Single-Dose Cyclophosphamide for the Prevention of Bladder Tumor Implantation in F344 Rats: Site of Drug Activity

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ABSTRACT

Previous studies demonstrated that single doses of systemic cyclophosphamide (CY) as low as 0.5 mg/kg are effective in preventing bladder tumor implantation in a rat model. In an effort to determine if the urinary bladder represents a unique site of CY activity, a series of experiments were performed to define the mechanism by which low-dose CY prevents bladder tumor implantation.

Potential sites for CY antitumor activity include direct tumor cytotoxicity resulting from serum delivery of drug to the tumor; tumor cytotoxicity resulting from tissue drug levels at the site of implantation; altered tumor cell adherence to the urothelial injury site; nonspecific urothelial cytotoxicity resulting from urinary excretion of the CY metabolites; tumor cell-specific cytotoxicity resulting from urinary excretion of the CY metabolite acrolein; and second-pass cytotoxicity resulting from urinary excretion of the active form of CY. Experiments were performed to determine if a single predominant site of activity could be defined.

Cyclophosphamide levels at the site of tumor implantation appeared to be the most important determinant of antimplantation activity. Only tumor recipients pretreated with CY had a significant decrease in bladder tumor implantation. In vivo and in vitro assays measuring the effect of blood-borne drug delivery directly to the tumor failed to demonstrate cytotoxic activity. Tumor cell adherence assays measuring in vitro adherence of CY-treated tumor cells and in vivo adherence of tumor cells in CY-treated recipients showed no difference in comparison to control groups. Interval histological comparison of CY-treated and control bladder failed to demonstrate any difference. Urinary levels of acrolein did not contribute to antimplantation activity. Preimplantation CY doses prevented tumor development in a s.c. implantation model, thereby excluding a second-pass effect resulting from urinary drug excretion.

These data suggest that the bladder is not unique in its response to systemic low-dose CY administered for the prevention of implantation-mediated tumor recurrence. Low-dose, perioperative chemoprophylaxis may be applicable to other tumor systems in which intraoperative tumor dissemination is felt to contribute to recurrence risk.

INTRODUCTION

Previous reports demonstrated the ability of single-dose, preoperatively administered, systemic CY to prevent bladder tumor implantation in a rat model (1). The timing of drug administration was shown to be important, with optimal activity observed when CY was administered immediately prior to or shortly after tumor implantation. Using this protocol, doses as low as 0.5 mg/kg resulted in a significant reduction in bladder tumor implantation compared to controls. The plateau of peak activity occurred at a dose of 2.5 mg/kg, and no drug-related mortality was observed at doses of 50 mg/kg or less. The remarkable low-dose activity of this regime, with a therapeutic index of 100 (maximal nonlethal/minimal effective), prompted questions as to its mechanism of activity. The purpose of this study was to determine the mechanism responsible for this low-dose efficacy.

When administered before tumor implantation, systemic CY achieves drug levels in both the tumor and the tissue at the site of implantation. In addition, renal excretion of the drug and its metabolites results in continued exposure of the bladder implantation site to urinary drug levels during the process of drug elimination. This series of experiments was designed to determine which, if any, of these potential causes of tumor cytotoxicity played a predominant role in the anti-bladder implantation effect of systemic CY.

MATERIALS AND METHODS

Tumors. The rat transitional cell bladder carcinoma 4909 was used in all experiments. This tumor, originally induced in F344 rats by the intravesical implantation of methylcholanthrene-impregnated wax pellets, has a moderately well-differentiated transitional histology (2). The tumor is maintained in vivo by s.c. passage in syngeneic animals.

Animals. Female F344 rats, 10–12 wk old, were utilized in all experiments (National Cancer Institute, Frederick, Maryland). Animals were maintained at a facility approved by the American Association for the Accreditation of Laboratory Care. The protocols used in these experiments received approval of the Animal Care and Use Committee prior to their performance. All animal manipulations were performed under general anesthesia using i.p. Nembutal (50 mg/kg).

