Kinetics of Transitional Tumor Cell Line 4909 Adherence to Injured Urothelial Surfaces in F-344 Rats

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ABSTRACT

Prior reports have described the mechanism initially responsible for transitional tumor cell adherence and implantation on injured urothelial surfaces. This study further quantifies variables that influence the size of tumor inoculum at the injury site and thereby affects bladder tumor recurrence risk. The surface area of urothelial injury, the concentration of tumor cells in the intravesical bathing medium, the viability of tumor cells, the time of urothelial exposure to tumor cells, and the intravesical pressure were the variables studied. Increasing the surface area of urothelial injury resulted in a linear increase in the size of the tumor inoculum ($r^2 = 0.805, P = 0.0001$). Tumor inoculum increased as a direct function of the concentration of tumor cells in the bathing medium. This relationship was linear at low cell concentrations ($r^2 = 0.64, P = 0.0001$). Higher concentrations of tumor cells appeared to result in saturation of the system, with a relationship best described by a log/log function ($r^2 = 0.975, r = 0.0001$). Viable and nonviable tumor cells appeared to compete for the effect of exposure time on the size of the tumor inoculum ($r^2 = 0.513, r = 0.0198$). Tumor inoculum increased as a linear function of the intravesical pressure ($r^2 = 0.314, P = 0.0125$). These results demonstrate a significant positive correlation between each of the experimental variables and the size of tumor inoculum. Manipulation of these variables in clinical practice may alter the size of tumor inoculum and thereby have an impact on tumor recurrence secondary to implantation.

INTRODUCTION

The ability of transitional carcinoma tumor cells to implant on urothelial surfaces is well established (1, 2). This phenomenon is felt to contribute to the high rate of intravesical tumor recurrence following transurethral tumor resection. Experimental studies of tumor implantation in a variety of systems suggest that it is similar to infectious processes, in that a key factor in determining outcome is the size of the initial inoculum (3). Prior work in our laboratory has suggested that the intravesical tumor inoculum occurring at the time of transurethral surgery results from tumor cell adherence to fibrin at sites of urothelial injury (4).

Although a number of authors have offered anecdotal clinical evidence implicating numerous factors as predetermining for or against implantation, the relationships between these variables and size of tumor inoculum (i.e., risk of implantation) remain undefined. The purpose of this study was to evaluate the kinetics of tumor cell adherence to sites of urothelial injury, in an effort to better understand variables influencing the size of the viable tumor inoculum. The variables chosen for this series of experiments were the surface area of urothelial injury, the concentration of tumor cells in the bathing medium, the viability of tumor cells in the inoculum, the duration of exposure to tumor cells, and the intravesical pressure at the time of tumor cell exposure.

MATERIALS AND METHODS

Animals. Ten- to 12-week-old syngeneic female F-344 rats (National Cancer Institute, Frederick, MD) were utilized for all experiments. Animals were given standard rat chow and water ad libitum up to the time of an experiment.

Tumor. The 3-methylcholanthrene-induced transitional cell carcinoma 4909 was utilized in all experiments (5). The tumor was 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 (6). The single-cell suspension was pulse labeled with [6-3H]thymidine (10 µCi/ml), [125I]iododeoxyuridine (100 µCi/ml), or 51Cr (150 mCi) for 1 h at 37°C, after which the cells were washed 3 times in RPMI with 10% fetal calf serum. 51Cr-labeled cells were then incubated at 4°C for 30 min, after which they underwent two additional washes. An aliquot of the final cell suspension was serially diluted for use in the generation of a cell number-radioactivity curve. Viability of the tumor cell suspension was determined by trypan blue exclusion and, except where noted, all concentrations are given as the number of viable cells/ml.

Experimental Model. The in situ catheterized rat bladder served as the model for cellular adherence (7). Anesthetized animals were catheterized with a PE-50 catheter to a distance of 2.5 cm from the external meatus. The bladder was exposed through a midline incision and a 4-0 surgical steel wire was advanced through the catheter until it was observed to contact the dome of the bladder. With the animal on a Bovie grounding plate (Sybrone Corp., Cincinnati, OH), electrocautery current was applied to the wire for a duration of 1 s. An aliquot of the labeled tumor cell suspension was then instilled into the bladder. Subsequently, the bladder was emptied, washed 3 times with aliquots of PBS, and removed by transection at the urethrov ejaculational junction.

