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Isolated Limb Perfusion in the Sarcoma-bearing Rat: A Novel Preclinical Gene Delivery System

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Abstract

Reliable site-specific delivery of genetic constructs remains a challenging component of gene-based therapy of solid tumors. Isolated limb perfusion (ILP) continues to be evaluated for treatment of locally advanced soft tissue sarcomas because this approach uniquely directs therapeutic agents into the tumor-bearing extremity without significant systemic leak. In light of these considerations, we tested the hypothesis that ILP could be used to deliver genes carried in viral vectors to the sarcoma-bearing rat extremity, resulting in demonstrable gene transfer into the tumor. ILP was performed in rats by cannulating the femoral artery and vein, isolating the hind limb from systemic circulation by tourniquet, and cycling perfusate for 15 min at a rate of 2.4 ml/min. Leakage into the systemic circulation was 7.5% of the total perfusate concentrated in the isolated limb, as determined by perfusion with technetium 99m-tagged RBCs. We used the ILP technique to perfuse rat hind limbs bearing syngeneic fibrosarcoma tumor nodules with the replication-defective adenovirus Ad5LacZ, which expresses the bacterial β-galactosidase. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside staining of the perfused limb tissues confirmed gene transfer to the tumor and peritumoral tissue, demonstrating that the tumor was part of the perfusion circuit and that gene therapy delivered via this method was feasible. These results suggest that adaptation of this preclinical gene delivery model to administer genetic constructs aimed at controlling tumor growth may prove beneficial to patients with extremity sarcomas.

Introduction

Soft-tissue sarcomas are a group of histologically diverse malignancies characterized by aggressive local growth and hematogenous metastases, most often to the lungs. Two-thirds of these tumors arise in the extremities, where local therapy usually consists of surgical resection with or without radiation therapy. Hyperthermic isolated limb perfusion has also been used to treat advanced extremity soft-tissue sarcoma, with intent to achieve limb salvage for patients who would otherwise require amputation (1).

Although small animal models of ILP have investigated the therapeutic effectiveness of various chemotherapy regimens (2-7), the technique of ILP raises intriguing possibilities for the emerging field of genetically based approaches to cancer treatment. The defining feature of ILP is the ability to deliver a therapeutic agent in high concentrations to a specific region of the body, in this case an extremity, while avoiding systemic leakage and toxicity. In contrast, a continuing limitation of gene therapy strategies is achieving efficient and specific expression of therapeutic gene constructs in their intended target tissue (8). This is a function of a number of factors, including the type of vector used to package and deliver genetic constructs, the route of delivery, the induction of immunogenic response to the gene therapy, and variability among tissues for efficient gene transduction (8). In this study, we considered whether ILP, investigated in a rat model, could be usefully combined with gene therapy by taking advantage of a focused route of delivery for a therapeutic agent.

Several factors make the rat limb an ideal model of ILP and a practical system for the study of gene therapy. Anatomically, the rat hind limb derives most of its blood supply from a single femoral artery and vein that are easily accessible and facilitate maximal vascular occlusion for selective delivery of therapeutic agents, like viral vectors, to the limb. By virtue of their size and availability, rats can conveniently be studied in larger numbers that may be required for appropriate statistical analysis. Physiologically, rat and human tumors are transplantable into appropriately selected rat strains, thereby allowing in vivo investigation of tumors that have already been well characterized. Pharmacologically, higher doses of gene therapy constructs than would otherwise be delivered systemically or by injection can be administered via ILP because of the exclusion from systemic circulation built into this model.

Using the approach of ILP, we demonstrate the feasibility of delivering viral vectors selectively into the rat sarcoma-bearing extremity, with confirmed gene transfer to the tumor and peritumoral tissue. This observation has promising impli-
cations for the adaptability of gene therapy methods in the treatment of extremity sarcomas. The presence of mutations in the p53 tumor suppressor gene within a tumor can affect tumor response to standard treatments (9-11), and restoration of normal p53 gene function augments chemosensitivity and radiosensitivity in some tumor systems (12-13), including sarcomas. In the future, it may be possible to selectively deliver genetic agents via ILP, which in combination with standard treatments may result in augmented therapeutic advantage to the patient burdened by soft-tissue sarcoma.