Experiment 1. In an implantation system with a steep dose/implantation response, small decreases in the effective tumor inoculum may result in large decreases in implantation. To better define the kinetics of tumor implantation as a function of tumor inoculum in the previously described model system, the following experiment was performed. Subsequent to bladder fulguration at three discrete sites, animals received 0.2 ml of tumor cell suspension. Groups of 10 animals each received concentrations of the tumor cell suspension at 10^5, 10^6, and 10^7 cells/ml using the previously described protocol (1).

Experiment 2. In our model, preimplantation systemic CY was administered so as to result in drug levels in both the tumor and the tissue at the site of tumor implantation. Both the tumor donor and the tumor recipient received an identical dose of CY. This is analogous to the clinical setting in which the patient receiving CY prior to transurethral tumor removal would have drug levels in both the tumor and the bladder tissue. The physical separation of tumor donor and recipient in this model allows the relative contribution of direct CY toxicity on the tumor (resulting from blood-borne drug delivery) and cytotoxicity at the level of the bladder to be determined. The following experiment was designed to determine if CY exerted its principal activity at either of these sites.

A s.c. tumor-bearing animal to be used as the tumor donor was prepared by injecting an equal number of tumor cells from the same tumor preparation into both the right and the left flank. When the tumors reached 1.5 cm in diameter, the animal was anesthetized and the right flank tumor was excised and used for the preparation of a "non-chemotherapy-treated" tumor cell preparation. Immediately following the excision of the first tumor, the animal was given a single i.p. dose of CY (2.5 mg/kg). This dose was based on previous experi-

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ments that demonstrated it to be the lowest dose resulting in activity on the plateau portion of the dose-response curve (1). One h subsequent to CY administration, the left flank tumor was excised and used for the preparation of a "chemotherapy-treated" tumor cell suspension. A group of 80 recipient animals was then randomized to receive either i.p. CY (2.5 mg/kg) or an equal volume of i.p. saline. One h later, each recipient group was further subdivided to receive either the chemotherapy-treated tumor cell suspension or the non-chemotherapy-treated tumor cell suspension (1 x 10^6 viable cells/ml). Tumor implantation and subsequent bladder evaluation were then performed as previously described (3). This protocol resulted in the following treatment groups: normal cells/saline recipient (n = 15); CY cells/saline recipient (n = 20); normal cells/CY recipient (n = 20); CY cells/CY recipient (n = 20).

Experiment 3. A modification of the MTT assay was used as an in vitro measurement of direct tumor cytotoxicity resulting from blood-borne drug delivery to the tumor (4). Chemotherapy- and non-chemotherapy-treated tumor cell suspensions were simultaneously prepared from a bilateral tumor donor as described in Experiment 2. Viable tumor cells from each cell suspension (1 x 10^6) was plated in quadruplicate into 96-well tissue culture plates (100-μl volume/well). A parallel experiment was performed using 1 x 10^4 total cells (viable + nonviable) per well. At 1.5, 17, 22, 55, and 68 h following plating, 25 μl of a single i.p. dose of CY was given to each CY recipient group. After 1 h, all animals underwent tumor cell adherence measurement to fibrin-coated plates. Ninety-six-well flat-bottom plates were precoated with fibrin at a concentration of 10 μg/cm^2 as described by Unkeless et al. (5). Sample absorbance was read at 595 nm using a Titertech multilple plate reader. The relative viability of each tumor cell suspension over time was expressed as sample absorbance at each time point divided by sample absorbance at 1 h.

Experiment 4. Tumor cell adherence to sites of urothelial injury is an important requisite step in the process of implantation-mediated bladder tumor recurrence (5). One mechanism by which urinary or bladder tissue levels of CY could effect tumor implantation would be by altering tumor cell adherence to sites of urothelial injury. We performed a series of experiments to determine if systemic CY altered tumor cell adherence to injured urothelial surfaces.