[6-3H]Thymidine-labeled specimens were placed in 23-ml liquid scintillation vials and processed by overnight digestion in 1 ml of 2 N sodium hydroxide at 37°C, neutralization with 1 ml of 2 N hydrochloric acid, and the addition of 19 ml of Aquasol-2 (Du Pont-New England Nuclear, Boston, MA). Specimens were counted in a Beckman LS3801 spectrometer (Beckman Instrument Co., Irvine, CA). Preparations using [125I]iododeoxyuridine- or 51Cr-labeled cells were placed in gamma counting vials and counted in a Beckman model 300 gamma counter (Beckman Instrument Co., Irvine, CA). The adherent cell number was calculated by comparing sample radioactivity to points on a cell dilution-radioactivity curve.

In experiments using mixtures of cells labeled with either 51Cr or 125I, single isotope counts were calculated using the channels ratio method ($125I/51Cr = \text{counts } 125I \times 1 + (1/\text{ratio of }51Cr \text{ channel counts to }125I \text{ channel counts when }51Cr \text{ is counted alone); }125I = \text{counts }125I - (\text{counts }51Cr/\text{ratio of }125I \text{ channel counts to }125I \text{ channel counts when }51Cr \text{ is counted alone}). \text{ The respective number of adherent cells was then determined by comparing the corrected counts to the appropriate standard cell-radioactivity dilution curve.}

Surface Area of Injury versus Adherence. An experiment was performed to define the relationship between injury surface area and the size of tumor inoculum. Catheterized animals were given bladder injuries at either one or three discrete sites. An additional group of animals were catheterized and sham-fulgurated. Injuries were performed under direct vision and in a standardized fashion so that the resulting injuries were roughly equal in area. Each animal then received 0.2 ml of labeled tumor cell suspension at a concentration of 1 x 10^6/ml. The tumor cell suspension remained in place for 30 min, after which the bladders were

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2 The abbreviations used are: PBS, phosphate-buffered saline; NBF, neutral buffered formalin.
washed 3 times with 0.2-ml aliquots of PBS and processed as described above. Eight animals received a single injury, seven animals were injured at three sites, and three animals were given a sham injury.

**Bathing Cell Concentration versus Adherence, Experiment 1.** The impact of tumor cell concentration in the intravesical bathing medium on the adherent tumor inoculum was studied. Following bladder full gation at three sites, animals received 0.2 ml of labeled tumor cell suspension at one of the following concentrations: 1 x 10³, 1 x 10⁴, 1 x 10⁵, 1 x 10⁶, or 1 x 10⁷ cells/ml. The cell suspension remained in place for 30 min, after which the bladder was emptied and washed 3 times with 0.2-ml aliquots of PBS. Bladders were removed and processed for liquid scintillation counting as previously described. Four animals were studied at each concentration.

**Bathing Cell Concentration versus Adherence, Experiment 2.** A second experiment utilizing higher cell concentrations and a single injury site was performed to determine if the injury site was saturable. Following single-site bladder injury, animals received 0.3 ml of labeled tumor cell suspension at one of the following concentrations: 5.5 x 10⁵, 5.5 x 10⁶, 5.5 x 10⁷, 5.5 x 10⁸, or 2.7 x 10⁹ cells/ml. The larger inoculation volume, higher cell concentration, and smaller surface area of injury resulted in a 12-fold increase in the absolute number of cells/injury area at the highest cell concentration. Bladders were processed as described. Two animals were studied at each of the two lowest concentrations and a single animal was evaluated at each of the three highest concentrations.

