Modulation of Fibronectin-mediated \textit{Bacillus Calmette-Guérin} Attachment to Murine Bladder Mucosa by Drugs Influencing the Coagulation Pathways

M'Liss A. Hudson, Eric J. Brown, Julie K. Ritchey, and Timothy L. Ratliff

Scott Department of Urology, Baylor College of Medicine, Houston, Texas 77030 [M. A. H.], and Division of Urological Surgery, Departments of Medicine, Microbiology and Immunology, Washington University School of Medicine and the Jewish Hospital of St. Louis, St. Louis, Missouri 63110 [E. J. B., J. K. R., T. L. R.]

ABSTRACT

Adjuvant intravesical \textit{Bacillus Calmette-Guérin} (BCG) has proved to be an effective treatment for superficial bladder cancer. Intraluminal attachment of BCG organisms via binding to the extracellular matrix protein, fibronectin (FN), appears to be required for expression of the antitumor efficacy of BCG against a murine bladder tumor. Initial studies demonstrated that radiolabeled FN localized to the acutely injured urothelium but not to intact urothelium. These studies also demonstrated that exogenous administration of FN enhanced BCG attachment to the injured but not to the intact urothelium. Because FN has been shown to be an integral part of clot formation at sites of urothelial injury, drugs known to affect fibrin clot formation were tested for their effects on BCG attachment and antitumor efficacy in a murine bladder tumor model. A stabilizer of fibrin clot formation was shown to enhance both BCG attachment and antitumor efficacy in the same model. An increased number of BCG organisms were also retained in the lymph nodes and spleens of mice receiving fibrin clot stabilizers, suggesting indirectly that immunological mechanisms are involved in the antitumor efficacy of BCG.

The data presented herein provide further support for the hypothesis that BCG attachment to the injured bladder is mediated by FN. Furthermore, modulation of BCG-FN attachment is demonstrated to be possible with drugs influencing the coagulation pathway. This attachment is shown to be required for the antitumor efficacy in a murine bladder tumor model, and thus modulation of BCG-FN attachment appears to have significant influence on the antitumor efficacy of BCG in the murine bladder tumor model.

INTRODUCTION

Intravesical BCG\textsuperscript{1} has proven efficacious in the treatment and prophylaxis of superficial papillary bladder tumors and carcinoma \textit{in situ} of the bladder (1-4). Several authors have suggested that close contact between BCG organisms and bladder tumor cells is necessary for the antitumor effect observed (1-4). Although the mechanism by which BCG exerts an antineoplastic effect on superficial bladder tumors is not fully understood, recent evidence has suggested that BCG binding to FN exposed on disrupted urothelial surfaces is required for the antitumor effect to be expressed (5, 6). Histological studies have shown that BCG binds only at sites of urothelial disruption, and electron micrographs have demonstrated not only attachment but internalization of BCG at these sites by both urothelial cells and macrophages. Previous \textit{in vitro} studies in our laboratory have demonstrated that BCG binds to FN-coated surfaces in preference to other matrix proteins and that this attachment can be blocked by antibodies to FN but not by antibodies to laminin or preimmune serum. Furthermore, in an \textit{in vivo} murine model, BCG-FN attachment can be inhibited by anti-FN antibodies (5, 6). Concomitantly, the antitumor effect of BCG on the MBT-2 tumor was abrogated by the presence of anti-FN antibodies (7).

