Single-Dose Preoperative Systemic Cyclophosphamide for the Prevention of Bladder Tumor Implantation in F344 Rats

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ABSTRACT

An implantable rat bladder tumor model using the rat transitional carcinoma cell line 4909 was used to evaluate the effect of single-dose, preoperative, systemic chemotherapy on the risk of intravesical tumor implantation. To simulate the clinical setting in which drug levels would be present in both the tumor and the site of implantation, both tumor donor animals and tumor recipients were given a single dose of cyclophosphamide (CY) 1 h prior to tumor harvest and implantation. This protocol resulted in a significant reduction in the incidence of tumor implantation, in tumor volume, and in the incidence of nodal metastases relative to control animals. Dose-response experiments demonstrated that 10 of 139 (7%) animals treated with single doses of CY ranging from 2.5-100 mg/kg developed tumors as compared to 46 of 66 (70%) animals with tumors in the control groups (P < 0.001). CY doses below 2.5 mg/kg were associated with an increased incidence of tumor implantation (19 of 45, 42%). No lethal toxicity was seen at doses of 50 mg/kg or less. Peak antitumor activity occurred when the CY was administered 1 h prior to tumor implantation as compared to 48 or 24 h before or 1 or 24 h after tumor implantation. Preoperative "chemoprophylaxis" may be an effective strategy for preventing bladder tumor recurrences resulting from tumor implantation.

INTRODUCTION

Seventy % of the 47,000 patients diagnosed with new cases of transitional cell bladder carcinoma in 1989 underwent definitive management via transurethral tumor resection (1, 2). Intravesical tumor recurrence subsequent to excision of the primary lesion constitutes a major cause of morbidity in this group of patients with 1-yr and overall recurrence rates of 56% and 70%, respectively (3, 4).

Potential causes of intravesical recurrence include incomplete resection of the primary lesion, missed synchronous lesions, progression of multifocal areas of dysplasia and neoplasia, or implantation of tumor cells shed from the primary lesion on areas of urothelial injury at the time of resection. Although an abundance of evidence supports each of these potential causes, their relative contribution to the overall recurrence rate has yet to be defined (5, 6). Techniques currently used to prevent tumor recurrence, including intravesical chemotherapy and immunotherapy, have proven only modestly effective (7).

As a potential factor involved in tumor recurrence, tumor implantation has received strikingly little attention. Three requisite steps must be met for implantation to occur: viable tumor cells must be present in the bladder, the tumor cells must adhere to the urothelial surface, and the milieu at the site of adherence must be conducive to tumor growth (8). Laboratory studies have demonstrated that these conditions are fulfilled during transurethral tumor removal (8-10). Therefore, iatrogenic interventions that alter the viability of tumor cells present in the bladder, preclude their ability to adhere to the urothelial surface, or provide a milieu prohibitive to tumor growth should prevent recurrence due to implantation.

The tissue milieu at the site of tumor cell adherence may represent a crucial variable influencing implantation. The risk of tumor implantation is, in some ways, analogous to the risk of wound infection after bacterial contamination. Both the size of the bacterial inoculum and their ability to grow in the wound environment are determinants of infection. Tissue levels of appropriate antimicrobials (i.e., altered growth milieu) have been shown to significantly reduce the risk of wound infection and to form the basis for perioperative antibiotic prophylaxis (11). In the same fashion, tissue levels of an appropriate chemotherapeutic agent present at the site of adherence during the period of implantation risk may effectively reduce implantation-associated tumor recurrences. The purpose of this study was to determine if a single dose of systemic chemotherapy administered immediately prior to the period of risk for tumor implantation would be effective in preventing tumor recurrence.

MATERIALS AND METHODS

Tumor

The rat transitional cell bladder carcinoma 4909 was used in all experiments. This tumor was originally induced in F344 rats by the intravesical implantation of methylcholanthrene-impregnated wax pellets and was obtained from the authors who described it (12). The tumor is maintained in vivo by s.c. passage in syngeneic animals. It is currently in its fifteenth passage and maintains a moderately well-differentiated transitional histology. Its in vivo doubling time during the period of exponential growth is 1.2 days, and s.c. inoculation with 1 x 10⁶ viable cells results in tumor in 50% of animals at 4 wk. Unless otherwise noted, all animals received the tumor in suspension at a concentration of 1 x 10⁶ viable cells/ml.

