Urol Res (2001) 29:345–349
DOI 10.1007/s002400100204

ORIGINAL PAPER

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Extravesical cryosurgical approach for VX2 bladder tumor in rabbits

Received: 23 October 2000 / Accepted: 29 May 2001 / Published online: 8 August 2001

Abstract This study characterized the VX2 bladder cancer model in rabbits and tested the feasibility of treating bladder cancer by extravesical cryosurgery. After the growth characteristics of the VX2 bladder tumor model were determined, the VX2 tumor was inoculated into rabbits at the dome of the bladder. One week later, three freeze/thaw cycles were followed by immediate surgical repair. The control group underwent a sham operation without freezing. When the VX2 tumor is injected into the bladder wall, invasion and central necrosis occurred within 1 week, lymphatic metastases by 2 weeks, and lung metastases by 3 weeks after inoculation. By 4 weeks, all control rabbits had large VX2 tumors in their bladders and advanced lung metastases. Nine of the ten rabbits in the cryosurgical group had mild to moderate degrees of lung metastases, and six of them had relatively small local recurrences. One rabbit had no tumor in the bladder and only microscopic lung metastasis. The extravesical approach to cryosurgery employing bladder inversion is well tolerated. Cryosurgery exhibits modest efficacy in treating local tumors and delaying lung metastasis in this aggressive tumor model.

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Keywords Cryosurgery · Rabbit bladder cancer model · VX2 tumor

Introduction

The choice between cystectomy and bladder sparing procedures in patients with locally advanced bladder cancer is controversial [5, 12, 23, 31, 33]. Combined-modality therapy aimed at organ preservation has been employed successfully in selected patients [13, 17, 27]. Nevertheless, complete bladder excision by radical cystectomy remains the most common treatment for locally advanced bladder cancer [10, 19]. Although radical cystectomy has the theoretical advantage of complete eradication of local disease, it is also associated with significant morbidity, mortality and functional compromise. Partial cystectomy offers the opportunity for bladder preservation but is associated with the complications of tumor seeding, and local recurrences may result from either residual disease or second primaries [18].

Cryosurgery can provide tissue destruction without the immediate disruption of the integrity of the treated tissue [21, 26] and has been used to treat both benign and malignant diseases [1, 2, 14, 32, 36]. Transvesical cryosurgical treatment of bladder cancer has been described [20, 28], but this open approach carries the risk of tumor seeding as a result of tumor manipulation in an open bladder.

In this study, we characterize a rabbit bladder tumor model using the VX2 tumor and describe an extravesical cryosurgical approach for the treatment of bladder cancer. All animals received humane care in accordance with the requirements of the United States Animal Welfare Act. The VX2 tumor is a highly malignant Shope virus-induced squamous cell carcinoma that consistently produces tumors in the bladders of rabbits [15, 16, 24, 25]. To prevent delayed bladder rupture following cryosurgery, we used a bladder inversion technique. This extravesical technique avoids opening the bladder, thereby minimizing the risk of tumor seeding.

Materials and methods

Preparation of VX2 tumor cell suspension

The VX2 tumor was obtained from the thighs of New Zealand White rabbits 14 days after transplantation. The tumor was minced and treated with 1% collagenase for 1 h. The digested tumor was then successively passed through 18-gauge, 20-gauge, and 22-gauge needles. The cells were concentrated by centrifugation and resuspended at a concentration of 1×10^7 cells/ml in minimum essential medium with glutamine, non-essential amino acids, and 10% fetal calf serum.

Tumor model

The rabbits were anesthetized by mask induction of 4% isoflurane in oxygen using high flow techniques. They were then endotracheally intubated, and anesthesia was maintained with 2–3% isoflurane in oxygen employing a semiclosed system. Isotonic saline was administered at a rate of 5 ml/kg per hour throughout surgery and during recovery from anesthesia.

The bladder was exposed through a low midline incision, and 100 μl of VX2 cell suspension (10^6 cells) was injected into the dome of the bladder using an extravesical approach with a 22-gauge needle. A successful injection produced a small bleb at the dome.

Cryosurgery

After anesthesia was induced, the bladder was exposed through a low midline incision. The VX2 bladder tumor was measured in two dimensions. The bladder was emptied by manual compression and then filled with 40–50 ml of air using a 25-gauge needle. A total of three freeze/thaw cycles were performed through an extravesical approach. The cryoprobe containing liquid nitrogen was directly applied to the area of the bladder harboring the tumor. A freezing cycle was completed when the tissue was grossly frozen. Purse string sutures of 3–0 chromic were used to outline the frozen area after the first freeze. These sutures were used as a guide to mark the extent of the subsequent two freezes. After the three freeze/thaw cycles, the treated portion of the bladder was inverted using the previously placed purse string suture. The inverted tissue was reinforced with additional sutures of 3–0 chromic.