**Cell Viability versus Adherence.** The impact of cell viability on tumor cell adherence was evaluated using an *in vivo* competitive binding assay. A tumor cell suspension prepared from a single donor was divided into two equal aliquots to be labeled with either ⁵¹Cr or ¹³¹I. Immediately following incubation with the isotope, ¹²⁵I-labeled cells were washed 3 times in the fixative 10% NBF, after which they were resuspended in 5% NBF for 30 min. The cells were then washed 2 times with RPMI. Following viability determination, the cells were resuspended in RPMI with 10% fetal calf serum, at a total concentration of 1 x 10⁶ cells/ml (viable plus nonviable). Preliminary experiments demonstrated that this protocol resulted in approximately 90% nonviability, as determined by dye exclusion. The nonviable cells retained both their size and radio nuclide label.

⁵¹Cr-labeled tumor cells at a total concentration of 1 x 10⁶ cells/ml (viable plus nonviable) were admixed with the ¹²⁵I-labeled nonviable cells in the following ratios: 9:1, 7:3, 1:1, 3:7, and 1:9. Animals with three bladder injury sites then received 0.2 ml of one of the cell mixtures. After a 30-min exposure, the bladders were processed as described. Four animals were evaluated at each ratio.

**Exposure Time versus Adherence.** The rate of tumor cell adherence to the injury site was determined. Animals with a single bladder injury site received 0.2 ml of labeled tumor cell suspension at a concentration of 1 x 10⁶ cells/ml. The tumor cell suspension remained in the bladder for periods of 5, 15, 30, or 60 min. The bladders were then rinsed and processed as described. Four animals were studied at 5 min and two animals were evaluated at each of the remaining time points.

**Intravesical Pressure versus Adherence.** Catheterized animals were given a single bladder injury as described above. A second transurethral catheter of PE-10 tubing was then passed into the bladder and attached to a calibrated Hewlett Packard 1295-A pressure transducer (Hewlett Packard, Waltham, MA). Intravesical pressures were recorded on a Hewlett Packard 7404-A oscillographic recorder. Labeled tumor cells were instilled into the bladder until the desired intravesical pressure was achieved. After administration, the tumor cell suspension remained in the bladder for 5 min, after which the bladders were emptied and washed 3 times with 0.3-ml aliquots of normal saline. Bladders were removed and processed as described. A total of 19 animals were studied at pressures ranging from 25 to 130 cm of water.

Results of the intravesical pressure experiments prompted further investigation into the effects of pressure on adherence. In an effort to visualize the pattern of adherence, the particulate dye India ink was substituted for the tumor cell suspension. Animals that had undergone sham bladder injury, cautery bladder injury, or yttrium-aluminum garnet laser bladder injury were given either rapid hand (high pressure, 30–60 cm H₂O) or Harvard pump (low pressure, 10–12 cm H₂O) intravesical instillations of 0.3 ml of an India ink suspension. After 5 min, the bladders were emptied, in situ distended with 0.3 ml of formalin, excised, and placed in fixative. Forty-eight h later, the specimens were opened, examined grossly, and photographed.

A change in volume-mediated pressure experiment was also performed using India ink. Sham-injured animals received a hand-delivered instillation of 0.3 ml (low pressure) or 0.5 ml (high pressure) India ink suspension. The specimens were then processed as described above.

The pattern of adherence observed in the India ink experiments was correlated to patterns of implantation observed following hand instillation of various volumes of tumor cell suspension into cautery-injured animals. Six weeks following instillation, the animals were sacrificed and their bladders were excised, examined, and photographed.

**Data Analysis.** The size of tumor inoculum was calculated by plotting sample radioactivity against a cell number-radioactivity line. A unique line was constructed for each labeled tumor cell suspension and was used only for the analysis of animals that received the corresponding labeled cells. Linear transformations, regression lines, correlation coefficients, and statistical analyses were performed on a Macintosh Plus personal computer (Apple Computer, Inc., Cupertino, CA), utilizing Statview 512+ software (BrainPower, Calabasas, CA).

**RESULTS**

**Surface Area of Injury versus Adherence.** Increasing the number of bladder injury sites resulted in a linear increase in the size of the tumor inoculum (adherent cells = 17,544-injury site number - 481; r² = 0.841, P = 0.0001). Mean cell adherence (±SD) in the one- and three-injury site groups was 15,436 ± 6,802 and 52,770 ± 12,498 cells, respectively. Animals without bladder injury had a mean cell adherence of 2,409 ± 1,195 cells.