FN is a glycoprotein with a molecular weight of 440,000 found in a soluble form in plasma and a matrix form in extracellular spaces and along basement membranes (8-13). In the urinary tract, FN has been localized to the urothelial basement membrane (13). FN is involved in the four stages of wound healing after surgical trauma such as that associated with resection of a bladder tumor (8-13). It constitutes 4-5% of the blood clot formed at the initial injury site and is cross-linked to the fibrin fibers by factor XIIIa. Grinnell et al. (11, 13) have shown the appearance of FN within the fibrin clot within 5 h after the acute injury. FN incorporated in the blood clot serves as both a chemotactic agent and an attachment site for macrophages to remove effete cells, cellular debris, and some extracellular matrix protein. Martin et al. (8) have demonstrated that tissue debris is coated with FN within 2 h after injury. Repair of the injury starts as fibroblasts attach to FN coating the fibrin scaffolding and begin synthesizing and secreting both collagen and FN. FN cross-links the collagen fibers and provides part of the new matrix for movement of new urothelial cells over the basement membrane. Additionally, myofibroblasts, which are necessary for wound contraction, are anchored by FN. Finally, as the remodeling phase of wound healing takes place, FN serves as an attachment site for myofibroblasts and provides a surface on which movement of the different collagenase-secreting cells occurs (8-13).

FN is also found at sites of inflammation, as may be induced by repeated BCG instillations in the bladder, and appears to be necessary for normal function of the reticuloendothelial system (8, 12, 14-16). Decreased plasma FN levels and increased tissue FN deposition have been described after severe injuries, burns, and sepsis (17). Specific inflammatory conditions of the urinary tract, in the absence of surgical trauma, have also caused localized tissue FN deposition (18). FN augments chemotactic activity for peripheral monocytes (14). FN-coated particulate aggregates bind to macrophages and monocytes and, in some instances, FN appears to enhance phagocytosis (14-16). Previous studies have also shown that the FN molecule contains binding sites for staphylococci, streptococci, treponemes, and mycobacteria (19-22). During the formation of intraperitoneal abscesses, bacteria have been demonstrated to be incorporated into fibrin clots (23). Breakdown of these bacteria-laden clots with plasminogen activators has been shown to prevent collections of bacteria with the fibrin clots (23).

It is our hypothesis that BCG organisms are incorporated into the fibrin clot at sites of urothelial disruption through binding to FN and that this interaction is necessary for the antitumor effect of BCG on superficial bladder tumors. Thus, factors affecting fibrin clot formation may also affect the BCG-
FN interaction and antitumor efficacy. 

These studies were undertaken to modulate BCG-FN binding through the use of drugs which affect fibrin clot formation and fibrinolysis. EACA, a drug often used in the urinary tract to control bleeding, blocks the activation of plasminogen to plasmin and thereby inhibits fibrinolysis (24). It is considered to be the specific antidote for an overdose of fibrinolytic agents such as urokinase or streptokinase. EACA may prevent early clot lysis and prolong the interaction between BCG and FN. A prolonged interaction between BCG and FN might allow for further enhancement of the antitumor effect of BCG on superficial bladder tumors.

Heparin sulfate acts at multiple sites in the coagulation pathway to inhibit clot formation (24). Heparin inhibits the conversion of prothrombin to thrombin and the aggregation of platelets by thrombin. Since heparin inhibits fibrin clot formation, it may also inhibit BCG-FN binding and prevent BCG from exerting its antitumor effect on bladder tumors. Another anticoagulant, crystalline warfarin sodium, inhibits the synthesis of vitamin K-dependent clotting factors and thus inhibits clot formation through a mechanism unrelated to the heparin effect (24). If BCG-FN binding does take place during clot formation, then warfarin would also be expected to inhibit BCG-FN attachment and antitumor efficacy.

Both in vitro and in vivo studies were performed to determine the effect of modulation of fibrin clot formation by the use of EACA or heparin on BCG-FN binding. Furthermore, in vivo studies were performed to determine whether enhanced BCG-FN binding would correlate with an enhanced antitumor effect of BCG in a murine tumor model.

MATERIALS AND METHODS

Bacteria. Lyophilized BCG, Pasteur substrain (120 mg/vial; Armand Frappier, Québec, Canada), was used in these studies.