Sham operation

In the control rabbits, all the procedures including bladder inversion were the same as for the rabbits receiving cryosurgery except that the cryoprobe did not contain liquid nitrogen.

Pathological studies

Four New Zealand white rabbits were used to determine the growth characteristics of the tumor in the VX2 model. The rabbits were killed at 7, 14, 21, and 28 days after tumor implantation, and both local tumor growth and distant metastases were evaluated.

Four rabbits were used for acute pathological studies to determine the early effects of cryosurgery. One week after the animals were inoculated with the VX2 tumor, extravesical cryosurgery was performed. The rabbits were killed 6 h, 24 h, 48 h and 1 week after cryosurgery. Detailed pathological changes were investigated.

Evaluation of cryosurgery

Twenty rabbits were inoculated with VX2 tumor cells in the bladder dome (10^6 cells/100 μl). The rabbits were randomized into control and cryosurgery groups ($n=10$ each), and both groups underwent surgery 7 days after tumor cell implantation. The animals were killed 4 weeks after tumor implantation and examined for local and metastatic disease. Bladder tumor volumes were calculated using the formula of a sphere ($4/3\pi\ r^3$). Lung metastases were estimated using transparent grid paper covering the surface of the lung. Statistical analysis was by non-paired Student's *t*-test.

Results

VX2 bladder tumor model

One week after bladder inoculation, the VX2 tumor cell suspension formed a 11×8 mm tumor in the dome of the bladder that invaded the bladder muscle and extended through the full thickness of the bladder wall. The iliac lymph nodes were free of metastasis on both gross and microscopic examination. The liver, kidneys, and lungs were all normal on gross examination.

Two weeks after bladder inoculation, the bladder tumor was 16×15 mm in diameter and exhibited central necrosis and invasion of adjacent tissue. The iliac lymph nodes were 2×3 mm in diameter and contained VX2 tumor. The liver, spleen, and lung remained grossly free of metastasis.

Three weeks after bladder inoculation, areas of necrotic tumor were noted along with ascites. Metastases were seen in the lymph nodes, the small and large intestines, the mesentery, and the lungs. The liver and spleen remained grossly free of disease.

At 4 weeks, the VX2 tumor was easily visible. The bladder tumor grossly invaded the anterior abdominal wall and the intestines. Multiple metastases were noted at the serosal surface of the small and large intestines and in the omentum. Extensive lung metastases were seen.

Acute changes after extravesical cryosurgery

Four rabbits were killed 6 h, 24 h, 48 h and 1 week after cryosurgery to observe acute changes following cryosurgery. The acute changes at 6 and 24 h are depicted in Figs. 1 and 2. Grossly, the infolded portion of the bladder appeared dusky within 2 days of cryosurgery and at 7 days was pale white. The reconstructed bladder wall on top of the inverted tumor appeared viable. Inversion of the treated part of the bladder resulted in the formation of a cystic mass within the bladder containing the necrotic VX2 tumor. Blood clots were seen in this necrotic tissue in some of the rabbits. Histopathologically, congested blood vessels, hemorrhage, and edema were noted in the areas with coagulative necrosis and in the adjacent tissue. There was no evidence of viable tumor on multiple histologic sections. No metastases were seen.

Evaluation of cryosurgery

At the time of treatment, mean tumor sizes in the experimental and control groups were 6.9 ± 0.2 and

