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Urinary trypsin levels observed in pancreas transplant patients with duodenocystostomies promote in vitro fibrinolysis and in vivo bacterial adherence to urothelial surfaces*

W. A. See[1] and J. L. Smith[2]

Departments of [1]Urology and [2]Surgery, The University of Iowa Hospitals and Clinics, Iowa City, Iowa, USA

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Summary. This study evaluated the role of activated urinary trypsin in mediating the increased incidence of infectious and hemorrhagic lower urinary tract complications in pancreas transplant patients with pancreatico-duodenocystostomies (PDC). The effect of trypsin concentrations corresponding to those observed in the urine of PDC patients were studied using an in vitro assay of clot lysis and an in vivo assay of bacterial-urinary tract adherence. Results were compared with those of parallel assays performed using urine from a pancreas transplant patient with a duodenocystostomy and control human urine. All trypsin concentrations studied demonstrated fibrinolytic activity. Fibrinolysis increased as a direct function of both trpysin concentration and duration of substrate exposure ($P < 0.0001$). Fibrin lysis resulting from the urine of the transplant patient was 4.6 times greater than that predicted based upon assays of total trypsin content in the sample. Fibrinolytic activity in control urine specimens was 0.16% of that observed in transplant urine specimens. Exposure of the rat urinary bladder to 200 μg/ml trypsin concentrations, or transplant urine, resulted in a significant increase in bacterial adherence over that seen in control urine from treated animals ($P < 0.05$). These findings demonstrate a significant effect of urinary trypsin on physiologic processes involved in hemostasis and the prevention of urinary tract infection. Active urinary trypsin may play an etiologic role in hemorrhagic and infectious lower urinary tract complications observed in patients with a PDC.

Key words: Pancreatic transplantation – Bladder injury – Trypsin – Infection – Hemorrhage

Whole organ pancreatico-duodenal transplantation is currently being utilized as treatment for selected patients with insulin-dependent diabetes mellitus [25, 26]. In these patients duodenocystostomy (DC) has evolved as the "diversion" method of choice for controlling the exocrine secretions of the transplanted pancreas [10, 23, 24]. While this technique has reduced the morbidity associated with pancreatico-duodenal transplantation, further experience has revealed persistent troublesome complications. In a recent report by Smith et al. a significantly higher incidence of infectious and hemorrhagic lower urinary tract complications was noted in pancreatic transplant patients with a DC than in patients receiving a pancreatico-duodenoenterostomy or an isolated renal transplant [22].

Activated proteolytic enzymes corresponding to trypsin concentrations of up to 200 μg/ml have recently been identified in the urine of these patients [18]. The role of these enzymes in general, and trypsin specifically, in mediating lower urinary tract complications is unknown. The purpose of this study was to evaluate the effect of urinary trypsin concentrations corresponding to those observed in the clinical setting on several important variables involved in urinary tract hemostasis and the initiation of lower urinary tract bacterial infections.

Materials and methods

Test samples

Human urine documented to be free of trypsin activity by chromogenic assays was used to prepare test urinary trypsin concentrations. Serial trypsin dilutions of 200, 125, 75, 25, and 5 μg/ml were prepared in trypsin-free human urine on the day of use.

A freshly voided urine sample was collected from a patient with a functioning pancreatic allograft drained by a DC. The protein content and pH of the specimen were determined. Trypsin-specific (trypsin inhibitor-sensitive), trypsin-"like" (trypsin inhibitor-independent), and trypsinogen (enterokinase activatable) activity of the specimen were measured as previously described [18]. Samples were aliquoted and stored at −70°C until the time of use.

Control urine was collected from a normal volunteer. The pH and protein content of the control sample were measured and adjusted to that of the PDC specimen using 1 *N* HCl and bovine serum albumin. Portions were aliquoted and stored at −70°C until the time of use. Immediately prior to use all samples were filtered through a 22-μm syringe filter.

Fibrinolysis

Initiation of the clotting cascade subsequent to urothelial injury cultimates in the formation of red cell-fibrin clot, and hemostasis, at the injury site. A delicate balance exists between clot formation and thrombolysis under normal circumstances. Uncontrolled clot formation, or unregulated thrombolysis, can be equally disastrous for the patient. This experiment examined the effect of various trypsin concentrations on fibrin clot integrity.