Animals

Female F344 rats, 10-12 wk old, were utilized in all experiments (National Cancer Institute, Frederick, MD). Animals were maintained at a facility approved by the American Association for the Accreditation of Laboratory Care.

Intravesical Implantation Model

The technique for intravesical bladder tumor implantation was a modification of that described by Soloway and Masters (13). On the day of use, the s.c. tumor is removed from its host, scissor-minced, and trypsin-digested with 0.05% trypsin at 37°C for 30 min. Cells from the first trypsinization are discarded, after which a second 30-min trypsinization is performed. The single-cell suspension yielded from this trypsinization is filtered through a gauze mesh sponge, after which it is washed 3 times in Roswell Park Memorial Institute Medium with 10% fetal calf serum. Cell viability is determined by trypan blue exclusion. Subsequent to the last wash, the cell suspension is diluted to the desired concentration expressed as the number of viable cells/ml.

Recipient animals are anesthetized with i.p. Nembutal (50 mg/kg) and given a concomitant dose of i.p. gentamycin (0.5 mg/kg). After animals are shaved of their dorsal and ventral hair, they are positioned...
on the grounding plate of a surgical electrocautery unit and a 22 gauge, 1-in. Teflon i.v. catheter (Critikon, Tampa, FL) is passed into the bladder via the urethra. Gentle pressure on the lower abdomen is used to empty the bladder, after which a lower midline incision is made and the bladder is delivered into the wound.

A 4-0 surgical steel wire, premeasured to protrude 1 mm from the end of the transurethral catheter, is passed through the i.v. catheter until it is seen to contact the bladder wall. Electrocautery current is applied to the wire for a duration of 1 s, resulting in a visually observed 1.5-mm-diameter burn at the site of contact. The injury is performed at three discrete sites in the dome of the bladder. With the wire removed, 0.2 ml of the previously prepared tumor cell suspension is instilled into the bladder via the catheter immediately following injury. The catheter is withdrawn, the surgical wound is closed, and the animal is returned to its cage. Reepithelialization of the injury site occurs within 1 wk. The mechanism of bladder injury, the resulting injury site, and the temporal sequence of injury with relationship to the presence of the tumor cell suspension used in this model are identical to those observed in the clinical setting of transurethral bladder tumor resection.

Four wk from the time of implantation, the animals are euthanized and their bladders are dissected in situ with formalin and then excised. After 48 h of formalin fixation, all surrounding connective tissue is dissected free from the bladder, after which the bladder is opened, dried by compression with a gauze sponge, and weighed. The luminal surface of the bladder is examined at x40 magnification for the presence of any urothelial abnormalities. Suspicious areas are excised and histologically evaluated. When it occurs, tumor implantation is limited exclusively to sites of prior bladder injury.

Systemic Chemotherapy Experiments

Single-Dose Recipient Cyclophosphamide. A "screening" study was performed to determine if CY\(^2\) was active in preventing bladder tumor implantation. Recipient animals were randomized to receive either single-dose CY or sham treatment with sterile saline. One h prior to tumor implantation, the animals were given an i.p. injection of 100 mg/kg CY (n = 9) or an equal volume of sterile saline (n = 9). Tumor implantation was then carried out as described above (3 x 10\(^6\) viable cells/ml). Tumor donors were not treated with CY in this experiment.

At 4 wk, the animals were sacrificed and their bladders were evaluated as described. At the time of sacrifice, the retroperitoneal, peri-aortic lymph nodes were excised and their bladders are distended in situ with formalin and then excised. The bladders were assessed for the presence or absence of tumor at 4 wk. This experiment was performed twice, and the results were pooled. Twenty animals received saline, 24 animals received 25 mg/kg CY, 26 animals received 50 mg/kg CY, and 25 animals received 75 mg/kg CY.