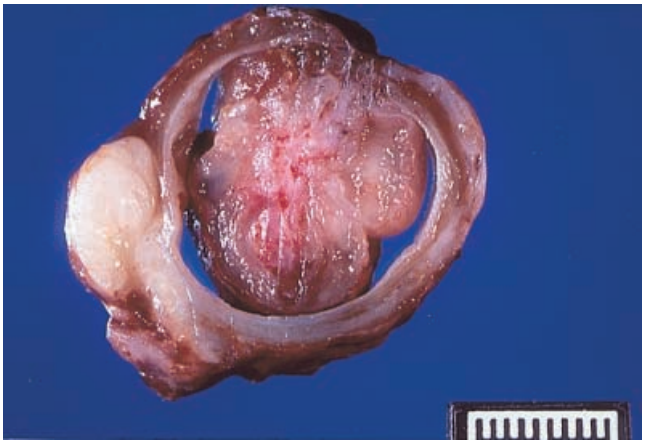

Fig. 1 Cross section of inverted bladder tumor 6 h after cryosurgery

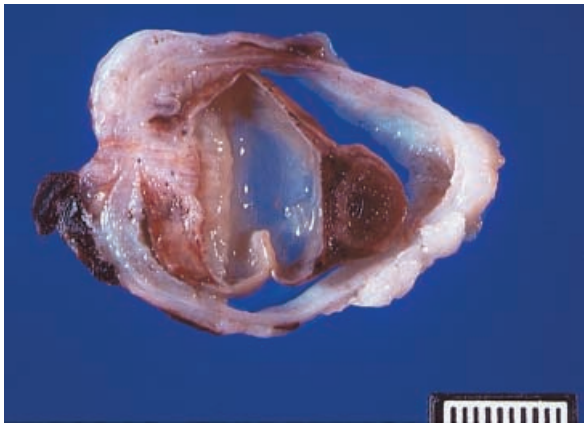

Fig. 2 Cross section of inverted bladder tumor 24 h after cryosurgery

6.7 ± 0.2 mm, respectively. These measurements were transformed into tumor volume with the assumption that the tumor was spherical. There were no operative deaths. Hematuria was common in the acute postoperative period. The pathologic findings 28 days after cryosurgery are summarized in Table 1.

Of the ten rabbits that underwent cryosurgery, four had no tumor remaining in the bladder. One of these four had microscopic lung metastasis and the other three had gross lung metastases. The remaining six rabbits had both bladder tumor recurrences and lung metastases. Peritoneal and abdominal wall seeding were noted in four rabbits all of which also had lung metastases, and all but one had local recurrences in the bladder. Bladder stones were noted in six of ten rabbits and in two of these rabbits the stones were attached to a recurrent tumor.

All ten rabbits in the control group had large tumors protruding into the bladder and moderate to extensive lung metastases. One control rabbit died several hours before it was due to be killed because of extensive lung metastases. Peritoneal and abdominal wall seeding was not noted in this group, and bladder stones did not occur.

Discussion

The VX2 tumor is a highly malignant rabbit squamous cell carcinoma. A 1-h digestion of VX2 tumor with 1% collagenase resulted in a high yield of viable tumor cells, and injection of 10^6 cells consistently resulted in a tumor mass within 1 week. At 2 weeks, the VX2 tumor was found in lymphatic vessels and in the regional lymph nodes. Early invasive and metastatic growth are likely to be critical factors responsible for the failure of localized treatment in this model. At 1 week, there was gross local disease without obvious metastases. However, because of the propensity for local invasion, the margin of treatment will need to be wider than that dictated by the gross appearance.

The lung is the most common site of metastasis and was responsible for the death of animals in a previous report [25]. The interval between tumor implantation into the bladder and the demonstration of multiple lung metastases has been reported to be 3–4 weeks. Rabbits seldom survive more than 40 days after bladder implantation of the VX2 tumor. Lymph node metastases have been reported to occur 3 weeks after implantation [25]. In our studies, lymph node metastases occurred as early as 2 weeks after implantation, and lung metastases were seen 1 week later. This early and aggressive growth may result from the high proportion of viable cells in our tumor preparation.

Prior studies evaluating aggressive local therapy for bladder cancer have been associated with bladder perforation due to necrosis of the bladder wall, with resultant limitations on the treatment because of concern for bladder perforation [3, 9]. Microwave hyperthermia, laser cauterization, electrocauterization, shock wave treatment, piezoelectric treatment, and cryotherapy have been evaluated as definitive therapies for local bladder cancer [3, 7, 9, 11, 15, 20, 22, 28, 30, 35]. Because the bladder wall is thin and because relatively high pressures occur during voiding, full thickness bladder wall necrosis can lead to bladder perforation.