Radiolabeling of Bacteria. One 120-mg ampul of BCG was cultured for 7 days in Younan's medium at 37°C in 5% CO₂. Bacteria were washed in PBS (0.1 M, pH 7.2) and resuspended in RPMI 1640 supplemented with 0.2% l-glutamine, 0.2% asparagine, and 0.005% ferric ammonium citrate. [¹⁴C]Uracil (0.1 ml; 10 mcCi/ml) was added to the BCG and incubated for 48 h. Bacteria were washed twice in PBS and resuspended in PBS. The number of organisms was determined by measuring the absorbance at a wavelength of 520 nm and comparing it with a standard curve quantitating CFU from absorbance. Quantitation of BCG was periodically performed by subculture on Middlebrook 7H9 agar for comparison with a previously established absorbance curve.

Purification of FN. Human plasma FN was purified as previously described by Pommier et al. (14). Briefly, 10% polyethylene glycol 3350 precipitate from EDTA, benzamidine, and phenylmethylsulfonyl fluoride-treated plasma was resuspended in a buffer of 150 mM NaCl, 50 mM KH₂PO₄/K₂HPO₄, and 10 mM EDTA, pH 7.4. This plasma fraction was then absorbed by passage over gelatin-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), and the FN was purified by elution from gelatin-Sepharose with 1 M arginine. All buffers used for chromatography and elution contained 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and/or 25 mM p-nitrophenyl p'-guanidinobenzoate to inhibit serum proteases. The purified FN showed a single band on immunoelectrophoresis versus whole antihuman serum and a single major band at M, 440,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Upon reduction of disulfide bonds, sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a closely spaced doublet, as has been previously reported for human FN. Antibodies raised against this FN in rabbits gave a monospecific response on immunoelectrophoresis and Ouchterlony double diffusion against whole human plasma.

Radiolabeling of FN. Purified FN (300 μg) was iodinated with 0.5 mCi of Na[¹²⁵]I (Amerham Corp., Arlington Heights, IL) in a glass test tube coated with lodogen (1 mg) in chloroform (1 ml) evaporated under N₂ on ice for 20 min. The radiolabeled protein was desalted by passage over a 10-m1 Sephadex G-25 column precoated with bovine serum albumin (30 μg/ml) and eluted with PBS. Fifteen 1-ml aliquots were collected, and radioactivity was assayed on a gamma counter. Two peaks were observed, the first peak representing bound [¹²⁵]I-FN, and the second peak representing free [¹²⁵]I.

Trichloroacetic acid assay was used to confirm binding of [¹²⁵]I to the FN. The specific activity of the [¹²⁵]I-FN was routinely 10⁶-10⁷ cpm/μg.

Drugs. EACA (Lederle Parenterals, Inc., Carolina, Puerto Rico) was used at a concentration of 14 mg/ml. Heparin sulfate (Invex Laboratories, Division of Lymphomed, Inc., Melrose Park, IL) was used at a concentration of 300 units/ml. Warfarin sodium (E. I. duPont Company, Inc., Wilmington, DE) was used at a concentration of 0.1 mg/mouse.

Mice. C57/B6 and C3H/HEN mice were obtained from Charles River, housed in a limited access area, and given food and water ad libitum.

Tumors. The murine bladder tumor MB49 was maintained as a transplantable tumor in C57/B6 mice (25).

Antibodies. Antibodies to FN were prepared in rabbits and gave a monospecific response on immunoelectrophoresis and Ouchterlony double diffusion against whole human plasma.

In Vitro Matrix-FN Assay. Immunun Removawells (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 120 μg FN or 120 μg human serum albumin (controls) for 1 h. Wells were washed with PBS and then pretreated with 200 ml of EACA (14 mg/ml), heparin sulfate (300 units/ml), or PBS (controls) for 2 h. Following removal of the drug, [¹²⁵]I-BCG (2.5 x 10⁶ CFU) were added to the wells and incubated for 2 h at 37°C in 5% CO₂. Alternatively, the [¹²⁵]I-BGG was pretreated with EACA (14 mg/ml) or heparin sulfate (300 units/ml) for 2 h and then added to the FN- or HSA-coated wells for 2 h at 37°C in 5% CO₂. In a similar manner, dose-response curves were determined by pretreating the [¹²⁵]I-BGG with heparin (35, 17.5, 8.75, 4.4, or 2.2 units) for 2 h (PBS and controls) and then adding it to FN- or HSA-coated wells for 2 h at 37°C in 5% CO₂. Dose-response curves were also determined by pretreating the [¹²⁵]I-BGG for 2 h with EACA (1, 4, 0.7, 0.14, or 0.07 mg) or PBS (controls), and binding was assayed in the FN- or HSA-coated wells. The wells were washed twice with PBS and transferred to vials, and attached [¹²⁵]I-BGG was quantitated by liquid scintillation methods. Results were reproducible in 2 separate experiments.

