Pathophysiology of Transitional Tumor Cell Adherence to Sites of Urothelial Injury in Rats: Mechanisms Mediating Intravesical Recurrence Due to Implantation

William A. See, Jeffrey S. Miller, and Richard D. Williams
Department of Urology, University of Iowa, Iowa City, Iowa 52242

ABSTRACT

The mechanism by which transitional tumor cells adhere to areas of urothelial injury and the means by which heparin prevents this phenomenon were studied. Scanning electron microscopy and a radiolabeled tumor cell adherence assay were used to assess the activity of heparin and a "nonglycosaminoglycan" thrombolytic agent, recombinant tissue plasminogen activator, in preventing tumor cell adherence to areas of urothelial injury. Systemically administered heparin and intravesical therapy with recombinant tissue plasminogen activator duplicated the activity of intravesical heparin. Scanning electron microscopy showed tumor cells entrapped at the injury surface in a RBC/fibrin clot, which was prevented by intravesical heparin. These data suggest that clotting cascade activation by urothelial injury is the mechanism by which particulate adherence to the urothelium occurs. Interruption of this process by local or systemic anticoagulation with heparin or shifting of the equilibrium of clot formation/lysis toward thrombolysis with recombinant tissue plasminogen activator prevents tumor cell adherence. Intravesical thrombolytic therapy may represent a new approach to recurrence prophylaxis for superficial bladder carcinoma.

INTRODUCTION

A number of investigators have implicated the GAG layer of the bladder as playing a crucial role in preventing the adherence of particulate matter to the bladder surface. Impairment or depletion of this functional layer is postulated to predispose the bladder to urinary tract infection through increased bacterial adherence or increased bladder tumor recurrence rates due to tumor cell adherence and implantation at the time of resection (1–5). Intravesical administration of exogenous glycosaminoglycan (heparin) into "GAG-depleted" bladders has been shown to decrease crystal, bacterial, or tumor cell adherence rates to the same as those of uninjured bladders. GAG reppletion by heparin has been theorized as the mechanism by which bladder antiadherencetion integrity is restored (4, 6–9). An alternative hypothesis suggests that the adherence of particulate matter to areas of urothelial injury is mediated by nonspecific entrapment in fibrin clot at the site of injury (10, 11). The purpose of this study was to elucidate the mechanism by which microscopic particles (tumor cells) adhere to areas of urothelial injury and to determine how heparin prevents this adherence.

MATERIALS AND METHODS

Animals

Ten- to 12-week-old syngeneic female F-344 rats (Simonson Laboratories, Gilroy, CA) were utilized for all experiments. Animals were given standard rat chow and water ad libitum.

Received 4/21/89; accepted 6/23/89.

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1 This work was supported in part by a grant from the American Foundation for Urologic Disease.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: GAG, glycosaminoglycan; SEM, scanning electron microscopy; rt-PA, recombinant tissue plasminogen activator; PBS, phosphate-buffered saline; AHC, antithrombin heparin cofactor.

Tumor

The 3-methylcholanthrene-induced transitional cell carcinoma 4909 was utilized in all experiments (12). The tumor is maintained by s.c. passage in syngeneic F-344 rats. Sterile single-cell suspensions for use in the cellular adherence assay were prepared as previously described (13). Viability of the tumor cell suspension was determined by trypsin blue exclusion. The concentration of viable tumor cells used in all experiments was 1 × 10⁶/m.

Experimental Model

The in situ catheterized rat bladder served as the model for cellular adherence (14). In this system all bladder manipulations are performed transurethrally. Animals anesthetized with i.p. Nembutol (65 mg/kg) are catheterized with a 1-inch, 22-gauge Jelco catheter (Critikon, Tampa, FL). The bladder is exposed through a midline incision and a 4-O surgical steel wire is advanced through the catheter until it is observed to contact the dome of the bladder. With the animal on a grounding plate (Bovie; Sybron Corp., Cincinnati, OH), electrocautery current is applied to the wire for a duration of 1 s. Depending upon the experimental protocol, labeled or unlabeled tumor cells are instilled into the bladder following the cautery injury. Variables influencing the size of tumor inoculum on the area of urothelial injury are evaluated using the tumor cell adherence assay.