The experiment outlined above was performed an additional 3 times for CY doses of 0 (n = 15), 5 (n = 15), 10 (n = 15), and 25 (n = 10) mg/kg; 0 (n = 14), 0.5 (n = 14), 2.5 (n = 15), and 5 (n = 14) mg/kg; and 0 (n = 15), 0.05 (n = 10), 0.1 (n = 15), and 0.5 (n = 12) mg/kg.

Dose-related Toxicity. The dose-response experiment described above was used to evaluate dose-related toxicity as measured by postoperative mortality. The numbers of animals at each CY dose surviving until the 4-wk evaluation were quantified. Animals expiring within 24 h of the initial surgery were excluded from analysis. Preliminary experiments showed no tumor-related mortality during this interval.

Timing of CY Administration. An experiment to determine the optimal timing of CY administration was performed using the minimal effective dose of CY defined in the above experiment. The design of this experiment was intended to mimic the clinical setting with respect to the biodistribution of preoperatively versus postoperatively administered chemotherapy. Individuals treated prior to tumor exposure (i.e., preoperatively) would have drug levels in both the tumor and the site of potential implantation. Individuals treated subsequent to tumor exposure (i.e., postoperatively) would have drug levels only at the site of implantation.

Four identical tumor donors were prepared as described above. Tumor donors were paired to recipients with respect to the timing of CY administration. Three tumor donors and their paired recipients received a single i.p. injection of CY (2.5 mg/kg) 48 h (n = 15 recipients), 24 h (n = 15 recipients), or 1 h (n = 12 recipients) prior to tumor implantation. The remaining tumor donor did not receive CY. This animal was used to prepare a tumor cell suspension for recipient animals that were treated with a single i.p. dose of CY 1 h (n = 15 recipients) or 24 h (n = 15 recipients) following tumor implantation. Tumor implantation was evaluated 4 wk later as described.

Data Analysis

Chi square analysis with continuity correction was used to test for differences in the rate of tumor implantation or survival between experimental groups. For statistical analysis of the dose-response experiments, the results from animals receiving identical doses of CY were pooled. Depending upon sample size, either the Mann-Whitney U or Student’s t test was used to compare tumor-bearing bladder weights. Results were considered significant at P < 0.05.

RESULTS

Single-Dose, Recipient Only, Cyclophosphamide. Seven of 9 animals in the CY-treated (100 mg/kg) group and all 9 animals in the sham-treated control group survived to the 4-wk evaluation. One hundred % of the animals in the sham-treated control group developed intravesical tumors with a mean bladder weight of 2.23 ± 2.02 (SD) g. Six of the animals in this group had histologically demonstrated retroperitoneal nodal metastases. None of the animals in the CY-treated group developed a bladder tumor or retroperitoneal nodal tumors. The mean bladder weight in the CY-treated group was 0.10 g. Both tumor implantation and bladder weight were significantly decreased in the CY group compared to controls (P < 0.001 and P < 0.024, respectively). The results of this experiment are shown graphically in Fig. 1.

Dose-Response Relationship. The results of the dose-response experiment are summarized in Table 1. All CY doses of 0.5 mg/kg or greater resulted in a significant decrease in tumor implantation compared to control. Ten of 122 surviving animals (8%) treated with 2.5 mg/kg CY or greater developed bladder tumors. Eight of these 10 tumors were observed in the first experiment, perhaps suggesting the influence of a variable unique to this experiment. In contrast, 37 of 57 surviving animals (65%) in the control group developed bladder tumors. The 0.5-mg/kg CY dose appeared to represent a transition dose in terms of antimplantation efficacy as 6 of 22 surviving
animals (27%) in this group developed intravesical tumors. Fig. 2 demonstrates the incidence of intravesical tumor implantation as a function of CY dose.

The mean weight of all tumor-bearing bladders in animals treated with a CY dose of 2.5 mg/kg or greater (all groups pooled, n = 10) was 0.14 ± 0.06 g. This was significantly less than the mean tumor-bearing bladder weight of 1.00 ± 1.09 g in control animals (n = 37, P = 0.018, Student's t test). The average weight of non-tumor-bearing bladders was 0.100 ± 0.06 g (n = 155). Tumor-bearing bladder weights as a function of CY dose are shown in Fig. 3.