Materials and Methods

**Animals.** Female Fisher 344 and female nude (strain nu/nu) rats weighing from 150 to 250 g were obtained from Harlan Sprague Dawley (Indianapolis, IN). Animals were maintained on a standard laboratory diet ad libitum in diurnal lighting conditions. Both rat strains were used in investigations of ILP technique; however, because of cost considerations, tumor was implanted in Fisher 344 rats for experimental studies. Animals received humane care in accordance with the Animal Welfare Act and the NIH “Guide for the Care and Use of Laboratory Animals.” The experiments were approved by the Institutional Animal Care and Use Committee at M. D. Anderson Cancer Center.

**Tumor.** A rapidly growing RFS originally induced by methylcholanthrene (14) was kindly provided by Dr. Joan Bull (The University of Texas Medical School, Houston, TX). The tumor was maintained in Fisher 344 rats by biweekly s.c. transplantation on the flank. Small tumor slivers (1 × 1 mm) from donor rats were implanted s.c. into the hind legs of experimental rats. The tumor was inserted by needle or small skin incision at the tarsal joint and deposited midway between the knee and tarsal joints. A tumor nodule approximately 7-10 mm in diameter developed within 7-10 days.

**Operative Technique: Isolated Limb Perfusion.** The operative procedure was performed under sterile conditions using sterile instruments and solutions and an operative microscope (×10; Zeiss Super-Lux 40, Andover, MA). The overall scheme of ILP is shown in Fig. 1A. Anesthesia was administered by i.m. injections of a ketamine (63 mg/ml), xylazine (3.6 mg/ml), and atropine (0.07 mg/ml) mixture based on rat weight (0.9 ml/kg), and buprenex (0.1 mg/kg). The rat was shaved circumferentially from mid-abdomen down, prepped with povidone-iodine, and secured to a heating pad. A tourniquet consisting of a 12-cm length of 0-nylon ligature was passed under the right leg at the hip joint, the two ligature ends were brought together above the inguinal region and secured temporarily by hemostat. A 1-cm horizontal incision was placed over the femoral triangle at a level just below the second nipple. An opening was created through the underlying fatty tissue to visualize the femoral vessels. A small retractor was introduced into the incision; retraction on the cephalad side exposed the inguinal ligament. One free end of the tourniquet ligature was passed underneath the inguinal ligament and then both ends of the tourniquet were again secured loosely by hemostat.

The femoral artery and vein were next dissected from the inguinal ligament to the branch point of the epigastric vessels, taking care to avoid the femoral nerve (Fig. 2). A 6-0 silk suture was passed around four points: proximally and distally under the femoral artery, and proximally and distally under the femoral vein. The proximal suture on the femoral artery was then permanently tied. Gently retracting only the proximal suture cephalad allowed accumulation of blood distally and improved ease of cannulation. A 24-gauge i.v. catheter (Becton Dickinson Vascular Access, Sandy, UT) was inserted, and the distal silk suture was tightened around the catheter. A 1-ml syringe attached to extension tubing (MEDEX, Inc., Hilliard, OH) containing heparinized saline (5 IU/ml) was connected to the cath-

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*Unpublished observations, laboratory of D. Yu and R. Pollock.*
eter hub, and 0.1 ml of this solution was flushed through the catheter. The procedure was repeated for the femoral vein.

The perfusion pump was positioned below the plane of the animal to allow venous outflow by gravity. The perfusion pump was a roller pump with flow rates adjustable from 0.08 to 3.0 ml/min (Instech, Plymouth Meeting, PA). Pump tubing was connected to vascular catheters, the tourniquet was tightened, and perfusion was started at 2.4 ml/min. Perfusate entered via the femoral artery and returned via the femoral vein. Perfusion with heparinized normal saline for 15 min was used in animals receiving no therapeutic agent. The perfusate and returned blood were oxygenated through a port in the reservoir using a 1 L/min oxygen stream. For animals receiving a therapeutic agent, a 1-min perfusion with normal saline was done, after which the viral vector was added directly to the reservoir for perfusion lasting 13 min. A final 1-min perfusion with heparinized saline was done to wash out residual drug, giving 15 min of total perfusion time.

At the end of the perfusion period, the vascular catheters were disconnected from pump attachments, the tourniquet was released, and a 6-0 silk suture was used to ligate the vessels just distal to their arteriotomy and venotomy sites. Although the femoral artery was ligated proximally, there was sufficient collateral circulation to preserve limb viability. The incision was closed with a single deep suture that reapproximated the fatty tissue over the femoral vessels, and a running 5-0 nylon suture was used for skin closure. The rat was allowed to recover from anesthesia in a warm environment and then returned to its cage. The animals were examined daily for wound status and leg function. The ILP procedure is depicted in Fig. 1B.