Tumor Cell Adherence Assay

In the tumor cell adherence assay, a single-cell suspension of the tumor is pulse labeled with [125I]iododeoxyuridine (50 μCi/ml) (Amersham Corp., Arlington Heights, IL) for 1 h at 37°C, after which the cells are washed 3 times in RPMI 1640 medium with 10% fetal calf serum. An aliquot of the final cell suspension is serially diluted for use in the generation of a cell number-to-radioactivity standard curve. Subsequent to bladder injury, and appropriately timed relative to the variable being studied, animals receive a transurethral intravesical instillation of the 125I-labeled tumor cell suspension. After experimental manipulation the bladder is removed by transection at the ureterovesical junction and placed in a gamma counting vial. Specimens are counted on a Beckman model 300 gamma counter (Beckman Instrument Co., Irvine, CA). The adherent cell number is calculated by comparing sample radioactivity to points on the standard curve.

Scanning Electron Microscopy

Specimens fixed in 2.5% glutaraldehyde were washed, dehydrated by passage through graded ethanol solutions, and critical point dried. Specimens were sputter-coated with gold-palladium in an EM-Scope SC-500 (EM-Scope, Cambridge, England). Scanning electron micrographs were obtained using a Hitachi S-570 scanning electron microscope (Hitachi, Mito, Japan).

Effect of Intravesical Heparin

Experiments 1 and 2 evaluated the impact of the timing of heparin administration on its ability to prevent tumor cell adherence to cauter-injured bladders.

Experiment 1. Preadherence Intravesical Heparin. Following bladder fulguration, animals received a 0.2-ml intravesical instillation of heparin (10 mg/ml in PBS) (six rats) or 0.2 ml of PBS (six rats) for a period of 15 min. At the end of this time the bladders were emptied and 0.2 ml of the 125I-labeled tumor cell suspension was instilled. Following a 30-min dwell time, the bladders were emptied, washed 3 times with
were in situ distended with 2.5% glutaraldehyde, ligated at the bladder to visualize the injury site in control and heparin-treated bladders. Experimental tumor cells were used. Following the final bladder wash, the specimens heparin or PBS remained in the bladder for 30 min and unlabeled were washed 3 times with 0.3-ml aliquots of either rt-PA (1 mg/ml) (six rats) or PBS (six rats). Each wash was allowed to remain in the bladder for a period of 10 min. After the last wash, the bladder was removed and processed for the radioactive adherence assay.

Effect of Systemic Heparin

Heparin prevention of tumor cell adherence by repletion of the GAG layer at sites of urothelial injury would require the heparin-containing solution to come in contact with the bladder surface. In the absence of any potential urinary excretion of a "GAG-repleting substance," systemic heparinization should have no impact on tumor cell adherence. However, if the antiadherence effect of heparin is mediated by its anticoagulant activity, systemic heparinization should be similar to intravesical heparin in its ability to prevent cellular adherence. Experiment 3 was designed to differentiate an antiadherence effect of heparin resulting from its anticoagulant activity from one resulting from "GAG repletion."

Experiment 3. Systemic Heparinization. Five min prior to bladder injury, animals were given an intra-vena caval injection of 0.5 ml of either heparin (1000 units/ml) (six rats) or normal saline (six rats). In order to preclude the possibility of urinary excretion of a GAG-repleting factor, animals underwent bilateral renal pedicle ligation immediately prior to the administration of the heparin or saline. Thirty min after bladder fulguration, the bladders were washed once with 0.3 ml of phosphate-buffered saline, after which 0.3 ml of the labeled tumor cell suspension was instilled for a period of 30 min. The bladders were then processed as described in experiment 1.