Experiment 4A. Prior work suggested that fibrin is an important adherence substrate at the injury site (5). An in vitro experiment was performed to determine if tumor treatment with CY decreased adherence to fibrin-coated plates. Ninety-six-well flat-bottom plates were coated with fibrin at a concentration of 10 μg/cm^2 as described by Unkeless et al. (6). Cyclophosphamide- and non-CY-treated tumor cell suspensions were prepared from a bilateral s.c. tumor donor as described in Experiment 1. Plates were placed at a concentration of 1 x 10^5 cells/well in a 100-μl volume. At intervals following plating, individual wells were vortexed under an air stream, the supernatant fluid was removed, and the wells were washed 3 times with 100 μl of Roswell Park Memorial Institute Medium. The remaining cells were recovered with 100 μl of medium. This procedure was performed sequentially on different wells at time points of 1, 3, 8, and 12 h following plating. Subsequent to the last time point, the MTT assay was performed as described. A standard cell number dilution curve was concomitantly performed. A separate cell number standard curve was constructed for each tumor cell suspension. Results were calculated as the number of adherent cells at each time point. All sample points were performed in quadruplicate.

Experiment 4B. An experiment was performed to determine if bladder tissue drug levels or urinary drug excretion affected tumor cell adherence. A non-chemotherapy-treated tumor cell suspension was prepared and labeled with [125I] as previously described (5). One h prior to intravesical tumor instillation, recipient animals were randomized to receive either i.p. saline or i.p. CY at a dose of 2.5 mg/kg. The in vivo tumor cell adherence assay was then performed as previously described (5). Seven animals were studied in each group.

Experiment 5. Acrolein is a metabolic conversion product of CY that is excreted in the urine and is responsible for the urothelial toxicity observed following high-dose systemic CY (7, 8). Nonspecific urothelial toxicity or specific tumor cytotoxicity secondary to urinary levels of acrolein represents a possible mode of action of CY in preventing bladder tumor implantation. We performed experiments to determine if low-dose systemic CY (2.5 mg/kg) resulted in histological urothelial alterations or if urinary levels of acrolein were responsible for the antimplantation effect of systemic CY.

Experiment 5A. Eight animals underwent i.p. injection with CY (2.5 mg/kg), and an additional 8 animals were treated with i.p. saline. One h later all animals underwent bladder electrocautery injury analogous to that used for the tumor implantation assay. Two animals each from the CY-treated and control groups were sacrificed at 24, 48, 72, and 120 h following CY instillation. At the time of sacrifice the bladders were distended with intravesical formalin and fixed in the same solution. Formalin-fixed bladder specimens were prepared for routine hematoxylin and eosin staining and histological evaluation. Both injured and normal urothelial sites were evaluated for histological changes.

Experiment 5B. Previous clinical and laboratory studies demonstrated that acrolein-mediated urothelial toxicity may be prevented by systemic administration of 2-mercaptoethane sodium sulfonate (Mesna) (9, 10). Following urinary excretion, Mesna binds to acrolein and other 4-hydroxy metabolites of CY to form nontoxic additive compounds. This experiment was performed to determine if binding of urinary acrolein with systemically administered Mesna altered the antimplantation efficacy of low-dose CY. The CY-treated tumor cell suspension was prepared as described. Recipient animals were randomized to receive CY at a dose of 2.5 mg/kg (n = 20) or CY at the identical dose plus Mesna at a dose of 30 mg/kg (n = 20). This dose of Mesna has been shown to prevent acrolein-mediated urothelial toxicity in a rat model (10). Recipient animals were treated with a respective drug combination 1 h prior to tumor implantation. At 4 and 8 h following implantation, animals were treated with additional doses of either Mesna (30 mg/kg) or phosphate-buffered saline. The 4- and 8-h treatments corresponded to the initial treatment group of all animals. Tumor implantation was evaluated at 4 wk, as described.