**Perfusion with Tc99m-labeled RBCs.** Two rats underwent isolated limb perfusion as described above. Donor rat blood was labeled with Tc99m using the UltraTag RBC preparation kit (Mallinckrodt, Chesterfield, MO). Tc99m-RBC mixture (0.3 mCi in 2.5 ml blood) was added to the reservoir containing heparinized saline. During perfusion, serial images were obtained by a gamma camera (General Electric Medical Systems, Houston, TX) at 5-s intervals for 2 min, followed by 1-min intervals for 28 min. The perfusion pump was then stopped, and the tourniquet was released. Images were obtained at 5-s intervals for the first 2 min after tourniquet release and then at 1-min intervals for an additional 28 min. Radioactivity in the isolated leg and the heart, liver, and total body area outside the perfused leg was determined from the gamma images. The percentage of leak was calculated from radioactive signals in the region of interest relative to radioactive signal retained in the perfused limb.

**Adenovirus Preparation.** The construction and properties of Ad5LacZ have been reported elsewhere (15, 16). The Ad5LacZ virus was kindly provided by Dr. Mien-Chie Hung (M. D. Anderson Cancer Center). Adenovirus was amplified in 293 cells, and aliquots of supernatant were stored as viral stock in PBS with 10% glycerol after three freeze-thaw cycles to lyse the cells. Viral titer was determined by plaque assay. The ability of Ad5LacZ to transduce RFS cells in vitro was determined by infecting monolayer cultures of RFS at increasing levels (10, 50, 100, 500, and 1000) of viral-to-cell MOI for 30 min, with brief agitation every 5 min. This was followed by the addition of culture medium and reincubation at 37°C. After 48 h, the monolayer of cells was fixed and stained with X-gal solution as described in the “Histology” section below. The percentage of cells expressing β-gal, and hence staining blue, was scored at ×20.

**Fig. 2** Anatomy of the femoral vessels from the inguinal ligament to the branch point of the epigastric vessels in the right leg of a rat. The loops indicate positioning of silk ligatures.
Gene Delivery with Isolated Limb Perfusion. Twelve rats bearing 1-cm RFS tumor nodule on the right leg underwent isolated right limb perfusion with adenovirus Ad5LacZ or saline. The rats were killed at designated time intervals after perfusion: 24 h (n = 1 saline, n = 2 virus); 48 h (n = 1 saline, n = 4 virus); 72 h (n = 2 virus); and 7 days (n = 2 virus). The total dose of Ad5LacZ delivered ranged from 1 × 10^9 to 5 × 10^10 plaque-forming units. An RFS tumor nodule was implanted in the opposite (left) extremity of each rat and was not perfused to provide information about the RFS tumor when grown in an undisturbed tissue environment.

Histology. Two sections each of perfused tumor, perfused leg muscle, nonperfused tumor, nonperfused leg muscle, liver, and lung were obtained at the specified time intervals following perfusion with Ad5LacZ. One sample was preserved in formalin, embedded in paraffin, and stained with H&E. The other sample was frozen in liquid nitrogen, and 8-μm cryostat sections were placed on glass slides. Freshly mounted slides were rinsed in PBS, immersed in 2% formaldehyde/0.2% glutaraldehyde for 5 min at 4°C, and then incubated for 4 h at 37°C in X-gal staining solution: 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 2 mM MgCl₂, and 1 mg/ml X-gal (Life Technologies, Inc., Gaithersburg, MD). Cells expressing β-gal cleave X-gal to yield a blue chromophore in the cytoplasm. Blue-staining areas in tissues were detected at ×10 and ×40 after slides were counterstained with H&E. Tissue histology was reviewed by an experienced pathologist.

Results and Discussion

A total of 37 animals underwent isolated limb perfusion. Eighteen rats underwent perfusion for technique design studies,
and 19 rats underwent perfusion as part of survival and limb viability studies. All 19 animals in this latter group survived the surgery. Overall limb viability was preserved in 19 of 19 rats in the survival group; however, two rats developed black toe tips or footpad discoloration in the perfused extremity, which demarcated and then healed without consequence. All rats developed mild postoperative swelling of the perfused extremity, which subsided within 48 h. After recovery from anesthesia, rats were mobile but limped on the perfused extremity or held it in a retracted position against the body. This resolved within 48–72 h with return of full leg function. There was minimal to no intraoperative bleeding. Blood loss was limited to washout of the limb intravascular volume, estimated to be less than 0.3 ml. There were no postoperative infectious complications.