Effect of Intravesical rt-PA

Tissue-type plasminogen activator is an enzyme present in human tissues which, in the fibrinolytic system, causes proteolytic conversion of the zymogen plasminogen to the active molecule plasmin. Plasmin then functions to lyse fibrin clot. Recently, the DNA segment coding for tissue plasminogen activator has been isolated and cloned (15). Pharmacological amounts of rt-PA are now available and have undergone clinical trials as thrombolytic agents (16). As a large protein molecule, rt-PA has none of the chemical properties of glycosaminoglycans. If fibrin clot formation subsequent to urothelial injury, rather than GAG depletion, mediates tumor cell adherence, intravesical rt-PA should be effective in preventing adherence. In experiments 4 and 5 rt-PA was administered intravesically in an effort to determine if fibrin clot lysis could decrease tumor cell adherence to areas of urothelial injury or remove already adherent tumor cells.

Experiment 4. Preadherence rt-PA. Following fulguration injury, animals received an intravesical instillation of 0.3 ml of either a 1 mg/ml solution of rt-PA (Genentech, San Francisco, CA) (six rats) or PBS (six rats). After a 30-min dwell time, the bladders were emptied and 0.3 ml of the radiolabeled tumor cell suspension was instilled. Thirty min later, the bladders were emptied, washed 3 times with 0.3-ml aliquots of PBS, and removed for gamma counting.

Experiment 5. Postadherence rt-PA. Following fulguration injury, animals received an intravesical instillation of 0.3 ml of the radiolabeled tumor cell suspension. After 30 min, the bladders were emptied and washed 3 times with 0.3-ml aliquots of either rt-PA (1 mg/ml) (six rats) or PBS (five rats). Each wash was allowed to remain intravesically for 10 min. Following the final wash, the bladders were removed for gamma counting.

Experiment 6. SEM of Cautery-injured Bladders with and without Heparin. In this experiment, scanning electron microscopy was used to visualize the injury site in control and heparin-treated bladders. Experiment 6 followed the same protocol as experiment 1, except that the heparin or PBS remained in the bladder for 30 min and unlabeled tumor cells were used. Following the final bladder wash, the specimens were in situ distended with 2.5% glutaraldehyde, ligated at the bladder neck, removed, and placed in 2.5% glutaraldehyde. The specimens were then processed for SEM as described above. Two animals received heparin and two saline. Additional controls consisted of a single animal which was catheterized but received no other manipulation, one animal which received a cautery injury and subsequently received a cell-free medium instillation, and one animal which was sham-cauterized after which it was processed as per the PBS treatment group in experiment 1.

Statistical Analysis

Tumor cell adherence data were analyzed with nonparametric methodology, using the Mann-Whitney U test. Differences between experimental and control groups were considered significant at P < 0.05.

RESULTS

The results of experiments employing the radiolabeled tumor cell adherence assay (experiments 1, 2, 3, 4, and 5) are shown in a combined graph in Fig. 1.

Experiment 1. Preadherence Intravesical Heparin. Mean tumor cell adherence ± SD was 1163 ± 824 cells/bladder in the heparin-treated group and 6397 ± 2782 cells/bladder in the saline-treated group (P < 0.02; Mann-Whitney U test).

Experiment 2. Postadherence Intravesical Heparin. Mean tumor cell adherence ± SD was 1917 ± 6017 cells/bladder in the heparin-treated group and 21892 ± 17247 cells/bladder in the saline-treated group (P = 0.11; Mann-Whitney U test).

Experiment 3. Systemic Heparinization. Mean tumor cell adherence ± SD was 3907 ± 1784 cells/bladder in systemically heparinized animals and 17540 ± 7918 cells/bladder in the saline-treated group (P < 0.004; Mann-Whitney U test).

Experiment 4. Preadherence rt-PA. Mean tumor cell adherence ± SD was 3770 ± 3045 cells/bladder in the rt-PA group and 26920 ± 13293 cells/bladder in the control group (P < 0.01; Mann-Whitney U test).

Experiment 5. Postadherence rt-PA. Intravesical rt-PA, administered subsequent to tumor cell adherence, was as effective as preadherence administration in reducing the size of the tumor cell inoculum. Mean tumor cell adherence ± SD was 3514 ± 2914 cells/bladder in the rt-PA group and 40528 ± 26920 cells/bladder in the control group (P < 0.05; Mann-Whitney U test).

