Mouse Models of Subcutaneous Spleen Reservoir for Multiple Portal Venous Injections to Treat Liver Malignancies

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Abstract

Dog and rat animal models have been developed for repeated intravascular administrations to the liver. However, mice have generally been considered too small to use for these models. This study describes the development of mouse models that permit the establishment of liver metastases that can be subsequently treated by repeated injections into the portal venous system. A mini-laparotomy is done to mobilize the spleen and transpose it to a s.c. pocket with its vascular pedicle intact. A suspension of single tumor cells is then inoculated into the portal vein to establish diffuse liver metastases. These tumors may be treated by simple percutaneous injections directly into the s.c. whole spleen reservoir. The ease of injection into the s.c. spleen permits repeated injections into the portal venous system. The usefulness of this model was shown in experiments revealing that multiple portal venous administrations of a replication-conditional, oncolytic herpes simplex virus mutant are more effective than a single portal venous administration. In a modification of this model, the spleen is first split into two, leaving intact the vascular pedicle for each half of the spleen. Tumor cells are inoculated into one hemi-spleen, which is then resected 10 minutes later. The other hemi-spleen is transposed to the s.c. position, thereby permitting subsequent repetitive portal venous injections via percutaneous injections into the s.c. hemi-spleen. These mouse models are useful for a wide range of studies. (Cancer Res 2005; 65(9): 3823-7)

Introduction

Intravascular regional therapies to treat patients with hepatic malignancies have been investigated for many decades (1, 2). Portal venous delivery and hepatic arterial delivery of chemotherapy to treat liver tumors have both been studied extensively (3, 4). Preclinical clinical in this field has been limited by the absence of suitable mouse models. Canine models that permit intravascular delivery of agents to the liver have been described; however, the absence of a transplantable canine tumor precludes the use of this model for cancer research (5). A rat model that permits administration of agent into the hepatic artery has been described (6). Although it is technically feasible to cannulate the rat hepatic artery for administration of an agent to treat liver tumors, this model has generally been used to evaluate single dose administrations (7). Long-term cannulation of the rat hepatic artery is technically demanding, and because of the complications associated with this approach, this model has not been widely adopted for multiple dose administrations. Mouse models have several advantages compared with rat models for cancer research; the scope of reagents for mouse studies is much broader than for other species, including tumor cell lines, published gene sequences, antibodies, and genetically defined models of disease.

Like many other investigators, we have used the spleen to access the portal bloodstream in mice (8). This route has been used to administer hepatocytes for transplantation (9, 10), and has been used to establish diffuse liver metastases (11, 12). We have used this model to show that treatment of diffuse liver metastases in mice with a single portal venous injection of an oncolytic Herpes simplex virus-1 (HSV-1) mutant results in a reduction in liver tumor burden, and prolongation of mouse survival (8, 13, 14). We have also shown that regional (portal venous) delivery is more effective than systemic (tail vein) delivery (15). These experiments were conducted by first establishing liver metastases via intra-splenic inoculation of tumor cells at laparotomy, and then subsequently performing a second laparotomy to inject the spleen again with the oncolytic virus. A major drawback to this model is that it permits only a single portal venous treatment, as mice cannot tolerate repeated laparotomies for repetitive splenic injections. In addition, tumor growth in the spleen complicates analyses of the effect of control of liver tumor growth on animal survival. Another drawback specific to oncolytic therapy is the possibility that tumor cells infected with HSV-1 in the spleen are a source for continued showering of the liver with infectious HSV-1 released from the spleen. Finally, in these models, it is difficult to determine if observed reductions in liver tumor burden result from destruction of liver metastases, or from destruction of tumor cells in the spleen, which reduces shedding of viable tumor cells from the spleen to the liver.

It is not technically feasible to insert an indwelling cannula into the mouse hepatic artery or portal vein and maintain patency for repeated injections over days or weeks. We therefore developed an animal model in which mice bearing diffuse liver metastases can undergo repeated administrations into the portal venous system.

