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ORIGINAL ARTICLE

The fibronectin attachment protein of bacillus Calmette-Guerin (BCG) mediates antitumor activity

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Abstract
Purpose The receptor responsible for the attachment of bacillus Calmette-Guerin (BCG) to fibronectin, fibronectin attachment protein (FAP), has been cloned. Studies targeting FAP as an inducer of immunity in mycobacterial infections suggest that FAP is a highly immunogenic protein. In light of these findings and the need to find effective alternatives to BCG treatment for bladder cancer, we tested the ability of FAP to induce antitumor activity.
Materials and methods The ability of FAP to bind to bladder tumor cells and the bladder wall was established using ^{125}I-FAP. For testing antitumor activity in vivo, mice were catheterized and 5×10^4 MB-49 bladder tumor cells were implanted orthotopically on day 0. Test groups were treated with PBS only, FAP, or BCG on day 1 and day 8. A subset of mice was preimmunized with FAP prior to treatment.
Results FAP was observed to bind to bladder tumor cells in a fibronectin-dependent manner. Attachment of FAP within the bladder followed the pattern established for BCG binding. Antitumor studies showed a significant reduction in tumor growth in FAP-treated mice that had been preimmunized with FAP. Tumor growth was not inhibited in naïve mice treated with FAP. Dose-response studies showed that FAP-induced antitumor activity is dose dependent, and experiments comparing BCG with FAP showed equivalent antitumor effects. In vitro experiments showed antigen-specific lymphocyte proliferation and a cytokine profile indicative of Th-1 polarization of the FAP-induced immune response. CD8+ T cells and natural killer cells were found to be required for the FAP-induced antitumor response.
Conclusions FAP is an effective antitumor agent that inhibits tumor growth at a level equivalent to that observed for BCG. This protein may thus provide an alternative to BCG for treatment of superficial bladder cancer.

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Introduction

Since its discovery in 1976 by Morales and associates, bacillus Calmette-Guerin (BCG) immunotherapy has become the treatment of choice for superficial bladder cancer [22]. In spite of the fact that BCG currently is the most efficacious treatment for superficial bladder cancer, treatments can induce severe toxicity in some patients [17]. Moreover, toxicity increases with multiple treatments, limiting a patient's tolerance for treatment regimens optimized for anticancer effects [19]. Additionally, long-term follow up of BCG-responsive patients has revealed a high recurrence rate over time—up to 70% over 10 years [7]. Given the toxicities associated with BCG treatment, the post-response recurrence rate and the fact that BCG ultimately has little impact on patient survival, the identification of alternative treatments for superficial bladder cancer is desirable.

Since most superficial bladder tumors readily respond to BCG treatment, the identification of alternative immunotherapy approaches that do not use viable bacteria may yield treatments that are more effective and less toxic. Although the mechanism of action of BCG is incompletely understood, its investigation to date has shown that the

elimination of tumors is dependent on T cell immunity, with laboratory and clinical studies having demonstrated a significant correlation between a BCG-activated Type I delayed type hypersensitivity (DTH) response and antitumor activity [15, 24, 29, 30]. Consistent with a role for DTH in antitumor activity, interferon-γ (IFN-γ) levels have been linked to successful therapeutic outcomes, both in murine models and in patients undergoing BCG therapy [10, 24, 28]. The effector mechanisms mediating BCG-induced antitumor activity have not been clearly identified, although the innate immune response has been shown to be an important component [21, 27, 35, 36]. Thus, identifying an immunotherapy regimen that requires T cells and induces Type I immunity is a primary focus of our efforts to improve treatments for superficial bladder cancer.