Tripling the number of injury sites resulted in a 3.4-fold increase in the size of the tumor inoculum (not significantly different from 3 at a 95% confidence level). The experimental data and the calculated regression line are shown in Fig. 1.

**Cell Concentration versus Adherence, Experiment 1.** At lower tumor cell concentrations, tumor inoculum increased as a linear function of tumor cell concentration (adherent cells = 0.015 tumor cell concentration + 1,722; r² = 0.64, P = 0.0001). Fig. 2 shows the raw experimental data and the best fit line described by the above equation in a log/log plot.

**Cell Concentration versus Adherence, Experiment 2.** The experiment utilizing larger numbers of tumor cells relative to the surface area of injury confirmed the saturability of the injury site. Tumor inoculum was found to increase as a log/log function of the concentration of tumor cells in the bathing medium.

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Fig. 1. Tumor cell adherence to the urothelium as a function of the number of bladder injury sites. ◇, experimental data with regression line.
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Fig. 2. Tumor cell adherence to an area of urothelial injury as a function of the concentration of tumor cells in the bathing medium. The nonlinearity of the log/log plot reflects the simple linear relationship (no binding site saturation) between the adherent tumor inoculum and tumor cell concentration under the conditions used in this experiment. □, experimental data with regression line.

Fig. 3. Tumor cell adherence to an area of urothelial injury as a function of the concentration of tumor cells in the bathing medium. Binding site saturation at effectively higher tumor cell concentrations is demonstrated by the linearity of this log/log plot. □, experimental data with regression line.

(log adherent cells = 0.216-log tumor cell concentration + 3.212; \( r^2 = 0.975, P = 0.0001 \)). Fig. 3 shows the raw experimental data and the fitted line derived from the above equation in a log/log plot.

Cell Viability versus Adherence. The NBF-treated \(^{125}\text{I}\)-labeled cell suspension was 87% nonviable immediately prior to the experiment. The \(^{51}\text{Cr}\)-labeled cells were 72% viable. Fig. 4 demonstrates the viability ratios of adherent cells determined experimentally and compares the observed values to those predicted assuming identical adherence kinetics for both viable and nonviable cells. The observed ratios closely approximated the predicted values.

The adherence of both viable \((^{51}\text{Cr}\)-labeled) and nonviable \((^{125}\text{I}\)-labeled) cells showed highly significant correlations to tumor cell concentration \((P < 0.0001)\), which were best described by log/log functions. The binding site affinity of each cell population was determined as the slope of the best fit regression line. These values, 1.161 for nonviable cells and 1.247 for viable cells, were not significantly different. Fig. 5 demonstrates the experimental data and calculated regression lines.

The total number of adherent cells in each experimental group was determined as the sum of both viable \((^{51}\text{Cr}\)-labeled) and nonviable \((^{125}\text{I}\)-labeled) cells. As predicted by the above results, no difference between groups in the total number of adherent cells was observed (Fig. 6).

Exposure Time versus Adherence. The rate of tumor cell adherence decreased as a function of time. A simple logarithmic relationship best described the effect of exposure time on the size of the tumor inoculum (adherent cells = 7170 - log exposure time - 593; \( r^2 = 0.513, P = 0.0198 \)). Fig. 7 shows the data from this experiment and the fitted line described by the above equation.

Intravesical Pressure versus Adherence. Tumor cell adherence in the intravesical experiment was found to increase as a direct function of intravesical pressure. The best fit line for the experiment was described by the equation: tumor inoculum = 908.353 - pressure + 18,316 \((r^2 = 0.314, P = 0.0123)\). Fig. 8 demonstrates the experimental data and the fitted line.

The pattern of particulate adherence in the India ink experiment is shown in Fig. 9. Relative to the pump-injected (low

Fig. 4. The viability ratios of adherent cells in each group (mean ± 1 SD). The experimentally determined ratios are compared to theoretical ratios calculated assuming that both viable and nonviable cells have an identical affinity for the binding site. ■, viable \((^{51}\text{Cr}\)-labeled)/nonviable \((^{125}\text{I}\)-labeled); □, predicted viable/nonviable; ■, predicted nonviable/viable; □, nonviable/viable.

