDS-PAGE, which showed that the first peak collected was the desired conjugate with a molecular weight of approximately 250 KDa and excellent purity; the second and third peaks collected were unconjugated Fab and SEA (Figure 1). The concentration of the conjugate was 0.18 mg/ml. The number of BDI-1 Fab molecules per SEA was 4–5 on average, as calculated in the HPLC profile. The dose of conjugate to be used in proliferation and cytotoxicity experiments was calculated according to the total amount of SEA.

Anchoring of the conjugate to bladder carcinoma cells

Conjugates were dissolved in human urine in order to determine the stability of conjugate anchoring to the cell membrane. Indirect immunocytochemical analysis indicated that most bladder cancer cells (BIU-87) in the conjugate experimental group had a brown positive signal located mainly on the cell membrane, whereas no detectable positive signal was discerned in the control group, which included Lovo colorectal cancer cells. Similarly, the immunofluorescence test demonstrated that the BIU-87, T-24 and E-J bladder cancer cell lines were mostly positively stained, whereas the Lovo cells were negatively stained. Both results indicate that the conjugate had achieved the targeted activity by anchoring only to bladder carcinoma (data not shown).

Conjugate-stimulated lymphocyte proliferation

The specialty of proliferation was quantified following stimulation of PBMC cultures with conjugate or SEA; FBS was used as the negative control. Significant proliferation of PBMC was observed when compared with the negative control, suggesting that the conjugate could stimulate lymphocyte proliferation *in vitro*. Although T-cell proliferation with the conjugate was similar to that with SEA, the difference between 3.6×10^{-4} nM conjugate and 3.6×10^{-1} nM SEA was significant, which indicated that the conjugate had reduced potency in terms of proliferative activity (Figure 2).

Cytokine release after stimulation with SEA or conjugate

Given that the difference in lymphocyte proliferation between 3.6×10^{-4} nM conjugate and 3.6×10^{-1} nM SEA was significant, the release of IL-2, IFN-γ, and TNF-α by stimulated PBMC was measured after incubation with 3.6×10^{-2} nM SEA or conjugate. Production of all three cytokines was markedly induced in the experimental groups compared with the control group, whereas the differences between the SEA and conjugate were insignificant, suggesting that the conjugate completely retained the ability of the SAg to induce lymphocyte cytokine release (Figure 3).

Expression of MHC II

The expression of MHC II (HLA-DR) was examined in BIU-87, T-24, E-J and Lovo cell lines by flow cytometry. HLA-DR was expressed at only a low level by the BIU-87 cell line (20.5 ± 2.0%), but not by the T-24, E-J or Lovo cell lines.