Materials and Methods

Cells and viruses. Vero African Green Monkey kidney cells were obtained from American Type Culture Collection (Manassas, VA). MC26 mouse colon carcinoma cells were obtained from the National Cancer Institute Tumor Repository (Frederick, Maryland). All cell lines were maintained in DMEM, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. rRp450 was kindly provided by E. Antonio Chiocca (Ohio State University, Columbus, OH), and is defective in expression of ICP6 (16). KOS strain was kindly provided by David Knipe (Harvard Medical School, Boston, MA). Viruses were propagated and titered on Vero cells, and heat-inactivation of virus was done as described (17).
Subcutaneous whole spleen reservoir model. Animal studies were done in accordance with the policies of the Massachusetts General Hospital Subcommittee on Research Animal Care. The operations to create whole spleen reservoirs were conducted as follows: 8- to 10-week-old BALB/c mice (Charles River Laboratories, Wilmington, MA) are anesthetized by i.m. injection of 100 μL of 2% ketamine (100 mg/mL) and 0.1% xylazine (100 mg/mL) in PBS, given at a dose of 0.1 mL per 10 g body weight. After removing the hair on the mid-left side of the abdomen and steriley prepping the area, a superficial 1.0 cm skin incision is made in the middle left abdomen to create a small pocket between the s.c. fat and muscle. The abdomen is entered through the same incision by creation of a small opening in the muscle and peritoneum. The spleen is gently mobilized from peritoneal attachments, carefully preserving its dual vascular pedicle while transposing it to the s.c. pocket. The defect in the abdominal wall is reaproximated using 6-0 Prolene (Ethicon, Somerville, NJ) to hold the spleen in position. The skin is closed with 6-0 Prolene and a second laparotomy for tumor injection done is 2 weeks later. Using the same general anesthetic conditions, hair on the upper, mid-abdomen is removed and the area is steriley prepped. Through a small incision, the portal vein is identified into which 5 × 10⁷ MC26 cells are slowly injected via a 28-gauge needle. Pressure is applied to the portal vein using a cotton-tipped applicator for 10 minutes after withdrawal of the needle. Warm saline is dripped onto the intestines during this time to avoid desiccation. The incision is closed, and saline is injected s.c. on the flank to help maintain perioperative intravascular circulating volume following the laparotomy.

Treatments with an oncolytic Herpes simplex virus-1. Mice were separated to three groups of three mice each and randomized to different treatments involving injections into the s.c. splenic reservoir. The first group received 1 × 10⁷ plaque-forming units of rRp450 every 3rd day for a total of four injections. The second group received a single injection of 1 × 10⁷ pfu of rRp450, followed by three subsequent injections with HBSS. The third group received four injections of HBSS. The mice were sacrificed 15 days after tumor implantation.

Subcutaneous split-spleen reservoir model. The operations to establish split-spleen reservoirs were conducted as follows: 8- to 10-week-old BALB/c mice are anesthetized, the hair on the left side of the abdomen is removed, and the area is steriley prepped. A superficial 1.0 cm skin incision is made in the mid-abdomen on the left side to create a small pocket between the s.c. fat and muscle. The abdomen is entered through a small opening in the muscle and peritoneum. The spleen is gently mobilized from peritoneal attachments. The mid-body of the spleen is divided between two 6-0 Prolene sutures that are placed around the spleen and tied down. A superficial 1.0 cm skin incision is made in the middle left abdomen to create a small pocket between the s.c. fat and muscle. The abdomen is entered through the same incision by creation of a small opening in the muscle and peritoneum. The spleen is gently mobilized from peritoneal attachments. The mid-body of the spleen is divided between two 6-0 Prolene sutures that are placed around the spleen and tied down. These sutures are placed between splenic vascular pedicles, such that each hemi-spleen has its own vascular pedicle. The spleen is divided between these ties (and between the two vascular pedicles, such that each hemi-spleen has its own vascular pedicle (Fig. 2F). These results showed the viability of the spleen as well as patency of the splenic vein. In addition, the injected substance is rapidly and uniformly distributed throughout the liver following inoculation into the spleen.