Fig. 1. Mean tumor cell adherence in injured and bladders in experiments varying the agent administered as well as the timing and route of anticoagulant given. Experimental groups are compared to their corresponding control group. (Preheparin, experiment 1; post-heparin, experiment 2; sys-heparin, experiment 3; pre-rtPA, experiment 4; post-rtPA, experiment 5).
Experiment 6. SEM of Cautery-injured Bladders with and without Heparin. Scanning electron micrographs of the injury sites of saline-treated bladders (Fig. 2) demonstrated a layer of RBC and fibrin overlying the area of coagulation. Enmeshed in this clot were numbers of visibly larger cells, consistent with tumor cells. These large cells were not observed in the bladder of the coagulated animal which received the cell-free medium only. The injury sites of heparin-treated animals were remarkable for the absence of both the clot and the larger cell population (Fig. 3).

DISCUSSION

Clinical treatment of superficial bladder carcinoma proceeds in a fashion highly analogous to our experimental model. Piecemeal transurethral tumor removal releases viable tumor cells into the intravesical fluid in direct contact with sites of cautery or mechanical injury. Although the relative contribution of tumor implantation to overall intravesical recurrence rates is unknown, there is a large body of literature to support the contention that it plays some role (17–19). Transitional tumor cell adherence to the urothelial surface is a requisite step in the sequence of events necessary for intravesical recurrence due to implantation (13). The ability of the intact uroepithelium to resist tumor cell adherence and the predilection of injured urothelium to serve as a site for adherence and implantation are well documented (4). The mechanism by which intravesical particulate matter adheres to areas of urothelial injury has been an area of controversy. Parsons et al. (20) have demonstrated that the innermost surface of the bladder is lined with a glycosaminoglycan layer, which they postulated was responsible for imparting an "antiadherence integrity" to the urothelium. Physical or chemical injury to the bladder is associated with a marked increase in the adherence of bacteria, tumor cells, or crystals to the urothelium, due in theory to depletion of the GAG layer (4, 20–22). The intravesical administration of exogenous heparin (a sulfated GAG) has been shown to restore antiadherence integrity to areas of urothelial injury (4, 21, 23).

Studies which have investigated the mechanism of action of heparin in preventing the adherence of particulate matter have yielded interesting results. Heparin has been shown to bind exclusively to areas of urothelial injury (21–23). Its activity is exerted by some effect on the bladder surface rather than on the particulate matter present in the bladder (4, 22). In the presence of protamine, the ability of heparin to prevent particulate adherence to areas of injury is ablated (24). Protamine alone had no effect on adherence (21, 24). Pretreatment of bladder injuries with heparin results in a much greater decrease in particulate adherence than does heparin treatment subsequent to exposure of the urothelium to the adherent material in question (25). In one study heparin alone, of a number of sulfated glycosaminoglycans, sulfated polysaccharides, and monosaccharides tested, was able to abolish crystal adherence to areas of urothelial injury (21). Other groups found additional molecules, with weak anticoagulant properties, which exert an antiadherence effect similar to heparin (25, 26).

One theory explaining the action of heparin is that, following binding to areas of urothelial injury, hydrophilic segments of the sulfated glycosaminoglycan bind to water molecules in a micelle fashion, thus forming a nondisplaceable water layer at the luminal interface. This layer is thought to be responsible for preventing the adherence of a variety of molecular and macromolecular species (27). This theory is supported by known chemical properties of sulfated glycosaminoglycans and the fact that heparin has been shown to bind to areas of urothelial injury.

An alternative explanation for adherence following urothelial injury is the presence of a "glueing substance" produced by injured cells or leaked from blood vessels at the site of injury (21). In this model, heparin would function to inactivate the glueing substance. Support for this theory comes from several scanning electron microscope studies in which cells and crystals were shown to be trapped on the luminal surface at the area of injury by a network of fibrillar material (11, 28). Immunofluorescent staining for fibrin demonstrated strong fluorescence only at sites of injury. These authors postulated that fibrin may be in part responsible for particulate trapping at sites of injury.