Dose-related Toxicity. Fifty-seven of 64 control animals (89%) survived to the 4-wk evaluation. A CY dose of 75 mg/kg was associated with a significant decrease in animal survival. Sixteen of 25 animals (64%) in this group survived 4 wk (P < 0.02). There was no difference in 4-wk animal survival at CY doses of 50 mg/kg or less compared to controls. An average of 89% of the animals treated with 50 mg/kg CY or less survived 4 wk (151 of 170). Animal survival as a function of CY dose is shown in Fig. 4.

Timing of CY Administration. When administered as a single dose of 2.5 mg/kg, the efficacy of systemic CY in preventing bladder tumor implantation was dependent upon the timing of drug administration with respect to tumor exposure. Of the surviving animals, 4 of 12 (33%) treated 48 h prior to tumor implantation developed tumors; 6 of 11 (54%) treated 24 h prior to implantation developed tumors. Animals receiving CY 1 or 24 h subsequent to tumor exposure developed tumors in 2 of 13 (15%) and 1 of 11 (9%) cases, respectively. No animal of the 10 in the 1-h preimplantation treatment group developed a tumor. Although the small number in each treatment group precluded direct group-to-group comparison, 5 X 2 chi square analysis demonstrated a significant difference in tumor implantation among the five groups (P < 0.05). The results of this experiment are shown graphically in Fig. 5.

DISCUSSION

In an effort to reduce the high rate of intravesical recurrence following transurethral tumor resection, genitourinary surgeons have investigated the ability of a number of intravesical chemotherapeutic agents to alter recurrence rates. In theory, local administration of antineoplastic agents affords site-specific therapy with higher concentrations in the target organ and minimal systemic toxicity (14). Unfortunately, there are several theoretical and logistical problems with postresection intravesical therapy when administered for the prevention of implantation-mediated recurrence: drug absorption through injured bladder mucosa is unpredictable, the duration of exposure to the agent is relatively short, and delayed drug administration requires the maintenance or reinserterion of a urethral catheter.

Practical concerns regarding uncontrolled systemic absorption of the drug through areas of denuded urothelium have

<table>
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<th>CY dose (mg/kg)</th>
<th>No. animals treated</th>
<th>No. surviving animals</th>
<th>% survival</th>
<th>No. animals with bladder tumors</th>
<th>% implantation</th>
<th>Mean tumor-bearing bladder weight (g)</th>
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<tr>
<td>0</td>
<td>64</td>
<td>57</td>
<td>89</td>
<td>37</td>
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<td>0.05</td>
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<td>9</td>
<td>90</td>
<td>4</td>
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<td>0.1</td>
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<td>14</td>
<td>93</td>
<td>9</td>
<td>64</td>
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<td>0.5</td>
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<td>22</td>
<td>85</td>
<td>6</td>
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<td>88</td>
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<tr>
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<td>26</td>
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<td>92</td>
<td>3</td>
<td>12*</td>
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</tr>
<tr>
<td>75</td>
<td>25</td>
<td>16</td>
<td>64*</td>
<td>2</td>
<td>12*</td>
<td>0.105 ± 0.005*</td>
</tr>
</tbody>
</table>

* Mean ± SD.

* Significant difference from control group (0 mg/kg), P < 0.05.

* Significant difference from control group (0 mg/kg), P < 0.01.

* Significant difference from control group (0 mg/kg), P < 0.02.
prompted most investigators to recommend delaying administration for periods of 1 to 7 days. Clinical and laboratory studies of transvesical drug absorption have confirmed the potential for systemic drug distribution, and cases of serious myelosuppression and systemic cytotoxicity have been reported. As currently utilized, intravesical chemotherapy for the prevention of intravesical tumor recurrence has yielded modest results, with reports ranging from 21% to 40% reduction in tumor recurrence (15).