In Vivo Localization of [¹²⁵]I-FN to the Injured Bladder. Mice were anesthetized with Nembutal i.p. (0.05 mg/g animal weight) and catheterized with a 24-gauge Teflon sheath (Viera, Division of Travenol Laboratories, Inc.). Bladder injury was induced by instillation of 0.1 ml of dilute acrolein or PBS (controls) for 30 min. Supraphysiological doses of soluble [¹²⁵]I-FN (250 mg/0.25 ml) or [¹²⁵]I-HSA (250 mg/0.25 ml) were injected into the tail vein. After 30 min, the mice were sacrificed, and the bladders were surgically removed and washed in PBS. The presence of [¹²⁵]I-protein was assayed by counting on a gamma counter.

Anticoagulation Studies. Mice were anticoagulated with either an i.v. bolus of heparin (30 units/tail vein) or crystalline warfarin sodium p.o. (0.1 mg/day) daily for 2 or 4 days. Prothrombin times and partial thromboplastin times were determined on pooled plasma samples from each group. Each group contained 10 mice, and the results were reproduced in 2 separate experiments.

In Vivo Adherence of BCG. Intravesical instillation of BCG was performed as previously described (21). Briefly, mice were anesthetized, and bladder injury was induced as described above. Mice then received [¹²⁵]I-FN (250 mg/0.25 ml) or [¹²⁵]I-HSA (250 mg/0.25 ml) were injected into the tail vein. Immediately after i.v. drug administration, the intravesical acrolein or PBS was aspirated, the bladder was washed with PBS, and then [¹²⁵]I-BCG (10⁶ CFU/0.1 ml) or PBS (controls) was instilled intravesically for 30 min. The mice were sacrificed, and the bladders were surgically removed and washed in PBS. The bladders were minced in 10 ml Scintiverse (Fisher Scientific Company), and adherence was determined by liquid scintillation counting. Results were reproducible in 2 separate experiments. In a separate
experiment, mice received either 0.1 mg warfarin p.o. or sterile water p.o. daily for 4 days prior to the induction of bladder injury or catheterization (controls). Mice were then received intravesically 0.1 ml acrolein to induce bladder injury or PBS (controls) for 30 min followed by 3H-BCG (10⁷ CFU/0.1 ml) for 30 min. The mice were again sacrificed; the bladders were removed, washed, and minced; and BCG adherence was counted by scintillation methods.

Dose-response studies were next initiated using heparin (30, 15, 5, or 2.5 units) and PBS (control) in 0.1-ml volumes injected per tail vein. Acrolein-induced bladder injury and 3H-BCG instillation were performed as described above. Dose-response studies were also performed using EACA (1.4, 0.7, 0.14, and 0.07 mg) and PBS (controls). Mice were injected with 0.1 ml of these solutions per tail vein. Acrolein-induced bladder injury and 3H-BCG instillation were also performed as described above. In the last adherence studies, mice had bladder injury induced by electrocautery injury and then received EACA (1.4 mg/tail vein) just prior to intravesical instillation of 3H-BCG treated with either rabbit anti-mouse FN IgG antibodies (83 μg/mouse) or rabbit IgG (78 μg/mouse) (controls).