Here we report on an immunogenic protein that is a component of BCG, i.e. the fibronectin attachment protein (FAP). FAP mediates BCG attachment to bladder tumor cells and bladder wall following intravesical instillation [34, 40, 41]. FAP has unique properties, which include its ability to mediate the internalization of BCG by bladder epithelial cells through the α_5-β_1 integrin receptor [1, 9, 13, 14]. Moreover, the nucleotide sequence for FAP has been shown to be identical to that of a *Mycobacterium tuberculosis* protein termed the Apa/45-47 complex, [8, 20, 41] and both are highly immunogenic proteins capable of inducing strong CD8 T cell immune responses [20]. The utility of other immunogenic proteins, such as keyhole-limpet hemocyanin (KLH) and the mycobacterial protein PstS1, in anti-bladder tumor therapy has been well documented, in both animal models and clinical trials, but each has been found to have some important limitations [11, 12, 18, 33, 37, 38]. Since FAP is a highly antigenic protein with the unique properties of binding to, and being internalized by, bladder cancer cells, we hypothesized that FAP would induce an effective T cell-mediated antitumor response. In this paper, recombinant FAP is tested for its ability to induce antitumor activity in an orthotopic bladder tumor model.

Materials and methods

Animals

All studies were carried out on female C57BL/6 mice at 8–10 weeks of age. Mice were given food and water ad libitum. All animal studies were performed in accordance with institutional guidelines.

FAP

Recombinant FAP was produced and purified as previously described [40]. Lipopolysaccharide (LPS) was removed with DeToxi gel (Pierce Biotechnology, Rockford, IL) and tested for LPS using the E-TOXATE reagent from *Limulus polyphemus* (Sigma Chemicals, St Louis, MO).

^{125}I-labeled FAP

Purified FAP was labeled with ^{125}I using the IODO-GEN reagent from Pierce Biochemicals (Rockford, IL) as described in the reagent information.

In vitro ^{125}I-FAP binding studies

Approximately 5×10^4 cells (MB-49 and T-24) were plated in flat-bottom 96-well plates and incubated overnight. Cells were washed and 5 μCi ^{125}I-FAP added to appropriate wells. Cells were incubated 4 h, washed, detached with 0.01 M EDTA and counted in a gamma counter. Wells were visually inspected to assure removal of all cells. When used, anti-FAP was added to the ^{125}I-FAP immediately before addition to cells, and anti-FN was added to the cells 15 min prior to ^{125}I-FAP addition.

In vivo ^{125}I-FAP binding studies

Mice were anesthetized with 100 μl of a 17.5 mg/ml ketamine, 2.5 mg/ml xylazine solution before catheterization with a standard 24-gauge catheter. Some mice were cauterized immediately after catheterization as previously described [41]. Ten micro curie ^{125}I-FAP was added to each bladder in a total volume of 100 μl. Mice were sacrificed 2 h later, and bladders were removed, washed and counted in a gamma counter.

FAP pre-immunizations

Two weeks prior to tumor implantation a subset of mice was immunized subcutaneously with 200 μg FAP in complete Freud's adjuvant (CFA). Naïve mice served as the control.

Tumor implantation

Mice were anesthetized with 100 μl of a 17.5 mg/ml ketamine, 2.5 mg/ml xylazine solution before catheterization with a standard 24 gauge catheter. Bladders were treated with 4 μl of a 0.3 M silver nitrate solution and immediately rinsed twice with PBS. 100 μl of a 2.5×10^5 cell/ml solution of MB49 tumor cells in RPMI was instilled into the bladder (day 0). The catheter was removed and mice were allowed to recover for 24 h before therapy was initiated. MB49 tumor cells were prepared as a single cell suspension from a subcutaneous tumor, following which they were implantated into the bladder as previously described [30].

FAP therapy

Therapy began 24 h after tumor implantation (day 1). Mice were again anesthetized with 100 µl of a 17.5 mg/ml ketamine, 2.5 mg/ml xylazine solution before catheterization with a standard 24 gauge catheter. Treatments included PBS, FAP (25–100 µg), BCG (10^7 cfu). All instillations were at a total volume of 100 µl. Therapy was repeated on day 8. Mice with blood in their urine after day 14 were sacrificed and examined for urothelial tumors. Ten mice were used per group.