Ideally, chemotherapy directed at decreasing implantation-associated recurrence should be timed to maximize its cytotoxic effects directly on the tumor and indirectly at the site of tumor cell adherence. The postinoculation administration of chemotherapeutic agents, particularly when delayed several days, may have a diminished ability to access sites of implantation and be less cytotoxic to cells with an established "foothold" in the host environment. In the analogous clinical situation of perioperative wound infection, studies have emphasized the crucial importance of drug tissue levels at the time of exposure to the bacterial inoculum (11). Systemic chemotherapy administered immediately prior to the implantation risk would fulfill the objectives for optimal implantation prophylaxis.

The use of systemic chemotherapy for the prevention of bladder tumor recurrence is not a new concept. Clinical and laboratory studies using systemic chemotherapy during the postoperative period have described several active agents. England et al. administered CY at a dose of 1 g/m² at 3-wk intervals. A complete response rate was achieved in 12 of 15 patients (16). Using the MBT2 model system, Soloway and Martino showed that both adriamycin and CY were active in preventing the growth of established tumors (17).

The use of perioperative chemotherapy specifically for the prevention of tumor implantation was suggested by Weyrauch and Crossfield in 1962 (18). These authors advocated periprocedural i.v. thiotepa beginning the day of surgery and for 2 days postoperatively. Unfortunately, no patient follow-up in terms of either bladder tumor recurrence or chemotherapy toxicity was discussed in the original or subsequent publications.

In this study, we have shown that a single dose of CY, when administered systemically immediately prior to the time of bladder exposure to tumor cells, is effective in preventing tumor implantation. Cumulatively, of the 139 surviving animals that received a prophylactic dose of CY ranging from 2.5–100 mg/kg immediately prior to tumor exposure, 10 (7%) developed bladder tumors. Forty-six of 66 surviving animals (70%) in the corresponding control groups developed tumors. Therapeutic doses of CY appeared to reduce not only overall implantation risk, but also tumor volume in the few animals that developed bladder tumors. The significant reduction in tumor volume, as evidenced by tumor-bearing bladder weights, in the CY treatment groups suggests a reduction in the size of the viable tumor inoculum, leading to tumor development in these animals.

Antiimplantation activity of CY over a dose range of 0.5 to 100 mg/kg was perhaps the most striking finding in this group of experiments. Doses more than 2 logs below those associated with a significant increase in lethal toxicity were effective in reducing bladder tumor implantation. This persistence of activity at very low doses is probably due, in part, to the timing of drug administration. Although the numbers are relatively small,
results of the experiment on the timing of CY administration suggest that peak activity is achieved when the drug is administered 1 h prior to the period of tumor exposure.

While clinical utilization of this regime is theoretically feasible at this time, optimal use will require further laboratory study. The mechanism by which systemic CY exerts its antiimplantation effect and its efficacy relative to other chemotherapeutic agents remain to be defined. It is possible that CY exerts its activity not only by a direct cytotoxic effect on the tumor and an effect on the tumor at the site of implantation, but also by a second-pass effect in which active CY metabolites excreted in the urine continually bathe adherent neoplastic cells at the site of implantation. In essence, the half-life of the drug in combination with a primary renal route of excretion result in prolonged exposure of the implantation site to the therapeutic agent. This contrasts to intravesical approaches in which therapeutic drug levels are achieved for a much shorter period.

The role of urinary excretion of CY and its metabolites in mediating an antiimplantation effect is an important issue. The ability of the CY metabolite acrolein to mediate urotoxicity and the capacity of N-acetyl cystine (Mucomist) and sodium 2-mercaptoethane-sulfonate (mesna) to prevent this toxicity are well established (19–21). If acrolein plays no role in the antiimplantation activity of systemically administered CY, it may be possible to prevent the urotoxic effects of CY while maintaining its antiimplantation activity. Further reduction of the potential risks associated with systemic CY would enhance its attractiveness as an option for the prophylaxis of superficial bladder tumor recurrence.

Conclusions. Using a rat model of bladder tumor implantation, we have shown that preimplantation chemoprophylaxis with systemic CY effectively prevents tumor recurrence resulting from implantation. The lowest effective dose observed in these experiments corresponds to a human dose that should result in minimal or absent toxicity. Preoperative chemoprophylaxis may prove effective in preventing intravesical bladder tumor recurrences resulting from implantation.

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