Experiment 6. The results of the first four experiments suggested that principal antitumor activity occurred at the level of the bladder and that this activity appeared not to be related to nonspecific urothelial toxicity or acrolein-related cytotoxicity. The following experiment was designed to determine if this activity resulted from tissue drug levels or urinary excretion of active drug resulting in protracted exposure of the implantation site to urinary drug levels.

The ideal experiment to address this issue would divert urinary levels of CY and its metabolites from the bladder without influencing local tissue levels of CY or the normal physiology of bladder emptying. Unfortunately, surgical manipulations resulting in urinary diversion cause fundamental alterations of the intravesical implantation model. An alternative, albeit an indirect approach was to study the antimplantation efficacy of CY in a s.c. implantation model. Through the use of appropriate controls and by comparing the ability of systemic CY to prevent s.c. implantation to its ability to prevent intravesical implantation, it may be possible to make some inference regarding the role of urinary drug excretion in mediating antimplantation activity.

Cyclophosphamide-treated and non-CY-treated tumor cell suspensions were prepared from a bilateral s.c. tumor donor as described in Experiment 1. Sixty recipient animals were randomized to receive a single i.p. dose of CY (2.5 mg/kg) or i.p. saline. One h later, saline-treated animals received a s.c. tumor inoculum with the non-CY-treated cell suspension. Cyclophosphamide-treated recipients received an identical inoculum of the CY-treated tumor cell suspension. Animals received doses of 1 x 10^3, 1 x 10^4, or 1 x 10^5 viable tumor cells in 0.2 ml of media. Ten animals in each group received one of the three tumor cell concentrations. Beginning 1 wk from the time of tumor implantation, animals underwent biweekly s.c. tumor measurement. Both the incidence of s.c. tumor implantation and tumor volume as a function of time were determined. Tumor volume was calculated based on the formula:

Tumor volume (mm^3) = tumor length x tumor width x tumor height
Tumor measurements were made using Vernier calipers. Measurements of tumor height were made by rotating the s.c. tumor nodule 90° from its original position.

Data Analysis. Intravesical and s.c. tumor implantation rates among different treatment groups were compared using chi square analysis with continuity correction. Differences in in vitro tumor viability as a function of time following CY or sham treatment in the MTT assay were evaluated using an analysis of variance for repeated measures. An identical analysis was used to compare the rate of s.c. tumor growth over time in each treatment group. In vitro tumor cell adherence was compared using the nonparametric Mann-Whitney U test.

RESULTS

Experiment 1. Five of nine, four of six, five of seven, and nine of nine surviving animals developed tumors in the $10^4$, $10^5$, $10^6$, and $10^7$ cells/ml inoculation groups, respectively. The average weight of tumor-bearing bladders for each of these groups was $0.98 \pm 0.47$ (SD), $0.27 \pm 0.06$, $1.42 \pm 1.08$, and $2.23 \pm 2.02$ g. These results are shown graphically in Fig. 1. Previous work showed that the 1000-fold range in tumor cell concentration used in this experiment corresponds to a 46-fold range for the adherent tumor inoculum (11).

Experiment 2. Nine of 14 surviving animals developed tumors in the control (normal cells/normal recipient) group. Six of 20, 11 of 17, and 4 of 16 surviving animals developed tumors in the normal cells/CY recipient, CY cells/normal recipient, and CY cells/CY recipient groups, respectively. There was a significant difference in implantation rates among all groups ($4 \times 2$ chi square, $P < 0.01$). Cyclophosphamide administration to both the tumor donor and the tumor recipient significantly decreased tumor implantation rates compared to both control animals and animals treated with CY to the donor alone ($P < 0.05$ and $P < 0.02$, respectively). Tumor implantation in the group receiving normal cells and CY was reduced compared to the group receiving CY-treated cells and sham treatment ($P < 0.05$). There was no difference in tumor implantation rates in animals treated with normal cells and CY compared to animals treated with CY cells and CY. There was no difference in the tumor-bearing bladder weights of any of the treatment groups (data not shown). Implantation results for each treatment group are shown graphically in Fig. 2.