**In Vivo Therapy Study.** C57/B6 mice were anesthetized and catheterized as described above. Anesthetized mice were secured on a ground plate, and an electrode consisting of an Amplatz curved guidewire (Cook, Inc., Bloomington, IN), insulated by a 24-gauge Teflon sheath, was inserted urethra into the bladder. The electrode was attached to a Bovie cautery unit. While the tip of the electrode was inside the bladder, the cautery apparatus was activated for 3 s at the lowest coagulation setting. Following removal of the electrode, a single cell suspension of MB49 tumor cells (10⁶ cells) was instilled intravesically. Twenty-four h after tumor implantation, therapy with intravesical BCG was initiated and continued for 5 weeks. Mice were monitored for the development of bladder tumors and were sacrificed at the end of 5 weeks. The presence or absence of bladder tumors was verified by gross and histological sections. Initially, dose-response studies were performed with BCG (10⁵, 10⁶, and 10⁷ CFU/instillation). Next, therapy studies were performed with the addition of EACA or heparin i.v. to the intravesical BCG regimen. Twenty-four h after tumor implantation, mice received either EACA (14 mg/ml) or PBS (controls) (0.1 ml/tail vein), followed by intravesical instillation of BCG. A suboptimal BCG dose (3.5 × 10⁵ CFU) was chosen to test for an enhanced antitumor effect in the presence of EACA. Mice were treated weekly for 5 weeks and monitored for the development of bladder tumors. At the end of 5 weeks, mice were sacrificed, and the presence or absence of bladder tumors was verified by gross examination and histological sections.

In a separate experiment, mice underwent tumor implantation as described above, and treatment was initiated 24 h later with either heparin sulfate (300 units/ml) or PBS (controls) (0.1 ml/tail vein), and intravesical instillation of BCG was begun at an optimal dose (10⁷ CFU). Mice were treated weekly for 5 weeks and monitored for the development of bladder tumors. At the end of 5 weeks, mice were sacrificed, and the presence or absence of bladder tumors was verified by gross examination and histological section. In the final study, mice again underwent tumor implantation as described, and treatment was initiated 24 h later with either heparin sulfate (300 units/ml) or PBS (controls) (0.1 ml/tail vein), and intravesical instillation of BCG was begun at an optimal dose (10⁷ CFU). Mice were treated weekly for 5 weeks and monitored for the development of bladder tumors.

**BCG Retention by Bladders, Lymph Nodes, and Spleens.** In the EACA therapy study the bladders, iliac lymph nodes, and spleens were harvested from tumor-free mice under sterile conditions and cultured for the presence of BCG at the conclusion of the study. The bladders were digested in 2 ml of RPMI with type 1V collagenase (1 mg/ml) and hyaluronidase (0.5 mg/ml) for 1 h. The lymph nodes and spleens were minced finely with scissors and crushed with a glass syringe plunger in 2 ml of Youman’s medium and 0.5 ml of 1.5% agar to form a semisolid agar. Tubes were incubated for 3 weeks at 37°C in 5% CO₂. The number of CFU per organ was then quantitated from the semisolid agar tubes. Statistics. Student’s t test was performed on the data from these studies.

**RESULTS**

**In Vitro Matrix-FN Assay.** These experiments demonstrated that neither pretreatment of the FN-coated wells nor administration of 3H-BCG with either EACA or heparin increased BCG-FN binding beyond that in PBS controls (Fig. 1). We noted an inhibitory effect of heparin on BCG-FN binding that was statistically significant at higher concentrations as demonstrated by dose-response curves. EACA did not appear to affect 3H-BCG-matrix-FN binding at any concentration.

**In Vivo Localization of 12SI-FN to the Injured Bladder.** In the absence of bladder injury, neither 125I-FN nor 125I-HSA localized to the bladder. However, after induction of bladder injury with acrolein, 125I-FN localized to the bladder to a greater degree than 125I-HSA (P < 0.05) (Fig. 2).

**Anticoagulation Studies.** A bolus of i.v. heparin (30 units) induced a prolongation of the partial thromboplastin time to more than 100 s. Administration of warfarin p.o. (0.1 mg/day) for 4 consecutive days also induced a prolongation of both the prothrombin time and the partial thromboplastin time to more than 100 s. Thus, both heparin and warfarin were proved to induce the expected anticoagulated state in mice in the doses given.