Table 1 Gross findings in rabbits 3 weeks after cryosurgery or sham surgery

	Cryosurgery	Sham operation	*P* value
Beginning bladder tumor volume (ml)	0.24 ± 0.28	0.19 ± 0.16	$P = 0.88$
Final bladder tumor volume (ml)	3.20 ± 8.00	11.79 ± 4.88	$P = 0.003$
Lung metastases at 4 weeks (median % involved with tumor)	4%	85%	$P = 0.001$

We employed a novel method to perform aggressive tumor destruction while maintaining bladder wall integrity. By inverting the treated area into the bladder lumen, we could apply aggressive local therapy without concern for bladder disruption. This strategy worked well. Despite evidence of extensive necrosis from the cryosurgery, no bladder perforations occurred. This strategy of immediate local repair is crucial for this model of aggressive administration of local therapy in the treatment of bladder cancer. While we evaluated this treatment in an open surgical model, it is just as applicable to a minimally invasive technique through a laparoscope.

In both the cryosurgery and control groups, a portion of the bladder was inverted and secured with a purse string suture and reinforcement sutures. The reconstructed bladders were completely healed within 4 weeks. Cryosurgery resulted in full thickness necrosis of the treated area. At 4 weeks, six rabbits had bladder stones replacing the inverted tissue, and the remaining four rabbits showed complete disappearance of the inverted, frozen tumor. The inversion technique that we used avoided bladder perforation despite complete necrosis and absorption of the treated tissue.

The acute pathological changes associated with cryosurgery were coagulative necrosis with vascular congestion, hemorrhage, and edema. No viable tumor was observed in the bladder after cryosurgical treatment in the acute studies. One week after bladder inoculation, the primary tumors had grown to a size of 4–12 mm (6.9 ± 2.4 mm) in diameter and were easily palpable. We have observed early tumor infiltration, seeding, and tumor emboli distant from the primary site in the bladder VX2 tumor model. Therefore, in this study cryosurgery was performed to encompass the gross tumor with a 1 cm margin of normal bladder. Despite this extra margin of therapy and microscopic documentation of efficacy in the acute experiments, locally recurrent tumor and metastases were noted. Local bladder recurrences occurred in six of ten rabbits in the experimental cryosurgery group.

Local recurrence can occur because of microscopic tumor infiltration of the grossly normal bladder outside of the treatment area or because of treated tumor that may have survived the freeze/thaw cycles. This latter occurrence is most likely at the periphery of the frozen tumor ice ball [4, 6, 8, 29, 34]. The complete area of cell killing is smaller that the gross area of freezing. A mean diameter of 3.2 ± 0.4 cm of gross freezing area was used to kill the tumor (mean diameter 0.7 ± 0.2 cm). Although, no viable tumor cells were found in the acute pathological studies, local recurrence (persistent primary tumor) was seen in six of ten rabbits. Additional modifications in the delivery of cryotherapy may be required to achieve complete cell killing. In addition to the size of the ice ball and number of freeze/thaw cycles, factors such as the velocity of freeze and the end freeze temperature will also be considered in future studies to improve the efficacy of cryosurgery [4, 29, 34].

Peritoneal and abdominal wall metastases were seen in the cryosurgery treated group but not in the control group. This finding may reflect altered lymphatic and vascular changes following cryosurgery or occult extravasation of viable tumor as a result of the bladder wall freezing.

The incidence of lung metastases in both the cryosurgery group (90%) and the control group (100%) was high. However, control rabbits had more extensive metastases. Lung metastases can result from early dissemination that occurred prior to the cryosurgery or from recurrent tumor after failure of local treatment. The fact that four rabbits with successful local treatment had lung metastases supports the hypothesis that lung metastases occurred before the cryosurgery. Local recurrences may have also contributed to the extent of the lung metastases.

The extent of lung metastases was significantly lower in the cryosurgical group than the control group, $P = 0.001$. This phenomenon may have resulted from two mechanisms. First, the destruction of the primary tumor may have either decreased or delayed the occurrence of lung metastases. Second, the cryosurgery may have induced an immunological response that decreased the growth of the lung metastases.

Conclusions

The VX2 bladder tumor model reliably produces bladder tumors within 1 week, with metastases occurring by week 2. Local cryosurgery with immediate bladder reconstruction was evaluated in this model. This technique has the potential benefit of bladder preservation through the application of aggressive local therapy while maintaining bladder integrity. However, high rates of local recurrence and lung metastases were noted, which likely reflect the aggressive biological behavior of the VX2 tumor and the technical problems in targeting the tumor. Immediate bladder reconstruction through bladder inversion provides effective protection from bladder disruption in the setting of aggressive local therapy. Future experiments will need to address early surgical intervention to avoid distant metastasis or widespread infiltration, a larger treatment area, and modifications of the cryosurgical technique to improve cell killing and provide a better therapeutic outcome.

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