Results

Establishing subcutaneous whole spleen reservoirs in mice. The whole spleen was transposed to a s.c. position using the technique described above (Fig. 1A). Moderate s.c. swelling around the spleen was observed in the first few days after surgery, which precluded accurate palpation of the spleen for percutaneous injection. However, by the 7th postoperative day, the swelling around the spleen had resolved, allowing localization of the s.c. splenic reservoir by palpation (Fig. 1B). The mice suffered no ill effects from this operation as assessed by oral intake, sleep patterns, activity, or grooming behavior. On the 10th postoperative day, methylene blue (50 μL) was injected into the s.c. spleen reservoir during laparotomy. Diffuse blue spots were observed on the liver surface within 10 seconds, and the entire liver was stained blue within 1 minute (Fig. 1C). These results show the viability of the spleen as well as patency of the splenic vein. In addition, the injected substance is rapidly and uniformly distributed throughout the liver following inoculation into the spleen.

Treatment of diffuse liver metastases by multiple injections of an oncolytic virus. We have shown that a single inoculation of a replication-conditional HSV1 mutant into the portal venous system via the spleen to treat diffuse liver metastases leads to a reduction in liver tumor burden and prolongation of animal survival (8). We used the s.c. whole spleen reservoir model to assess the effect of a single treatment compared with four treatments with the oncolytic virus rRp450. Mice bearing diffuse liver metastases and a s.c. splenic reservoir were randomized to treatment with four injections done once every 3 days: (a) four injections of HBSS, (b) one injection of rRp450 followed by three injections of HBSS, and (c) four injections of rRp450. The difference in tumor burden was striking when the mice were sacrificed 15 days after tumor implantation (Fig. 1D). Although 20 days had passed from the initial operation to translocate the spleen, the whole spleen reservoir functioned well. In a repeated experiment with more mice, the liver weights of five mice receiving only a single injection of rRp450 were significantly greater than that of five mice receiving four rRp450 injections (5.20 ± 1.69 versus 2.97 ± 0.36 grams; P < 0.043; t test). Use of this model permitted demonstration that repeated dosing of rRp450 is more effective than a single dose.

None of the mice died following the initial operation involving translocation of the spleen. However, the operative mortality associated with the second operation was relatively high, with 20 mice out of 34 dying within 48 hours during the initial experiment. The most common finding at mouse necropsy following early postoperative death was necrotic bowel, presumably from portal vein thrombosis. Whereas inoculation of tumor cells into the portal vein thrombosis. Whereas inoculation of tumor cells into the portal vein eliminated the problem of subsequent development of a splenic tumor, the needle trauma to the delicate murine portal vein apparently led to frequent thrombosis. We tried using a smaller gauge needle, but observed no improvement in outcome. We therefore modified the model to split the spleen, and use half of the spleen to establish diffuse liver metastases, and the other half of the spleen for translocation to the s.c. position to create a split-spleen reservoir.

Establishing subcutaneous split-spleen reservoirs and diffuse liver metastases in mice. We modified our original model by splitting the spleen into two relatively equal parts, each with its own vascular pedicle (Fig. 2A). Tumor cells were inoculated into one hemi-spleen to establish diffuse liver metastases. This...
hemi-spleen was then resected 10 minutes later to prevent subsequent tumor growth in the spleen. The other hemi-spleen was transposed to a s.c. pocket. We were initially concerned that immediate removal of the hemi-spleen into which tumor was injected would lead to either a small number of liver metastases, or a high SD of liver tumor burden. However, neither of these was observed when mice were sacrificed 11 days following tumor implantation (Fig. 2B). Dozens of tumor nodules were reliably established throughout the liver, and the SD of liver weights was low. The mean tumor weight of the five livers shown was 5.0 grams, with a SD of 0.9 grams. Although only half of the spleen is transposed to the s.c. position, we had no difficulty palpating it for percutaneous injections. We injected methylene blue into the s.c. hemi-spleen, and again observed blue staining of the entire liver within 1 minute. The operative mortality of this procedure was substantially lower than that observed following tumor cell injection into the portal vein, with only one mouse death out of our first 21 split-spleen reservoir operations. These results show the feasibility of using just one-half of the spleen to establish diffuse liver metastases of consistent nature. These results also show the viability of the s.c. hemi-spleen as well as patency of the splenic vein.