**Fig. 1. A, FN-coated wells were pretreated with 200 μl of EACA (14 mg/ml), heparin sulfate (300 units/ml), or PBS (controls); 3H-BCG were then added to the wells. No statistically significant differences in BCG-FN binding were noted between EACA or control groups. Heparin inhibited BCG-FN binding to a significant degree in series 1 (P < 0.05); B, 3H-BCG were pretreated with EACA (14 mg/ml), heparin sulfate (300 units/ml), or PBS controls and then added to FN-coated wells. An inhibitory effect of heparin sulfate on BCG binding was noted in comparison to the effect on PBS- or EACA-treated groups and was statistically significant in some experiments (P < 0.05; series 2).**

**Fig. 2. A, BN-BCG (CPM X 1000) Bound 3H-BCG (CPM X 1000) Series 1 Series 2 B**

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MODULATION OF FN-BCG ATTACHMENT TO MURINE BLADDER

**Fig. 2.** In the presence of bladder injury, ^{125}I-FN localized to the bladder to a significantly greater degree than did ^{125}I-HSA (P < 0.05).

**Fig. 3.** In the presence of bladder injury, exogenous soluble FN significantly enhanced ^{3}H-BCG attachment to the bladder (P < 0.05).

**In Vivo Adherence of BCG.** In the absence of bladder injury, neither FN nor HSA demonstrated an effect on ^{3}H-BCG attachment to the bladder. However, in the acutely injured murine bladder, administration of exogenous soluble FN enhanced ^{3}H-BCG attachment to the bladder significantly more than in controls (P < 0.05) (Fig. 3). In the absence of bladder injury, EACA and heparin showed a minimal effect on ^{3}H-BCG binding to the murine bladder. However, in the presence of acrolein-induced bladder injury, EACA significantly enhanced ^{3}H-BCG binding to the murine bladder (P < 0.05) (Fig. 4A). EACA enhancement of ^{3}H-BCG binding was blocked in the presence of anti-FN antibodies but not control IgG (Fig. 4B).

Heparin significantly inhibited ^{3}H-BCG binding to the acrolein-injured murine bladder (P < 0.05) (Fig. 4A). Inhibition of ^{3}H-BCG binding was also demonstrated in mice anticoagulated with warfarin (P < 0.005) (Fig. 4C). Heparin inhibited ^{3}H-BCG attachment to the injured murine in a dose-dependent manner, while EACA enhanced ^{3}H-BCG attachment in a dose-dependent manner.

**In Vivo Therapy Study.** In vivo therapy studies testing the effects of varying doses of BCG on the outgrowth of the murine MB49 tumor were performed in 3 separate experiments to determine the optimal BCG dose (10^7 CFU). Therapy studies were next initiated with the addition i.v. of EACA or heparin to the intravesical BCG treatments. Experiments were performed in duplicate, and the results were combined. These studies demonstrated that BCG at a suboptimal dose of 3.5 × 10^7 CFU intravesically was effective in preventing tumor out-
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Fig. 5. A combination of two separate experiments showed a statistically significant difference between antitumor efficacy in mice receiving intravesical BCG plus i.v. EACA and its efficacy in those receiving intravesical BCG plus i.v. PBS. EACA administered alone i.v. showed no effect on prevention of tumor growth.

Fig. 6. In A, with an optimal dose of BCG (10^7 CFU), i.v. heparin sulfate was found to abrogate the antitumor efficacy of intravesical BCG as compared to the efficacy of intravesical BCG with i.v. PBS (P < 0.005) (two studies combined). In B, with an optimal dose of BCG (10^7 CFU), warfarin p.o. was found to abrogate the antitumor efficacy of intravesical BCG as compared to the efficacy of intravesical BCG therapy with sterile water p.o. (P < 0.05).

growth in treated mice as compared to control mice (Fig. 5). Addition of EACA (1.4 mg i.v.) plus BCG intravesically significantly enhanced antitumor activity, while EACA without BCG did not affect tumor outgrowth. A suboptimal BCG dose was necessary in these studies to show the enhanced effect in the presence of EACA.