The ^{125}I-fibrin lysis assay was used to determine the impact of trypsin on fibrin clot integrity [27]. ^{125}I-labeled substrate human fibrinogen (Sigma Chemical Co., St. Louis, Mo) was prepared by the lactoperoxidase-glucose oxidase method, as follows. ^{125}I-Human fibrinogen is placed into 96-well, flat-bottom culture plates at a concentration of 10 μg/cm^2 in a volume of 20 μl. Plates are air dried at 45°C for 24 h, after which the plates are exposed to 100 μl volume of Roswell Park Medium RPMI, containing 10% fetal bovine serum. Excess thrombin in the serum results in fibrinogen conversion to fibrin with a total radioactivity of 30,000–60,000 counts per minute per well. At the time of assay, the plates were washed two times with phosphate-buffered saline (PBS), after which 100 μl test urine is placed in each well.

^{125}I-fibrin plates were exposed to each control and test sample for 5, 10, 15, 30, and 60 min. Each sample concentration/exposure time was assayed in duplicate. Urine from the transplant patient was assayed both undiluted and following 50-fold dilution. At the end of the exposure time, well supernatants containing solubilized fibrin were collected. Supernatant radioactivity was measured using a Beckman 4000 gamma counter (Beckman Instruments, Inc., Fullerton, Calif.). Results were expressed as the percent of maximal releasable radioactivity [(soluble radioactivity/total radioactivity per well) × 100].

Bacterial adherence

The adherence of bacteria to the urothelial surface is a crucial step in the initiation of urinary tract infections. A spectrum of bladder alterations have been shown to promote bacterial adherence. Prior reports by Smith et al. suggest that the bladders of patients with duodenocytostomies are predisposed to infections with atypical urinary pathogens including *Staphylococcus epidermidis* [22]. This experiment studied the effect of transient urothelial exposure to trypsin on the adherence of *S. epidermidis* to the bladder surface.

S. epidermidis were maintained in a log phase of growth by serial passage in LB broth media (tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, 5 g/l; NaOH 1 *N*, 1 ml/l). On the day prior to the experiment bacteria were passaged into media containing 5 μCi/ml ^{3}H-thymidine. Twenty-four hours later, at an optical density corresponding to a 3 McFarland standard (approximately 9×10^8 bacteria/ml), the bacteria were washed three times in PBS and resuspended in PBS at a 2 McFarland optical density (approximately 6×10^8 bacteria/ml). A 200-μl aliquot of the bacterial suspension was removed for later liquid scintillation counting. A second aliquot was used to prepare serial dilutions for use in pour plate quantification of actual bacterial number. Log dilutions of the test bacterial suspension were plated onto LB broth, 4% bactyl agar (General Biochemicals, Chagrin Falls, Ohio) plates. Forty-eight hours later pour plate bacterial colony counts were determined and the starting bacterial concentration calculated.

The previously described in situ catheterized rat bladder model was used to assess adherence of the ^{3}H-thymidine-labeled bacteria to the urothelial surface [4]. Syngeneic, age-matched, female F-344 rats were anesthetized by inhalational metaphane followed by IP Nembutol (50 mg/kg). Animals were catheterized using a 22-gauge Teflon angiocatheter and the bladder emptied using gentle lower abdominal pressure. Trypsin, at a concentration of 200 μg/ml, was prepared in "trypsin-free" human urine. Animals received an intravesical instillation of 0.2 ml of the trypsin-containing ($n = 5$), DC ($n = 5$) or control ($n = 5$) human urine. After a 1-h exposure the bladders were emptied and washed three times with 0.2 ml aliquots of PBS. Animals then received 0.2 ml of the radiolabeled bacterial suspension intravesically. After 30 min the bacterial suspension was removed and the bladder washed with three 0.2 ml aliquots of PBS. Bladders were removed by sharp transection at the uretero-vesical junction and prepared for liquid scintillation counting.

Bladder specimens and the test aliquot of the bacterial suspension, were placed in 22 ml glass liquid scintillation vials. Specimens were digested at 37°C in 1 ml of 2 *N* NaOH for 24 h and subsequently neutralized with an equal volume of 2 *N* HCl, after which 20 ml of 3a70 complete scintillation cocktail was added to each sample (Research Products International Corp., Mt. Prospect, Ill). Specimens were counted using a Beckman LS 3801 liquid scintillation counter. Bacterial radioactivity (counts per minute/colony forming unit) was determined based upon the activity of the sample aliquot and pour plate bacterial quantification [bacterial radioactivity = (counts per minute/ml)/(colony forming units/ml)]. This experiment was repeated on two separate occasions and the results pooled for statistical analysis ($n = 10$/group).

Statistical analysis

The effects of time and trypsin concentration on fibrinolysis were analyzed using two-factor analysis of variance. Results were considered significant at $P < 0.01$. Bacterial adherence in control and trypsin-exposed bladders was compared using the non-parametric Mann-Whitney U-test. High and low bacterial adherence values in each group were edited prior to analysis. Differences were considered significant at $P < 0.05$.