In vitro proliferation/ELISA assay

Mice were immunized with 200 µg FAP in CFA or CFA alone 2 weeks prior to sacrifice. The spleen from each mouse was harvested and a single-cell suspension was prepared. In vitro proliferation assay: Spleen cells (2×10^5 cells per well) were incubated with the indicated concentration of FAP or anti-CD3 for 48 h. Cell cultures were subsequently incubated with 1μCu of ^{3}H-thymidine for 18 h before cells were collected and counted. ELISA: spleen cells were plated at 5×10^6 cells/well and incubated with 100 µg/ml FAP, 100 µg/ml BSA or 5 µg/ml anti-CD3 for 24 h. Supernatants were collected and frozen at −20°C until assayed for IFNγ or IL-4 by ELISA (BD Biosciences, San Jose, CA). Each data point is an average of the values obtained from 3 mice.

Depletion studies

Three days prior to tumor implantation (day −3), mice were i.p. injected with 100 µg of one of the following depleting antibodies: GK1.5 ($CD4^+$ T-cells), 2.43 ($CD8^+$ T-cells), PK136 (NK1.1 cells) or SFR8 (isotype control). Antibody injections were repeated on days −2 and −1. Designated mice were sacrificed before tumor implantation on day 0 to confirm specific cell depletions. Maintenance antibody injections of 100 µg were administered i.p. on day 7 and continued 2 times per week for the entire experimental period. Ten mice per were used for each group.

Results

FAP attachment to cells in vitro and in vivo

Previous studies have shown that FAP effectively blocks both BCG attachment to bladder tumor cells in vitro and binding to the bladder wall in vivo [41]. One interpretation of the blocking experiments is that under the experimental conditions, FAP acts as a competitive inhibitor of BCG for binding sites. To determine whether FAP actively binds to bladder cancer cells, binding of ^{125}I-labelled FAP was followed. In order to assess whether FAP binding utilizes the previously identified fibronectin-binding motif, RWFV, this experiment was performed with two different FAP constructs: one contained the RWFV amino acid-binding sequence (wtFAP), and another lacking this sequence (4dFAP) [41]. Data in Fig. 1a show that although FAP binds to the bladder tumor lines T-24 and MB-49 in a manner that is not completely dependent on the presence of the RWFV domain, this fibronectin-binding motif is required for a significant proportion of the binding that was observed. This finding is consistent with what has been observed for BCG previously [41]. As reported for BCG binding to T-24 bladder tumor cells, FAP binding to this same line was inhibited by a rabbit polyclonal antibody to FAP. In addition, the binding was inhibited by a rabbit polyclonal antibody to the FAP ligand, fibronectin (FN;