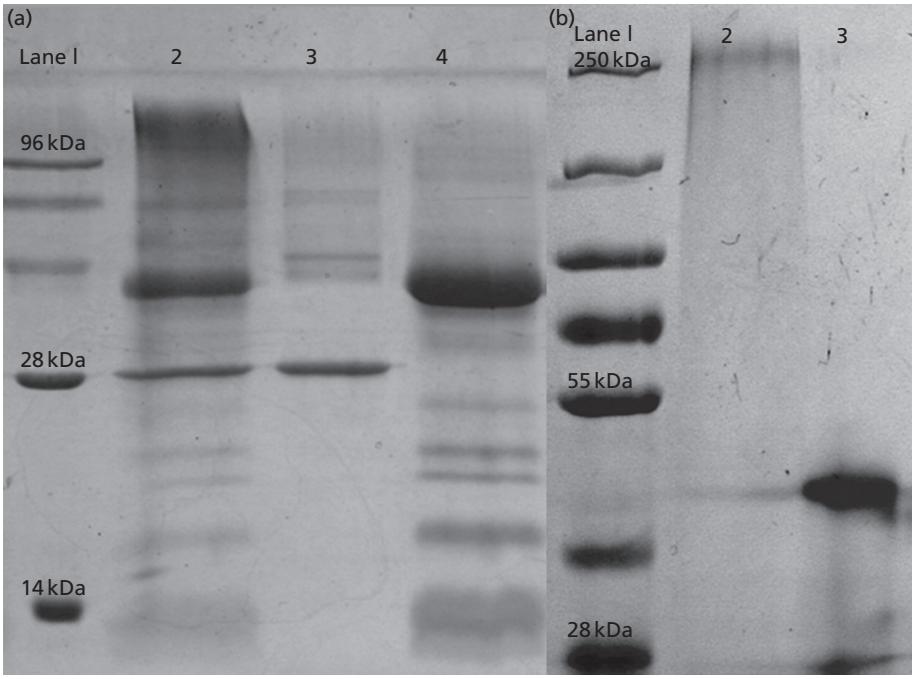

Figure 1 Analysis of the different products of SDS-PAGE. (a) Products before purification, lane 1: marker with a maximum apparent molecular weight (MW) of 96 KDa; lane 2: unpurified conjugate, which contains three groups of proteins with MW > 96, 50 and 28 KDa; lane 3: SEA protein control (28 KDa); lane 4: Fab fragment control (50 KDa). (b) Molecular mass and purity analysis of conjugate after purification, lane 1: marker with a maximum apparent MW of 250 KDa; lane 2: the first collected peak (conjugate), MW approximately 250 KDa; lane 3: the second and third collected peaks which contain two groups of proteins, MWs 28 and 50 KDa.

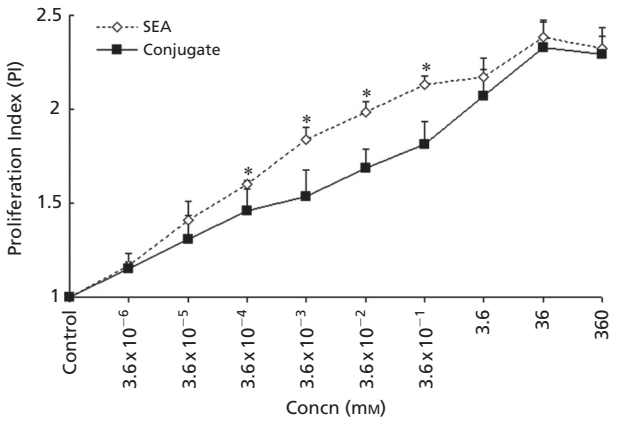

Figure 2 Effect of the conjugate and SEA on proliferation of lymphocytes. $*P < 0.05$ vs SEA group at same concentration (Kruskal–Wallis test followed by Dunn's test; $n = 6$).

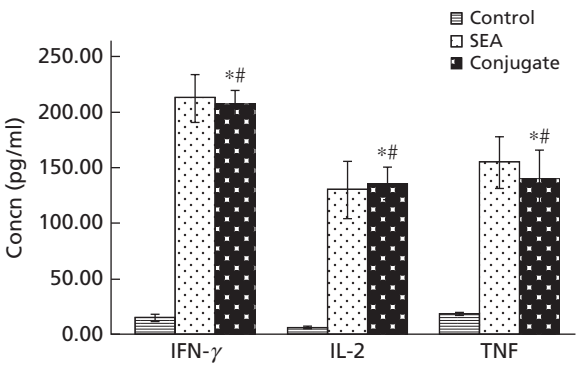

Figure 3 Conjugate- and SEA-induced cytokine release. $*P \geq 0.05$ vs control group; $^{\#}P < 0.05$ vs SEA group at same concentration (one-way ANOVA followed by Dunn's test; $n = 6$). IFN-γ, interferon γ; IL-2, interleukin-2; TNF, tumour necrosis factor.

Effect of conjugate against carcinoma cells *in vitro*

Compared with the SEA group, the conjugate improved PBMC-mediated cytotoxicity against the bladder carcinoma cells (BIU-87, T-24 and E-J; Figures 4a–c; $P < 0.01$) whereas a mixture of unconjugated SEA and BDI-1 Fab fragment did not have the same effect (Figure 4a). Improved cytotoxicity against bladder carcinoma cells was induced by the conjugate at concentrations as low as 0.036 nM, whereas the mixture of unconjugated SEA and BDI-1 Fab did not have this effect at any concentration (Figure 4a). Furthermore, this effect of the conjugate was not observed in Lovo colorectal cancer cells (Figure 4d), showing that the increased cytotoxicity mediated by the conjugate was restricted to bladder carcinoma cells.