Fig. 5. Tumor cell adherence as a function of the concentration of tumor cells in the inoculum. The experimental data points and corresponding regression lines for both viable and nonviable cells are shown. □, nonviable cells; *, viable cells.

The pattern of particulate adherence in the India ink experiment is shown in Fig. 9. Relative to the pump-injected (low
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pressure) group, the hand-injected (high pressure) group uniformly demonstrated a visible increase in the amount of adherent dye. One animal in the hand-injected group (laser injury) demonstrated an area of submucosal extravasation distant from the injury site. In the change in volume experiment, the higher pressures associated with volumes of 0.5 ml resulted in linear patterns of submucosal extravasation in a significant number of animals. Fig. 10 demonstrates a representative adherence and implantation pattern in animals receiving 0.5 ml acutely. These linear patterns of extravasation were rare but did occur in animals given smaller volumes. Implantation in this group occurred predominately at the site of cautery injury.

DISCUSSION

Theories regarding the etiology of the high rate of recurrence of transitional cell bladder carcinoma have been proposed since the turn of the century. Tumor cell implantation as a potential cause of intravesical recurrence was first suggested by Albarran and Imbert in 1903 (8). Since that time a substantial body of literature has accumulated in support of this concept. A number of authors have published anecdotal reports ascribing transitional cell tumor recurrence to implantation. Clinical reports by Hinman (9), Kiefer (10), and Boreham (11) all noted tumor recurrence in the prostatic fossa of urethra following combined prostatectomy and tumor resection. Boyd and Burnand’s (12) 5-year follow-up of 32 patients with well differentiated transitional cell carcinoma showed recurrent involvement of the bladder vault in 90% of patients, only 6% of whom had a primary lesion at this site. They interpreted this as indicative of a preferred site of implantation following transurethral resection. Page et al. (13) also compared the location of the primary lesion to the site of recurrence. Seventy-seven % of 56 patients in the series had their primary lesion within 5 cm of the ureteral orifices, while only 20% of recurrences were at this site. In contrast, no patient had a primary lesion in the bladder dome, whereas a high percentage had recurrences at this site.

Intravesical pressures generated during the course of bladder tumor resection, and subsequent fluid extravasation, were studied by Bodner et al. (14). Using an inflow pressure of approximately 30 cm of water, patients developed peak intravesical pressures ranging from 27 to 81 cm of water. Even at these modest pressures, 70% of patients demonstrated radiographic evidence of postoperative extravasation. Correlation of their findings in a cadaveric model demonstrated that, following incision of the mucosa, bladder distention resulted in progressive separation of muscle fibers and fluid leakage. These authors postulated that high intravesical pressures at the time of resection might increase the likelihood of tumor spread.

Laboratory studies have given strong support to the aforementioned clinical observations. McDonald and Thorson (15) were the first investigators to demonstrate the ability of transitional carcinoma tumor cells to implant on urothelial surfaces. Subsequently, Soloway and Masters (2), using a syngeneic mouse model, demonstrated that fulguration injury to the urothelium predisposed to tumor cell implantation. We have demonstrated that fulguration bladder injury results in increased tumor cell adherence, compared to noninjured bladders, and that this adherence is specific for the site of urothelial injury (16). Clotting cascade activation and subsequent fibrin formation at the site of injury appear to mediate tumor cell adherence to the injury site (4).

The effects of the tumor inoculum on subsequent implantation rates have been studied. Porter et al. (3) have shown that tumor implantation increases as a function of the number of cells in the tumor inoculum. Woodruff and Dunbar (17) found that for a given total cell dose the likelihood of an animal developing a tumor was not altered by distributing the dose in 10 sites as against concentrating it in 1. Additional studies in the syngeneic rat bladder tumor model have shown that the probability of tumor take increases as a function of the tumor inoculum size.
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Fig. 9. The pattern of adherence of India ink in hand- (high pressure) and pump- (low pressure) infused bladders with various types of urothelial injury.

Fig. 10. The pattern of India ink adherence (upper) and tumor implantation (lower) seen in animals given a hand (high pressure) instillation of 0.5 ml of fluid.