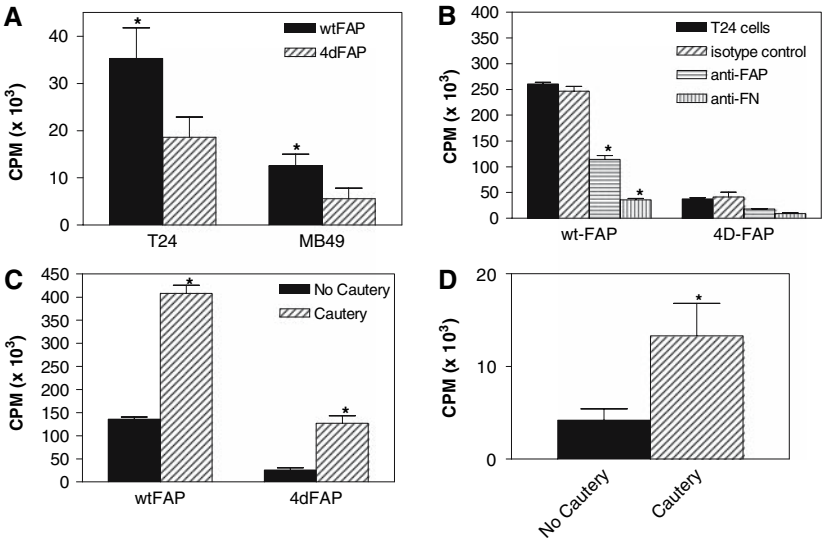

Fig. 1 Attachment of FAP to bladder cancer cells and retention within the bladder after intravesical instillation. FAP was purified and labeled with ^{125}I. **a** Binding of ^{125}I-FAP to T-24 and MB-49 bladder tumor cells; **b** fibronectin dependence of ^{125}I-FAP binding to T-24 bladder tumor cells; **c** retention of ^{125}I-FAP within the bladder after intravesical instillation; **d** retention of ^{14}C-labelled BCG within the bladder after intravesical instillation. * Statistical significance at $P \leq 0.05$ using the two-tailed Wilcoxon Rank Sum test

Fig. 1b) [14, 41]. These in vitro studies demonstrate that FAP binds to bladder tumor cells and suggest that, like BCG, FAP applied by intravesical instillation would result in binding to the bladder. To directly test this hypothesis, in vivo binding studies were performed with ^{125}I-FAP. Mice were anesthetized, catheterized and either subjected to electrocautery or left untreated. Subsequently, either ^{125}I-wtFAP or ^{125}I-4dFAP was instilled via the inserted catheter. Thirty minutes after instillation, mice were euthanized and their bladders removed, washed and tested for ^{125}I-FAP binding. The data show that ^{125}I-wtFAP is preferentially retained in the bladder (Fig. 1c). Further, as observed for BCG, electrocautery increased ^{125}I-wtFAP retention (Fig. 1c, d). Taken together, these data show that FAP binds to bladder tumor cells in vitro and that it is retained within the bladder after intravesical instillation, suggesting that it has the potential to become a useful tool for inducing an antitumor response.

FAP-induced antitumor activity

Previous studies have shown that some bacterial proteins, such as KLH, require prior immunization to provide effective antitumor activity, whereas others, such PstS1, do not [33, 37]. Thus, studies were initiated to assess the antitumor potential of FAP in immune and non-immune mice. Mice were preimmunized with FAP as described in "Materials and methods", and 2 weeks later MB-49 tumor cells were implanted in the bladder (day 0). Subsequently, FAP was instilled intravesically at concentrations of 25, 50 and 100 μg, on both day 1 and day 8. The data show a dose-dependent antitumor response, with FAP significantly inhibiting tumor growth at 50 and 100 μg but not at 25 μg (Fig. 2a). As was previously reported for KLH treatment of bladder tumors, FAP-induced antitumor activity required prior immunization [18, 33, 37].

Further studies were performed to compare the antitumor activity of FAP with that of BCG. FAP-immunized and naive mice carrying MB-49 tumors were treated with FAP (100 μg) or BCG (10^7 colony forming units) as outlined in "Materials and methods". Preimmunization with FAP followed by treatment (intravesical instillation) with FAP was found to be as effective as BCG therapy in inhibiting MB-49 tumor growth (Fig. 2B). Consistent with the results in the dose-response experiment (Fig. 2a), no significant FAP-mediated antitumor activity was observed in naive mice. In contrast, BCG was as effective at inducing antitumor activity in naive mice as in FAP preimmunized mice.

The requirement of FAP preimmunization for effective antitumor activity suggests that T cells participate in its stimulation. To test this theory, we performed in vitro experiments assessing levels of antigen-specific T-cell proliferation in response to FAP administration. Mice were first primed with FAP, and 2 weeks later spleen cells were isolated and assessed for their recognition of FAP in vitro using a ^{3}H-Thymidine proliferation assay. The data show that FAP exposure leads to immunization-specific and dose-dependent proliferation (Fig. 3a). For a positive control, we stimulated both cultures to proliferate in response to anti-CD3 (data not shown). To determine whether FAP induces a Type 1 or Type 2 response, we measured IFNγ (indicative of Type 1 cytokine response) and IL-4 (indicative of Type 2 cytokine response) levels. Antigen-specific IFNγ production was observed in splenocytes isolated from mice that had been both preimmunized and stimulated with FAP (Fig. 3b, c), and these levels were 5-fold higher than those observed in FAP-stimulated naive mice. The data for IL-4, on the other hand, did not reveal an increase in response to FAP exposure. Taken together these in vitro studies show that FAP immunization induces an antigen-specific Type 1 immune response. Furthermore, they formally demonstrate that FAP preimmunization primes mice for this reaction. However, they do not assess the contribution of immunity to the antitumor response.