Results

The urine sample from the pancreas transplant patient had a pH of 5.7 and a protein content of 2.02 mg/ml. Trypsin-specific, trypsin-like, and trypsinogen activity were calculated to be 135 μg/ml, 150 μg/ml, and 7 μg/ml, respectively.

Fibrinolysis

Both time and trypsin concentration were significant independent variables influencing in vitro fibrinolysis ($P < 0.0001$). In addition, these variables demonstrated a significant interactional effect ($P < 0.0001$). The percent of maximal fibrinolysis at 60 min was 40% and 100% for trypsin concentrations of 5 and 200 μg/ml, respectively. The 200 μg/ml trypsin concentration resulted in rapid fibrinolysis with 84% of fibrin solubilized at 5 min. A 1-to-50 dilution of the pancreas transplant patient urine solubilized 90% of the available radioactivity at 60 min. This corresponded to an equivalent trypsin fibrinolytic activity of 1310 μg/ml in the undiluted urine. In contrast, the control urine sample lysed 16% of the available fibrin after 1 h. The trypsin equivalent fibrinolytic activity of the control urine (2.17/μg/ml) was 603-fold less than that of the transplant patient urine. In vitro fibrinolysis over time is graphically illustrated for each trypsin concentration and test sample in Fig. 1.

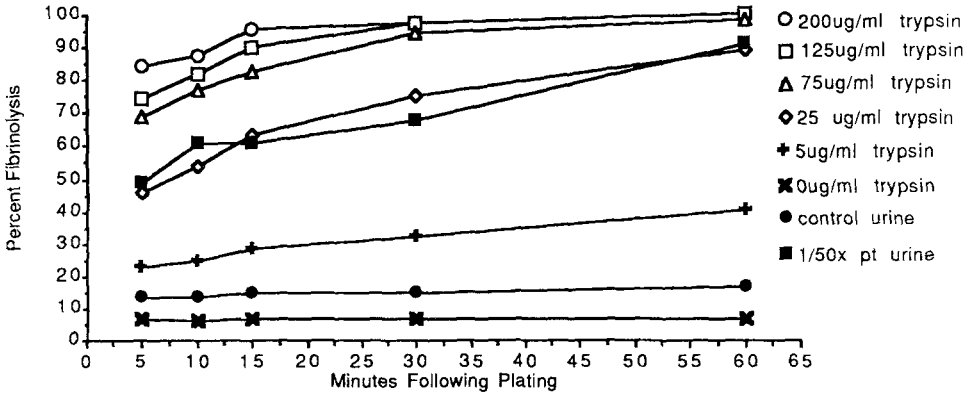

Fig. 1. Fibrin lysis, over time, resulting from test and control samples. Lysis observed in control urine and 50-fold diluted urine from a pancreas transplant patient with a DC is simultaneously plotted

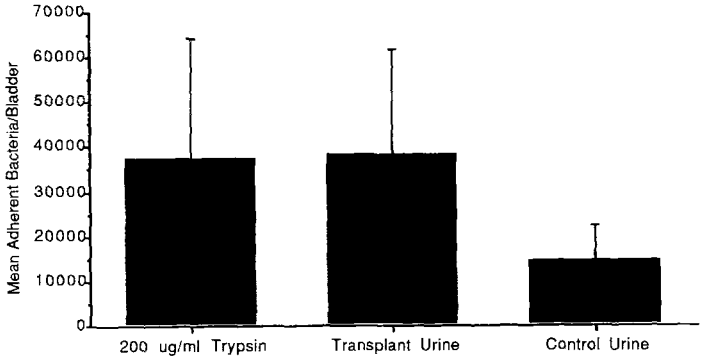

Fig. 2. Bacterial adherence to control urine, pancreas transplant urine, and trypsin exposed rat bladders

Table 1. Summary of publications reporting increased particulate adherence to bladder surfaces following specific bladder manipulations

Particulate species	Author	Bladder treatment
Bacteria	Parsons et al. 11]	Acid
	Bagley et al. [1]	Electrocautery
	Iverson et al. [9]	Bladder irrigation
	Chang et al. [3]	Povidone iodine
	Bodenstab et al. [2]	Carcinogens
	Ruggieri et al. [15]	Distension, ischemia, outflow obstruction
	Parsons et al. [12]	Ammonia
	Parsons et al. [13]	Protamine
Crystals	Gill et al. [5]	Acid, detergents, EDTA, cyclophosphamide, papain, neuraminidase
	Gill et al. [7]	Renograffin, hypertonic solutions
	Gill et al. [6]	Acetic acid, formaldehyde, DMSO Thio-TEPA
	Grenabo et al. [8]	Bacterial infection
Tumor cells	See et al. [20]	Electocautery
	See et al. [16]	Nd-YAG laser
	See et al. [17]	Acid
	See et al. [21]	Elevated intravesical pressure

Bacterial adherence

A 1-h bladder exposure to trypsin concentrations of 200 μg/ml or pancreas transplant patient urine resulted in a significant increase in bacterial adherence to the urothelial surface compared with control animals ($P = 0.024$ and $P = 0.01$, respectively). Average bacterial adherence (± 1 standard deviation) to control bladders was 14366 ± 7825 bacteria/bladder. Average bacterial adherence to trypsin- and transplant-urine-exposed bladders was 37586 ± 26860 and 38196 ± 23450 bacteria/bladder, respectively. These results are shown graphically in Fig. 2.