Experiments using heparin (30 units i.v.) or warfarin (0.1 mg p.o.) to inhibit clot formation supported the hypothesis that BCG attachment and subsequent antitumor activity are associated with the fibrin clot. Addition of either heparin or warfarin concomitantly with intravesical BCG at the optimal dose abrogated the antitumor activity of BCG alone (P < 0.05) (Fig. 6).

BCG Retention by Bladders, Lymph Nodes, and Spleens.

Seven days after the final treatment with BCG, mice were sacrificed, and the BCG content of bladder, lymph nodes, and spleens was quantitated. These studies demonstrated a significantly greater number of BCG organisms in the lymph nodes and spleens of mice receiving EACA i.v. plus intravesical BCG than in mice receiving PBS i.v. and intravesical BCG (P < 0.05) (Table 1). BCG organisms were observed in 1 of 6 bladders in the EACA group, while none were found in BCG/PBS bladders. BCG organisms were not observed in the spleens or lymph nodes of mice receiving intravesical BCG therapy without concomitant i.v. administration of EACA.

DISCUSSION

Although the mechanism by which BCG exerts an antitumor effect on superficial bladder tumors is not fully elucidated, the original paper by Morales et al. (1) suggested that close contact between the BCG organisms and tumor cells was important to the expression of the antitumor effect. Furthermore, a vigorous inflammatory response to BCG has been correlated with a positive antitumor response in patients with bladder tumors (1–4). Histological studies have demonstrated BCG attachment primarily at sites of urothelial disruption. In vitro studies have demonstrated that BCG binds preferentially to FN as opposed to other matrix proteins (5, 6). Further, in vitro studies have demonstrated that anti-FN antibodies can block the attachment of BCG to matrix-FN, and in vivo studies have confirmed that anti-FN antibodies can block BCG attachment to injured murine bladder. Finally, blocking the attachment of BCG to the

Table 1  BCG retention by bladders, lymph nodes, and spleens after BCG therapy with and without i.v. EACA

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bladder abrogates the antitumor effect of BCG on the murine MBT-2 tumor (7).

FN is a glycoprotein with important functions in both the reticuloendothelial system and fibrin clot formation and subsequent wound healing (8–22).

Earlier laboratory work suggested that the antitumor effect of BCG on superficial bladder tumors is initiated by attachment to FN (5–7). The data presented herein further support this hypothesis. First, in the in vitro matrix-FN assays, BCG is again noted to attach to FN more than to HSA controls. As expected, drug modulation of the coagulation pathway did not appear to enhance BCG-FN binding in the absence of the normal components of the coagulation pathway necessary for fibrin clot formation. The apparent inhibitory effect of heparin on matrix-FN-BCG binding may be due to a specific binding site for heparin on the BCG molecule located near the binding site for the BCG or to heparin attachment to the FN molecule which occurs in the region of presumed BCG attachment. In vivo localization studies with radiolabeled FN demonstrated accumulation of FN but not of radiolabeled HSA in the injured bladder but not in the intact bladder. Next, in the in vivo BCG adherence model in which the murine urothelium was chemically or electrically injured and all the components of the coagulation pathways were operational, EACA markedly enhanced BCG attachment to the injured bladder, presumably through stabilization of the fibrin clot. Anti-FN antibodies were demonstrated to directly inhibit the EACA enhancement of H-BCG attachment to the acutely injured bladder (Fig. 4B). Heparin sulfate, which has an inhibitory action at several sites in the normal coagulation pathways, was shown to inhibit BCG binding to the injured urothelium in vivo. Although heparin may also directly inhibit BCG attachment to FN, we believe that, under the conditions of these in vivo adherence studies, the observed inhibitory activity was associated with inhibition of clot formation (Fig. 4A). To further test this hypothesis, another anticoagulant, crystalline warfarin sodium, which inhibits clot formation through a mechanism different from that of heparin, was tested and also found to prevent BCG attachment to the injured murine bladder (Fig. 4C). Furthermore, these drugs appeared to have minimal effect on BCG attachment in the absence of bladder injury. Both heparin and warfarin were confirmed with pooled plasma samples to induce an anticoagulated state in mice at the dose given. These data further support the hypothesis that BCG-FN binding takes place at sites of urothelial disruption and that BCG may be incorporated into the fibrin clot through attachment to FN. Furthermore, it appears that BCG-FN binding can be modulated through drugs which modulate fibrin clot formation/fibrinolysis.