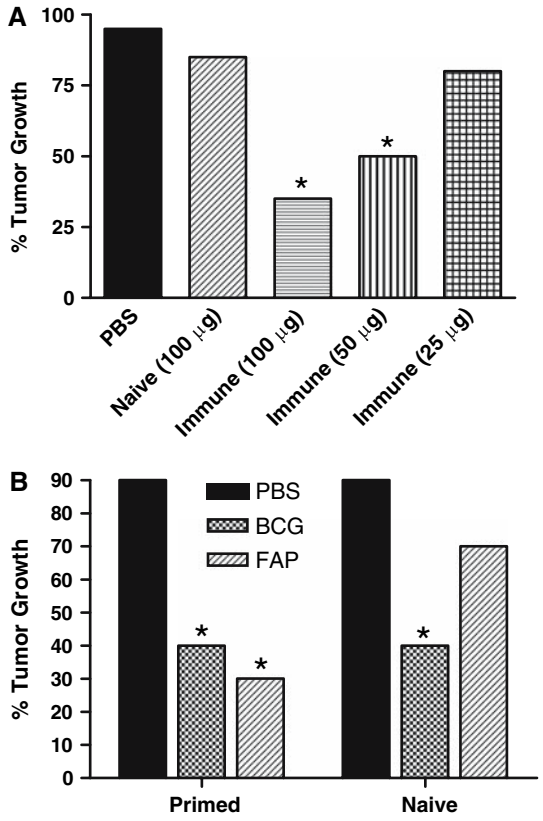

Fig. 2 Antitumor activity of FAP in an orthotopic bladder tumor model. **a** Effect of varying concentrations of FAP on MB-49 bladder tumor growth; **b** comparison of the antitumor activity of FAP and BCG. * Statistical significance at $P \leq 0.05$ using the Fisher's Exact Chi Square test

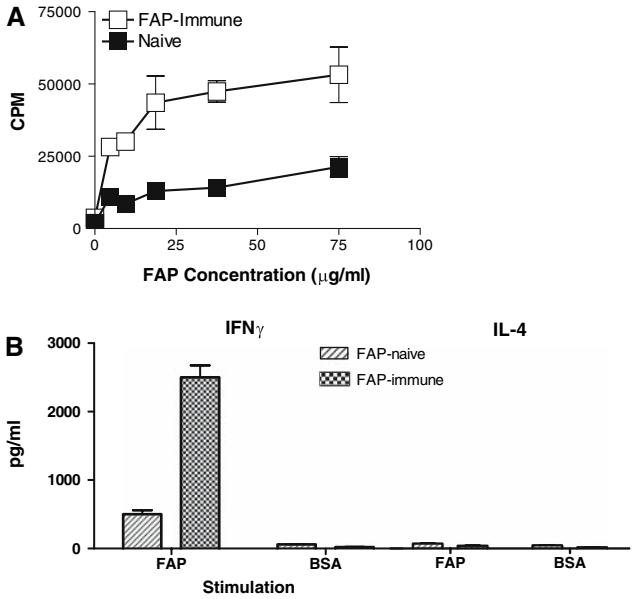

Fig. 3 In vitro immune response to FAP. **a** proliferation of spleen cells from mice previously immunized with FAP monitored by the uptake of ^{3}H-thymidine; **b** IL4 produced after antigen-specific stimulation of FAP immune spleen cells; **c** IFNγ produced after antigen-specific stimulation of FAP immune spleen cells