Fig. 2. Bladder injury site of an animal given intravesical saline prior to exposure to tumor cells. RBC, with an intermixed population of large cells with ruffled membranes, are present at the luminal surface. Right, 2 × enlargement of the bordered area on the left (RBC diameter, 3.5 μm).
In considering the above evidence, several questions arise. What is heparin binding to at the injury site? How does heparin inactivation by protamine impair its antiadherence activity? Of all the studied glycosaminoglycans, why do those effective in preventing adherence all have some anticoagulant activity? Although less effective than pretreatment, how does postadherence exposure to heparin decrease adherence?

All of these questions can be addressed if activation of the clotting cascade with ultimate formation of a fibrin clot, and entrapment of macromolecular particles, occurs at sites of urothelial injury. Thrombin is the enzyme responsible for both the conversion of fibrinogen to fibrin and the subsequent polymerization of the fibrin molecule. Heparin functions by binding to AHC. AHC is an essential cofactor for the action of heparin and, in its absence, neutralizes the activity of thrombin by slowly forming a 1:1 enzyme-inhibitor complex. Heparin binding to AHC results in a conformational change which accelerates the rate of formation of the enzyme-inhibitor complex approximately 1000-fold (29). As a result, the presence of heparin in almost instantaneous inactivation of thrombin and thereby prevents fibrin formation.

Following urothelial injury resulting in activation of the clotting cascade, heparin could be expected to bind to AHC and, subsequently, thrombin at the injury site. Thrombin inactivation would preclude the formation of polymerized fibrin and the entrapment of particulate matter. Protamine inhibition of heparin binding to AHC would interrupt this cascade, thereby allowing fibrin formation and particulate adherence to proceed. Heparinization subsequent to adherence would be anticipated to shift the equilibrium of the clotting cascade in favor of fibrinolysis and clot breakdown, potentially releasing trapped particles from the injured surface.

The present study was designed to delineate the mechanism responsible for tumor cell adherence at sites of urothelial injury and define the role of heparin in preventing adherence. We have demonstrated in this study that systemic anticoagulation with heparin, in the absence of any urinary excretion, significantly decreases tumor cell adherence to injured urothelium. Recombinant tissue plasminogen activator, a large protein molecule without GAG-repleting properties, was effective in preventing tumor cell adherence when administered intravesically. SEM of heparin- or saline-treated bladders exposed to tumor cells showed tumor cells enmeshed in a RBC clot overlying the site of injury in saline-treated bladders. These data suggest that tumor cell adherence occurs by entrapment of tumor cells in a RBC/fibrin clot resulting from activation of the clotting cascade subsequent to urothelial injury. Systemic or local anticoagulation with heparin or rt-PA prevents tumor cell adherence by preventing clot formation.

Although the experiments in this study were not designed to study the relative efficacy of intravesically administered anticoagulants, it is of interest to compare the ratio of adherent cells in experimental to control groups among the different treatment regimes. The intravesical administration of either heparin or rt-PA following injury but prior to tumor cell exposure would be anticipated to prevent, or decrease, clot formation and thereby decrease subsequent tumor cell adherence. The intravesical administration of heparin subsequent to tumor cell entrapment by clot should prove to be less effective than preadherence administration, because heparin prevents further clot formation but is not actively thrombolytic. In contrast, rt-PA as an active inducer of thrombolysis should be more effective than intravesical heparin in the post-adherence setting. Fig. 4 illustrates the ratio of the number of adherent cells in the intravesical anticoagulant-treated groups to the number of cells adherent in their corresponding control group and supports the postulated relationships. Future studies designed to specifically address the issue of relative efficacy will be necessary to confirm these findings.

Conclusions

Tumor cell adherence to sites of urothelial injury results from tumor cell entrapment in RBC/fibrin clots that are formed subsequent to clotting cascade activation by urothelial injury. We propose clot-mediated particulate entrapment as a general mechanism responsible for luminal surface adherence of intravesical macromolecular species subsequent to urothelial injury. Intervention in this pathway in the clinical setting should allow the relative contribution of tumor implantation in overall
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