Discussion

Diagnosis of bladder carcinoma, the most common genitourinary cancer in China, is increasing, the majority of cases being NMIBC.[26] Recurrences following TUR occur in 60–70% of NMIBC because of incomplete removal or implanted cancer

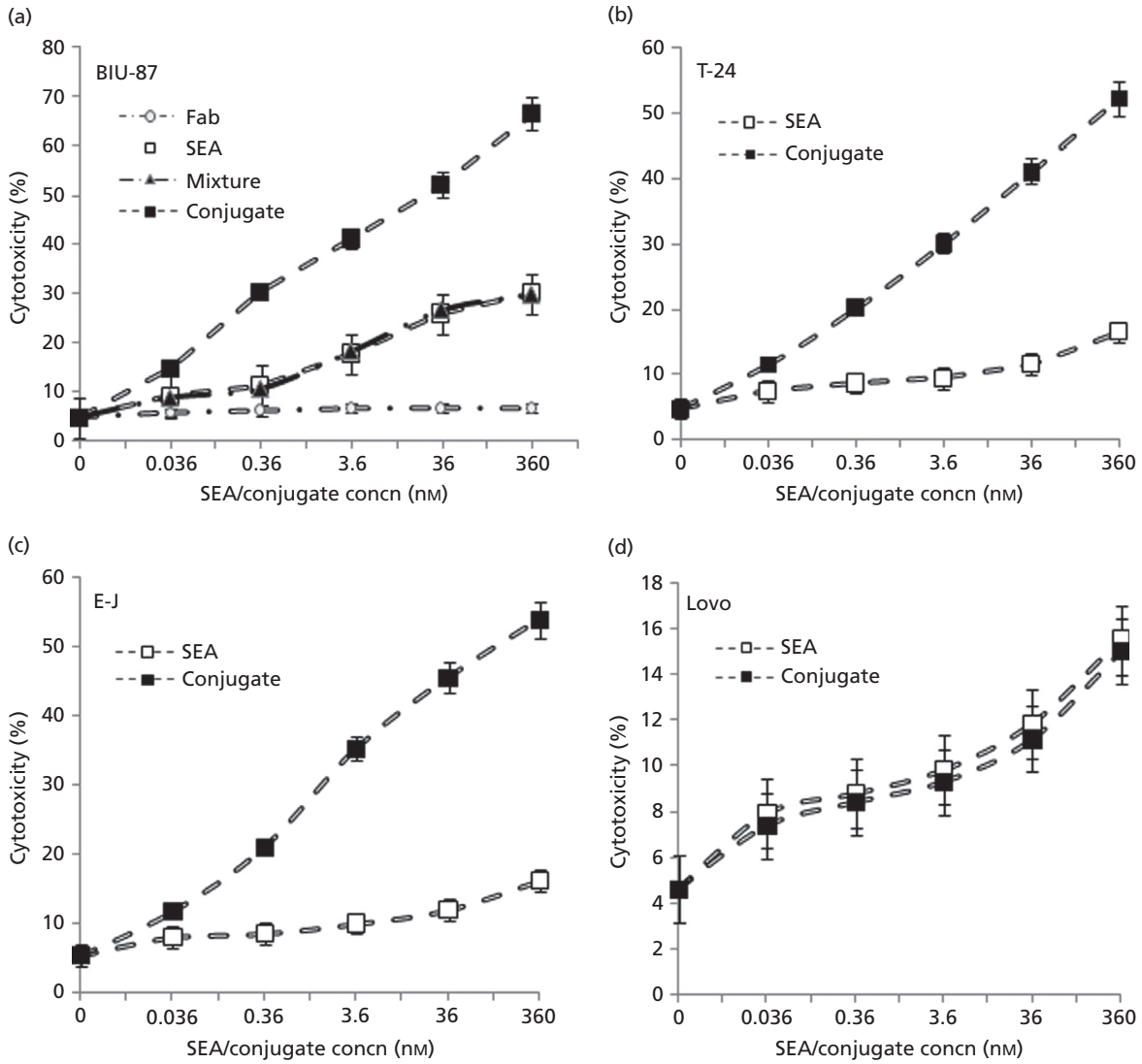

Figure 4 Effect of the conjugate and SEA against carcinoma cells *in vitro*. Cytotoxicity of the conjugate and SEA in (a) BIU-87 (in the absence and presence of conjugate, SEA, BDI-1 Fab fragment and a mixture of SEA and BDI-1 Fab fragment), (b) T-24 and (c) E-J human bladder cancer cell lines and (d) the Lovo colorectal cancer cell line at various concentrations with an effector : target ratio of 10 : 1. Data are from triplicate experiments. Note that the figures have different scales.

cells, and 20–30% progress to a higher stage or grade. The high recurrence rate and the unpredictability of the progression pattern of NMIBC have led to the widespread use of intravesical therapy, including chemotherapy and immunotherapy, as an established supplement to TUR.[3] However, current chemotherapeutic agents have limited efficacy, and the benefits of BCG, the most effective immunotherapeutic agent currently available, are outweighed by occasional severe local and systemic side-effects, necessitating a search for alternative therapeutic modalities.[27]