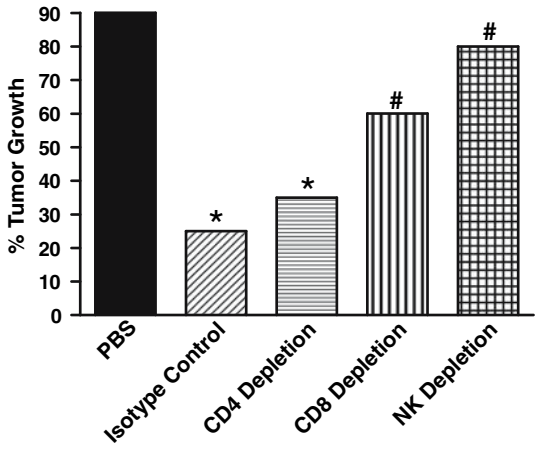

Fig. 4 Identification of immune effector population mediating FAP-induced antitumor activity. Mice were depleted of CD4+, CD8+, and NK cells as described in "Materials and methods". * Statistical significance comparing treatment groups to PBS control at $P \leq 0.05$ using the Fisher's Exact Chi Square test. # statistical significance comparing isotype control with effector cell-depleted groups at $P \leq 0.05$ using the Fisher's Exact Chi Square test

To dissect the relative contributions of T cells and NK cells to FAP-induced antitumor activity, we performed experiments in which CD4+, CD8+, and NK cells were individually depleted in vivo. Mice were first primed with FAP, and 12 days later they were treated with the appropriate lymphocyte-depleting antibody. MB-49 tumors were implanted on day 14, and FAP treatment was initiated 1 day later. We found that the depletion of either CD8+ T cells or NK cells abrogated FAP-induced antitumor activity (Fig. 4), whereas the depletion of CD4+ T cells had no significant effect on the FAP-induced antitumor response.

Discussion

Data reported herein demonstrate that the BCG-derived fibronectin attachment protein (FAP) has an effective antitumor activity. Specifically, FAP was shown to bind directly to bladder tumor cells and to be effectively retained in the bladder after intravesical instillation. Antitumor activity was found to be dependent on the immune system, requiring both CD8+ and NK cells for its elimination of tumors. Surprisingly, CD4+ effector T cells do not appear to contribute to the FAP-induced antitumor response. Notably, the FAP-induced antitumor activity observed was found to be equivalent to that of live BCG in terms of its potency, suggesting that it may be possible to use FAP as an alternative to BCG in therapeutic applications.

Currently, BCG is the treatment of choice for superficial bladder cancer [7]. Whereas BCG is effective in most patients, approximately 30% of patients fail to respond to the initial treatment with BCG [2, 7]. Moreover, over time tumors reappear in up to 70% of the patients who are initially responsive [7]. While many individuals who fail to respond to BCG initially do respond to further treatment with either BCG alone or BCG plus interferon, others never become responsive, which creates a dilemma concerning patient management. Furthermore, systemic toxicity can become an issue and, although rare, has resulted in death [16]. Several other factors limit the usefulness of BCG treatment. For example, the potential for systemic infection with BCG precludes its use in immunocompromised patients, including transplant patients as well as individuals positive for human immunodeficiency virus [17]. Also, the fact that treating recurrent low-grade bladder tumors with a higher-risk treatment such as BCG shifts the risk: benefit ratio, preventing some clinicians from proceeding with this approach [4]. Finally, local toxicities can impose limits on sequential BCG treatments—to lower doses in some cases, and to the cessation of BCG treatment altogether in some others [7, 25]. Taken together, these issues highlight the need for new and less toxic anti-bladder tumor agents. Since, in the studies reported herein, FAP appeared to be as effective as BCG at inhibiting tumor growth, FAP may represent a lower-risk alternative to BCG.