The precise mechanism by which tumor cells adhere to sites of urothelial injury remains undefined. While our earlier work suggesting that clotting cascade activation and fibrin formation at the injury site are requisite steps, the nature of the cell's participation is unclear. The finding reported herein, that cell viability as defined by dye exclusion following formalin fixation does not play a role in adherance, suggests that the cell may be a passive participant in the process of adherence. Further evidence in support of this premise comes from the experiments that demonstrated the adherence of India ink to injury sites and from several reports which have demonstrated the ability of inorganic urinary crystals to adhere to bladder injury sites (19, 20). However, these observations do not preclude the possibility that tumor cell surface receptors with affinity for molecules present at the injury site are preserved during formalin fixation and function to mediate adherence. Irrespective of the exact means by which the tumor cell adheres, the clinical implication of these results is that the size of the viable tumor inoculum is affected by the proportion of nonviable cells present in the inoculum. By competing for limited binding site availability, nonviable cells proportionately reduce the viable tumor inoculum. Clinical manipulations that result in a relative increase in the number of nonviable tumor cells should reduce implantation recurrence risk.

The surface area of urothelial injury is an additional critical variable that may be manipulated to determine the size of the tumor inoculum. Based on the observed kinetics of tumor cell adherence, a given injury area represents a saturable site to which a limited number of cells may adhere. Tumor inoculum increases as a linear function of the area of injury. However, the ultimate size of urothelial injury is governed not only by direct injury with electrocautery but also by indirect injury resulting from elevated intravesical pressures. The impact of intravesical pressure on tumor adherence and de novo urothelial injury is evidenced by both the increase of tumor cell adherence as a function of pressure and the pattern of particulate adherence and tumor implantation at different pressures.

The rate of saturation, or absolute number of adherent cells on a given area of injury, is governed by kinetics that differ depending upon the variable in question. Adherence has been shown to increase as a log/log function of cell concentration, as a logarithmic function of time, and as a linear function of intravesical pressure. The size of tumor inoculum is modifiable by manipulation of each of these variables, as well as the surface area of injury.

A valid question of these results is whether an anecdotal correlation can be made with clinical predictors of bladder tumor recurrence risk. The concentration, or absolute number, of tumor cells released into the intravesical fluid at the time of tumor removal should increase as a direct function of tumor inoculum (18). These data imply that increasing or decreasing the size of the tumor inoculum will have a similar impact on the likelihood of tumor take.

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volume (i.e., size and multiplicity). Similarly, the total surface area of urothelial injury, both direct and indirect, should correlate with the size and number of tumors resected and the duration of the procedure. Both the size and number of bladder tumors at initial presentation have shown a positive correlation with recurrence risk (21).

The ability of neoplastic cells to implant on tissue surfaces is well recognized. Open cancer surgery requires techniques to avoid tumor spillage in an effort to prevent local recurrence secondary to implantation. It is not unreasonable to think that intravesical neoplasms exhibit a similar phenomenon on their "native soil." Despite the lip service paid to the risk of intravesical implantation as early as 30 years ago, strikingly little clinical study has been directed toward this problem. A lack of understanding of the factors governing the size of tumor inoculum and subsequent risk of implantation may be partly responsible for this. The results presented in this study provide quantitative data in support of the anecdotal observations and theories of early clinical investigators and hopefully will serve as the foundation for controlled clinical investigation and further study of tumor cell interaction with tissue injury sites.

CONCLUSIONS

Sites of urothelial injury serve as saturable areas for tumor cell adherence. Maximal tumor inoculum is dictated by the surface area of injury resulting from direct (fulguration) or indirect (intravesical pressure) manipulation. The ultimate size of the tumor inoculum on a given area of injury is governed by the intravesical pressure, the duration of exposure to tumor cells, and the tumor cell concentration in the bathing medium. Nonviable tumor cells demonstrate the same binding site affinity as their viable counterparts and may effectively reduce the size of the viable tumor inoculum by competing for available binding sites. While their impact on intravesical recurrence rates remains to be determined by additional laboratory and clinical study, these variables represent factors that may be manipulated in the clinical setting in an effort to reduce tumor recurrence resulting from implantation.

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