Experiment 3. Immediately following preparation, the normal and CY-treated tumor cell suspensions had viabilities of 78% and 86%, respectively, as determined by trypan blue exclusion. Fig. 3 compares relative tumor cell viability at each time point for the CY-treated and non-CY-treated tumor cell suspensions plated as both equal numbers of viable and total cells. Values are expressed as the ratio of sample absorbance at each time point relative to the 1-h absorbance value. There was no decrease in cell viability in the CY tumor compared to the control tumor.

Experiment 4A. In vitro tumor cell adherence of CY or sham-treated tumor cell suspensions to fibrin-coated plates as a function of time is shown graphically in Fig. 4. No difference in adherence was noted up to 12 h following plating.

Experiment 4B. There was no difference in tumor cell adherence to the cautery-injured bladders of CY-treated recipients compared to controls. Mean tumor cell adherence in the control groups was 21,057 ± 18,561 (±1SD) cells/bladder compared to 17,656 ± 7,982 cells/bladder in the CY-treated group.

Experiment 5A. The bladder injury sites of both CY and sham-treated animals demonstrated acute and chronic inflammatory changes. There was no difference between groups. No histological alteration at urothelial sites remote from the area of injury was observed at any time point in either the CY-treated or control groups.

Fig. 3 compares relative tumor cell viability at each time point for the CY-treated and non-CY-treated tumor cell suspensions plated as both equal numbers of viable and total cells. Values are expressed as the ratio of sample absorbance at each time point relative to the 1-h absorbance value. There was no decrease in cell viability in the CY tumor compared to the control tumor.
Fig. 4. In vitro adherence of CY-treated and control tumor cells to fibrin-coated plates. Each point represents the average value from quadruplicate samples.

Experiment 5B. The addition of Mesna to the CY treatment regime failed to result in any statistically significant change in tumor implantation rates. Three of 13 surviving animals in the CY-treated group developed tumors compared to 0 of 16 surviving animals in the CY plus Mesna group.

Experiment 6. Tumor implantation rates for each treatment group, at specific inoculums of 1 × 10³, 1 × 10⁴, and 1 × 10⁵, are shown in Figs. 5A, 5B, and 5C, respectively. A similar comparison of average tumor volume as a function of time postinoculation is shown in Figs. 6A, 6B, and 6C. “Preoperative” CY administration resulted in a significant delay in tumor growth for all tumor inoculum sizes (P < 0.009, P < 0.04, and P < 0.004 for the 10³, 10⁴, and 10⁵ inoculum groups, respectively). However, differences in actual tumor implantation rates were observed only in animals receiving the 1 × 10³ tumor cell inoculum. Five wk from the time of inoculation, 6 of 10 animals in the control group had developed s.c. tumors compared to 0 of 10 in the CY treatment group (P < 0.02). No animal in the 10³ CY treatment group developed a tumor during a 15-wk total observation period.

DISCUSSION

Cyclophosphamide is a cyclic ester of mechlorethamine and functions as a non-cell cycle specific alkylating agent. It requires conversion to its active form by the hepatic microsomal (P-450) mixed function oxidase enzymes and thus must be systemically administered. Renal excretion eliminates 10% of the unchanged drug and 50% of metabolites within 24 h (12). The plasma half-life appears to range from 4–6.5 h. The activity of CY against urothelial cancers has been demonstrated in both the clinical and the laboratory setting. Logothetis et al. reported a significant survival advantage in high-risk patients treated with an adjuvant chemotherapy regime including CY, cis-platinum, and doxorubicin compared to a concomitant high-risk control group (13). In an animal model, Soloway and Martino demonstrated that CY administered at 10-day intervals beginning on the fifth day following intravesical tumor implantation prevented the development of bladder tumors (14).