Previous studies have reported the use of mycobacterial products to treat bladder cancer [3, 23, 33, 37]. Cell-wall extracts from *Mycobacterium phlei* (MCWE) inhibit bladder tumor growth in both a murine model and clinical

trials [3, 23]. However, in contrast to the observations for FAP-induced antitumor activity, where comparative studies showed FAP to be as potent as BCG in pre-immunized mice, MCWE was significantly less potent [3]. Although the mechanism whereby MCWE induces antitumor activity has not been clearly defined, it appears to be distinct from that of FAP. Like FAP, MCWE modulates immunity through cytokine induction. However, the mechanism of action relevant to its antitumor activity was reported to be an induction of apoptosis specifically in bladder tumor cells [5, 31]. Our findings that FAP-induced antitumor activity depends on both CD8+ T cells and NK cells, and our failure to detect any evidence for cell death in T-24 cells treated with FAP, even at FAP concentrations of up to 100 μg/ml, indicate that antitumor activity induced by FAP is mechanistically distinct from that induced by MCWE.

Other efforts to identify therapies to combat bladder tumors have focused on the soluble mycobacterial protein PstS1, for which Sanger and associates reported significant antitumor activity in a bladder tumor model [33]. Similar to our observations with FAP, these investigators demonstrated a Type I response, with PstS1 also inducing IL12 and IFNγ production in normal human peripheral blood lymphocytes. These data suggest that a Type I response is required for antitumor activity. However, cytokine analyses in the animal model were not performed, leaving the physiological relevance of these findings unclear. Unlike our observations with regard to FAP, effective tumor control with PstS1 was observed only in naive mice. Surprisingly, PstS1 was not active in preimmunized mice. The reason for this is not clear. However, given that the T-cell effector population for FAP-induced antitumor activity is CD8+ T cells, it is possible that PstS1 priming primarily activates CD4+ T cells instead. It is also possible that, in the animal model, PstS1 preimmunization induced a Type II response rather than a Type I response, and the latter appears to be important in bladder tumor therapy [10, 24, 28]. Mitigating against the explanation of a Type II response is data from other investigators, showing that PstS1 induces a strong Type I response [39].

Yet another set of studies has evaluated KLH as an anti-bladder tumor agent [12, 18, 37]. Early clinical studies suggested that the systemic administration of this protein mediates antitumor activity in bladder cancer patients [26, 32]. Subsequent clinical studies, as well as studies in animal models, have clearly demonstrated effective antitumor activity when KLH is administered intravesically [18, 37]. Animal model studies have further shown that, as for FAP, KLH requires preimmunization for optimal antitumor activity, and that it is as potent as BCG in inducing antitumor activity [18, 37]. However, whereas some clinical studies indicated that KLH treatment is more effective than chemotherapy, others failed to confirm this [6, 12]. The reason(s) for the variability in antitumor activity are not clear, but may relate to variations in KLH preparations. With regard to this possibility, it should be noted that KLH exists in multiple forms of varying molecular weight [38]. In addition, studies in an orthotopic bladder cancer model have suggested that the disassociated form of KLH is less effective in inhibiting bladder tumor growth than the associated form [37]. The production of FAP as a recombinant protein eliminates the potential for such variations in the final product, while maintaining effective antitumor activity. As in the case of PstS1, the mechanism whereby KLH induces antitumor activity has not been entirely established. As with FAP, augmentation of NK cells has been reported and suggested to contribute to the KLH antitumor effect, but direct evidence for the participation of this lymphocyte population in KLH-induced antitumor activity is lacking [18]. Furthermore, a role for T-cell immunity in KLH-induced antitumor activity has not been studied. Thus, the potential of KLH for remains limited.

In conclusion, the experiments reported herein investigate the usefulness of FAP in the treatment of bladder cancer. Our findings show that FAP inhibits bladder tumor growth in a dose-dependent manner, and that the antitumor activity in FAP-preimmunized mice is equivalent to that of BCG. In conjunction with the fact that FAP is easy to produce and, more importantly, its application is unlikely to result in severe adverse side effects such as those associated with the instillation of live BCG, they validate further investigation into the use of FAP in the treatment of superficial bladder cancer.

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