We used this split-spleen model to examine the therapeutic efficacy of HSV-1 oncolysis. Mice received inoculations of MC26 cells into one hemi-spleen, which was removed 10 minutes later. The other hemi-spleen was transposed to the s.c. position. Mice then received one injection every 3 days for a total of four injections, but were randomized into three treatment groups: (a) four injections of saline, (b) one injection of KOS strain of HSV-1 followed by three injections of saline, (c) four injections of KOS. Just prior to sacrifice, methylene blue was injected into the s.c. spleen of mice that were treated with HSV-1 to observe whether it was still effectively delivered to the liver. We observed that four doses of KOS were more effective than a single dose (Fig. 2C). We also observed that the methylene blue was still effectively delivered to the entire liver, even after four doses of KOS had been injected into the s.c. spleen.

Discussion

We developed experimental mouse models of s.c. spleen reservoirs for repeated portal venous administrations. These models were developed out of necessity to compare the efficacy of multiple portal venous administrations of an oncolytic virus
relative to a single dose for treatment of diffuse liver metastases. Both of the models were useful because they allowed us to compare multiple doses of HSV-1 to a single dose. Importantly, these models also enabled us to show that the efficacy of HSV-1 against liver metastases results directly from a reduction in established liver tumor burden, rather than reduction in continued shedding of tumor cells from the spleen to the liver. Although we developed this model specifically for testing efficacy of HSV-1 oncolytic mutants against established liver metastases, it is useful for any experimental agent that requires repeated administrations into the portal vein (e.g., portal venous administration of fluorouridine for treatment of diffuse colon carcinoma liver metastases).

For investigators seeking to deliver agents repeatedly to the portal vein without first establishing liver tumors, the s.c. whole spleen reservoir model is an excellent model. Transposition of the entire spleen to the s.c. position is an easier operation to perform compared with division of the spleen and transposition of a hemi-spleen. The method we describe is quite simple and can be done quickly through a single incision. This seems to be easier than another model described in rats involving two different incisions (6).

However, if an experimental animal model with liver tumors is required, the s.c. whole spleen model is not ideal. We experienced unacceptably high operative mortality with our original model of a s.c. whole spleen and inoculation of tumor cells directly into the portal vein, with the mortality apparently related to mesenteric venous thrombosis and bowel necrosis. It is feasible to establish single liver metastases by direct inoculation of tumor cells under the liver capsule rather than by portal venous inoculation (8). Unfortunately, despite keeping pressure on the liver capsule injection site for an extended time, we observed that carcinomatosis occurs quite frequently with this technique.

The split-spleen approach solves several problems. Half of the spleen can be used to reliably establish uniform, diffuse liver metastases without risking carcinomatosis or portal vein thrombosis. Resection of this half of the spleen during the same laparotomy avoids the consequences of splenic tumor growth. The other half of the spleen can be transposed to a s.c. pocket to permit repeated portal venous delivery with the ease of a percutaneous splenic injection.

Other animal models in which the spleen is used for repeated portal vein injection have been described, including models in rats, and one in dogs (5, 6). We found no descriptions of mouse models for repeated portal venous delivery to treat liver tumors. Because mice are smaller and less tolerant of complex surgical procedures, the procedure we developed is purposefully simple. A single laparotomy is required to establish liver metastases, resect the hemi-spleen, and implant the other hemi-spleen in a s.c. pocket. The need for a single laparotomy (rather than two) also reduces pain and suffering of the experimental mice. Whereas some familiarity with small animal surgery is clearly a prerequisite, little practice is required to comfortably master the split-spleen model. The operative mortality for this procedure is very low. The s.c. spleen reservoir seems relatively durable in the mouse model. Inflammation and scarring around the spleen and multiple injections into the spleen did not compromise the integrity of the vascular pedicle. Patency of a s.c.
spleen reservoir has been shown 177 days following its creation in a dog model (5).

We developed these mouse models to compare single versus multiple dose administrations of an oncolytic herpes virus. However, these models are useful for a wide range of studies, including cancer therapy, cancer imaging, toxicology, and hepatocellular transplantation.

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