The SAgs of microbial or viral protein toxins have potent immunostimulatory properties and hold great promise for the intravesical therapy of NMIBC. SAgs have mitogenic activity; they bind to both regions of MHC II molecules outside the classic antigen-binding groove and particular TCR β-chain variable segments simultaneously.[28] In contrast to BCG, this interaction triggers the activation and proliferation of more subsets of T lymphocytes and leads to the release of higher levels of various cytokines,[11] generating some encouraging results in the intravesical therapy of bladder carcinoma.[12] However, the clinical application of SAgs in the treatment of bladder carcinoma is seriously restricted because activated T-cells, particularly cytotoxic T-lymphocytes (CTLs), and the cytokines secreted, result in toxicity to non-malignant cells. Lysis of bladder cancer cells directed by CTLs will also be limited because of the low expression of MHC II molecules on bladder cancer cells.[29,30] Since MAb–SAg-mediated cytotoxicity is probably independent of MHC II,[29] the use of MAbs that react with bladder cancer cells to target activated T-cells and produce cytokines mainly in the area of remnant or implanted carcinomas

after TUR and to lyse bladder cancer cells represents an attractive strategy for intravesical study. Compared with complete MAb, Fab fragments of MAb have a smaller volume and can penetrate through tumour tissues more easily and also avoid non-specific conjugation to non-malignant cells with Fc receptors. For these reasons, we attempted to prepare MAb Fab-targeted SAgs. MAbs targeted directly at the bladder tumour include C595,[31] Mac387,[32] 7E12H12[33] and BDI-1. Murine BDI-1 was initially prepared at Peking University and has been used extensively in cytotoxic[34] and intravesical[35–37] studies in China. It was raised against the BIU-87 human bladder cancer cell line and exhibits strong specific reactivity with most bladder carcinoma tissue samples and cell lines.

Unlike the common systemic use of MAbs, intravesical instillation avoids the need for humanisation of murine MAb and facilitates the immediate application of murine MAb. Thus, through the papainic digestion method, the Fab fragment of MAb BDI-1 was obtained at an enzyme : antibody ratio of 1 : 100 and conjugated to SEA by the chemical conjugating reagent SPDP.

Considering that extremely low concentrations of SAgs are able to activate a number of resting T-cells, thereby inducing massive cytokine release, which may even induce significant systemic reactions such as fever, disseminated intravascular coagulation, hypovolaemia and shock,[38,39] we focused more on targeted and MHC-II-independent selectivity than on superantigenic stimulation activity. With a premixed Fab-PDP : SEA-SH ratio of 5 : 1, the desirable conjugate was prepared successfully. It retained the targeted activity anchoring to the bladder carcinoma, with a slight reduction in stimulatory potency. Limitations in the purification method, however, meant that non-specific conjugate was not removed completely, which contributed to the continuous bands in SDS-PAGE analysis.

To evaluate the cytotoxicity of the conjugate targeting MHC II-negative bladder carcinomas *in vitro*, the surface expression of HLA-DR on a series of bladder carcinoma cell lines, to be targeted or not by conjugate, was detected before the cytotoxicity experiment. BIU-87, T-24, E-J and Lovo cell lines either did not express MHC II antigen, or had low expression, and a significant difference in antitumour effects between conjugate and SEA was observed in BIU-87, T-24 and E-J cells, but not in Lovo cells, indicating that in comparison with SEA alone, treatment of MHC II-negative carcinomas with the conjugate was enhanced, but only in bladder cancer. As earlier studies have demonstrated that the therapeutic effect of SAgs most likely involves SEA-reactive pseudo-specific T-cells as well as cytokines released to lyse tumour cells, these T-cells do lack the activity to lyse MHC II-negative cells.[29] In the present study, the lysis of MHC II-negative bladder carcinoma cells mediated by conjugate-reactive CTLs is likely to be responsible for the improved cytoxicity in BIU-87, T-24 and E-J cells. This is based on the fact that the stimulatory activity of the conjugate is only comparable or even reduced compared with SEA.

Conclusions

We have developed a novel intravesical instillation drug for NMIBC without any complicated genetic manipulation. Significantly augmented antitumour effect and selectivity for bladder carcinoma were observed for the conjugate *in vitro*. We therefore conclude that the conjugate of BDI-1-targeted SEA has been prepared successfully, and represents a potential agent for intravesical therapy of bladder carcinoma.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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