Single doses of systemic CY administered circa the time of tumor inoculation have been shown to be extremely effective in preventing tumor implantation in a rat bladder tumor model (1). The timing of drug administration is important in that maximal efficacy requires drug administration shortly before or shortly after tumor inoculation. This series of experiments attempted to further define the mechanism by which single-dose systemic CY prevented bladder tumor implantation.

The experiment designed to correlate intravesical tumor inoculum with the incidence of tumor implantation showed that a 3-log reduction in inoculum concentration, corresponding to a 46-fold decrease in the adherent tumor inoculum, resulted in less than a 2-fold decrease in implantation. Given that the implantation experiments use an inoculating tumor cell concentration of 1 × 10⁷ cells/ml (the highest concentration evaluated in the tumor inoculum/implantation experiment), a 50% reduction in implantation would require more than a 50-fold reduction in the effective, adherent inoculum.

Experiments designed to separate the effect of tumor drug levels, implantation site drug levels, and second-pass urinary drug excretion were performed. Tumor tissue levels of CY failed to alter tumor cell viability as measured by an in vitro assay. Similarly, tumor tissue levels of CY did not alter intravesical
tumor implantation rates or tumor volumes in the in vivo implantation model. Cyclophosphamide treatment of recipient animals appeared to be the most important variable in preventing intravesical tumor implantation and growth. Animals receiving systemic CY immediately prior to tumor implantation had significantly decreased implantation rates compared to non-CY-treated recipients. This effect was independent of whether or not the animals were inoculated with normal or CY-treated tumor cell suspensions.

Cyclophosphamide activity at the level of the bladder could be explained by alterations in tumor cell adherence to the injury site, second-pass-specific or nonspecific cytotoxicity resulting from urinary excretion of CY or its metabolites, or tissue drug levels at the site of tumor implantation. In vitro and in vivo experiments studying tumor cell adherence failed to show any effect of CY treatment on tumor cell adherence. Although we did not specifically evaluate the adherence of CY-treated tumor cells to the injury sites of CY-treated recipients, the implantation data do not support a need for combined tumor and tissue drug levels in order to mediate an effect. The role of urinary excretion of CY metabolites in general and of acrolein specifically on tumor implantation was determined. At a dose of 2.5 mg/kg, systemic CY failed to cause histologically discernible urothelial injury. The addition of Mesna to the CY treatment regime in doses that previously were demonstrated to prevent CY-associated, acrolein-mediated urotoxicity failed to increase tumor implantation.

As a final indirect measure of the role of urinary drug excretion in preventing tumor implantation, the effect of systemic CY in preventing s.c. tumor implantation was evaluated. Because the precise number of tumor cells remaining in the bladder, which constitutes the “tumor inoculum,” was unknown, it was necessary to evaluate the efficacy of CY across a range of tumor cell inocula. Irrespective of inoculum size, systemic CY significantly delayed s.c. tumor growth, as measured by tumor volume, compared to the respective control (P < 0.05). These conclusions are in agreement with our earlier studies which showed that CY maintained its activity when administered subsequent to tumor inoculation. The importance of this observation is that the antiimplantation activity of CY is not unique to the bladder. It may be possible to expand the utilization of this approach to the prevention of implantation-mediated tumor recurrence and other organ systems. One clinical study that supports this hypothesis has been published. A Scandinavian multiinstitutional trial prospectively compared recurrence and survival rates in a group of breast cancer patients randomized to receive a brief, immediate postoperative course of CY or no therapy (15). The chemotherapy-treated patients had a significant decrease in tumor recurrence and a significant survival advantage that remains consistent for up to 10 yr following mastectomy. In patients in whom the chemotherapy regime was delayed by 3 weeks, no survival or recurrence advantage was demonstrated.

Conclusion. Systemic single-dose CY when administered circa tumor inoculation is effective in preventing bladder tumor implantation. The principal site of activity is at the level of the bladder and appears to be related to tissue levels of CY. Perioperative, low-dose chemophrophylaxis may represent an effective strategy for application to other cancers in which surgical manipulation may predispose to implantation-mediated recurrence.

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