As a further extension of the hypothesis that BCG-FN binding is necessary for the antitumor effect of BCG on superficial bladder tumors and that modulation of attachment may enhance antitumor activity, studies were undertaken to determine whether enhanced BCG-FN binding correlated with an enhanced antitumor effect of BCG. In the MB49 bladder tumor model, tumor outgrowth was prevented in 16 of 20 mice receiving EACA i.v. plus intravesical BCG (3.5 × 10^8 CFU) and in only 9 of 20 mice receiving suboptimal intravesical BCG and i.v. PBS. This difference was statistically significant (P < 0.05). Studies were also undertaken to determine whether inhibition of BCG-FN binding through inhibition of clot formation with anticoagulants would correlate with abrogation of the antitumor effect of BCG. Administration of heparin sulfate i.v., concomitant with intravesical BCG (10^7 CFU) therapy, was ineffective in preventing tumor outgrowth in 20 of 20 mice using the MB49 bladder tumor model. In comparison, tumor outgrowth was prevented in 7 of 20 mice receiving PBS i.v. (0.1 ml) and intravesical BCG (10^7 CFU). Warfarin administration, concomitant with intravesical BCG (10^7 CFU) therapy, was also ineffective in preventing tumor outgrowth in 9 of 10 mice using the MB49 bladder tumor model. In comparison, tumor outgrowth was prevented in 9 mice receiving sterile water and BCG therapy.

Taken together, the EACA and anticoagulation therapy studies demonstrate that stabilization of clot formation correlates with enhanced antitumor efficacy while inhibition of clot formation abrogates the antitumor efficacy of intravesical BCG.

It has been suggested that an immunological response to BCG antigens is important for expression of the antitumor effect of BCG on superficial bladder tumors (2, 26). Both a delayed hypersensitivity reaction to purified protein derivative and granulomatous inflammation in the bladder following intravesical BCG therapy have been correlated with a tumor-free response to this therapy (2, 26). Augmented natural killer cell activity has been correlated with the antitumor activity of BCG (27), and the presence of T-lymphocytes has been shown to be a requirement for the antitumor effect of BCG in a murine bladder tumor model (28). Further indirect evidence that BCG are ingested and presented as antigens to the immune system is suggested by the presence of BCG organisms in the lymph nodes and spleens of mice achieving a tumor-free response following intravesical BCG administration (Table 1). Furthermore, there is a direct correlation between the number of BCG organisms retained in the lymph nodes and spleens and the enhanced antitumor activity noted with i.v. EACA to intravesical BCG therapy. These observations provide further indirect evidence that immunological mechanisms are involved in the antitumor effect of intravesical BCG on superficial bladder tumors.

In conclusion, these studies add further support to the hypothesis that BCG-FN attachment is a requisite for expression of the antitumor effect of BCG. These data further suggest that BCG attach to FN incorporated in the fibrin clot and that modulation of this interaction with drugs such as EACA enhances the effectiveness of BCG. Taken together, these data suggest that the clinical efficacy of intravesical BCG against bladder tumors may be enhanced by enhancing BCG attachment to the bladder at sites of urothelial disruption.

REFERENCES


* Unpublished observation.


Modulation of Fibronectin-mediated *Bacillus Calmette-Guérin* Attachment to Murine Bladder Mucosa by Drugs Influencing the Coagulation Pathways


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