Discussion

As the storage site for liquid waste, the luminal bladder surface is continually bathed by urine and its constituents. The intact urotelium plays an important role in normal bladder physiology by compartmentalizing bladder contents. In doing so, it simultaneously prevents the egress of urine and its soluble constituents back into the systemic compartment, and the ingress of systemic components into the bladder. In addition, the urothelial surface plays a crucial role in keeping the urinary tract free of particulate matter. This particulate matter may be present as a result of spontaneous nucleation and crystal growth of urinary solutes or the retrograde introduction of bacteria during the act of voiding.

Urothelial injury disrupts urothelial integrity, potentially allowing two-way exchange of constituents present in the bladder and systemic compartments, and the persistence of pathogenic particulate material within the urinary tract. Table 1 lists the spectrum of bladder manipulations which have been shown to alter urinary tract physiology in such a way as to promote the adherence of particulate matter including bacteria, crystals, and tumor cells [1–3, 5–9, 11, 12, 15–17, 20, 21, 23]. All these publications highlight the fact that the physiology of the intact urothelium is a delicately balanced process exquisitely adapted to the needs of the organism. Pathologic or iatrogenic processes that disrupt this balance have the potential for serious sequelae far beyond that of the initial injury.

Following PDC, a spectrum of digestive enzymes that are "non-physiologic" in the sense of their location are

introduced into the urinary tract. These enzymes, although secreted by the pancreas as inactive proenzymes, appear to be rapidly converted to their active form in the bladder [19]. As a result of the bladder's storage function, these substances maintain prolonged contact with the urothelial surface. Their impact on normal bladder function is unknown and represents a focus of this study. Although association does not imply causation, the finding that patients with a PDC are at an increased risk for lower urinary tract complications and have high levels of active urinary trypsin suggests that the two phenomena may be interrelated.

Formation and maintenance of fibrin clot is crucial to establishing hemostasis subsequent to injury. Similarly, the ability of the bladder to resist bacterial adherence is an essential component of host defenses against urinary tract infection. The results of this study demonstrate that urinary trypsin impairs these processes. Urinary trypsin concentrations 15 times less than the average level observed in transplant patients with duodenocystostomies cause relatively rapid fibrinolysis. Brief urothelial exposure to 299 μg/ml trypsin concentrations increased bacterial adherence. Virtualy identical results were achieved when urine from a patient with a PDC was tested. Given that the patient urinary tract is chronically exposed to trypsin, these findings may underestimate the impact of urothelial-pancreatic enzyme contact.

Duodenocystostomy has evolved as the exocrine diversion method of choice in patients undergoing whole organ pancreas transplantation. Relative to alternative methods of diversion, this technique affords fewer serious complications and rapid, non-invasive access to amylase secretion as a monitor of rejection [14]. Recent publications suggesting an increased incidence of lower urinary tract complications following DC are concerning but must be viewed relative to the risk of morbidity following alternative diversions.

The observations that the urine of these patients contains high levels of active proteolytic enzymes, and that these enzymes cause in vivo and in vitro physiologic alterations corresponding to clinical complications, may be important in addressing these problems. If trypsin plays an etiologic role in hemorrhagic and infectious lower urinary tract complications, it may be amenable to manipulations designed to diminish its injury potential. Interventions designed to prevent enzyme secretion, activation, or enzyme-substrate interaction may prove useful. Alternatively, it may be possible to address the problems of clot lysis or bacterial adherence specifically. Additional research into the issue of digestive enzyme-urinary tract interaction and approaches to prevent sequelae from it is warranted.

Conclusions

Urinary trypsin concentrations corresponding to those observed in patients with PDC promote fibrin clot lysis and bacterial adherence to the bladder surface. Urine from a patient with a duodeno-cystostomy reproduced these results. Active urinary trypsin may play a causative role in the increased incidence of hemorrhagic and infectious lower urinary tract complications observed in these patients.

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William A. See, MD
Department of Urology
The University of Iowa Hospitals and Clinics
Iowa City, IA 52242-1089
USA