Having defined the technical aspects of the perfusion, it was pertinent to determine the effectiveness of limb isolation from the systemic circulation. Perfusion with Tc99m-labeled RBCs demonstrated a uniform and concentrated uptake throughout the perfused limb, including the tumor. Four time-lapse images during the first 30 min of perfusion are depicted in Fig. 3. Tc99m-labeled RBC leakage to the systemic circulation at 15 min was 7.5% when radioactive signal from the entire body area outside the perfused limb was included. The predominant concentration of the leaked perfusate was in the liver and portal system. This implies that systemic toxicity of a therapeutic agent delivered via ILP may ultimately depend on the hepatotoxicity of that agent. This leakage pattern was not appreciated in studies reported previously (2–3), which relied on cardiac puncture to sample leakage of radioactive serum albumin administered to the isolated rat limb. By the cardiac puncture method, the radioactive leak rate in these earlier reports was less than 2% (3). When the Tc99m leak rate was calculated as a percentage of radioactive signal only from the heart and its blood volume, a comparable value of 1.4% was determined for the experiments reported here.

Next, it was important to determine if the ILP circuit delivered perfusate into the tumor growing in the rat extremity, and whether viral vectors carried in this perfusate could be incorporated into these tumors. The replication-deficient adenoviral vector Ad5LacZ was chosen for this purpose because it can easily be detected in histological preparations. The transducibility of RFS cells was confirmed in vitro with Ad5LacZ; 10% of RFS cells treated in vitro with Ad5LacZ at MOI 100 and 50% of cells at MOI 500 expressed β-gal and stained blue, indicating a relatively low transduction efficiency for this tumor type. In spite of this finding, the RFS tumor was used because it consistently yielded uniform size tumor nodules when implanted in rats, and because qualitative assessment of gene transduction would be sufficient to prove the feasibility of the ILP delivery system for future gene therapy purposes.

ILP administration of Ad5LacZ to rats bearing RFS tumor in the right hind limb confirmed that the tumor was part of the perfusion circuit. Three of four rats at 48 h, two of two rats at 72 h, and one of two rats at 7 days demonstrated blue staining in the perfused tumor/peritumoral tissue (Fig. 4). Rat necropsy revealed extensive tumor neovascularization originating from branches of the popliteal vessels directly extending from the femoral vessels; this no doubt accounts for the direct access to the tumor via the ILP circuit (Fig. 5). In contrast, no β-gal staining was apparent in the muscular tissues of the perfused limb. Lung tissue and the nonperfused tumor and muscle likewise demonstrated no β-gal staining. Only the liver had detectable expression of β-gal (four of eight rats), which seemed to be confined to Kupffer’s cells. Histologically normal architecture was observed in the liver, lungs, and muscles of both legs (Fig. 6, A–D). The muscle of the perfused leg appeared viable at all four time points, and the only detectable difference compared to nonperfused muscle was the presence of small inflammatory cell infiltrates. Tumors that underwent perfusion tended to have a necrotic center with a peripheral rim of viable cells, whereas nonperfused tumors tended to have uniform viability throughout the tumor nodule (Fig. 6, E–F).

A successful model of ILP will incorporate a reproducible surgical technique that results in uniform perfusion of the limb and survival of the animal with preserved viability and function of the limb. As described in this report, the technique itself can be consistently executed and performed relatively rapidly; total operating time lasted less than 40 min for a typical 15 min perfusion, with a 2-min ischemic period from the point of ligation of vessels to start of perfusion. The perfusate remained highly concentrated in the isolated limb with minimal systemic leakage rate. The longest surviving rat is currently 5 months after perfusion and has normal leg appearance and function.

Four important observations are noted regarding the technical aspects of this model: (a) it is feasible to cannulate femoral vessels in rats as small as 150 g, and vessel size did not change...
Fig. 6  Histological sections of tissues stained with H&E from rats killed 7 days after isolated limb perfusion: liver (A), lung (B), nonperfused muscle (C), perfused muscle (D), nonperfused tumor (E), and perfused tumor (F).

in rats older than 7 weeks or in different strains (Fisher versus nude rats); (b) the use of regular i.v. catheters allows for significantly faster cannulation compared to reported methods (2, 3, 6, 7), requiring insertion of relatively inflexible polystyrene tubing into arteriotomy and venotomy sites; (c) effective cannulation of the femoral vein with resultant brisk outflow is critical for success of perfusion and the reduction of postoperative extremity swelling; and (d) the ability to perform this technique in nude rats will facilitate investigation of human tumor xenografts in a host environment that more closely approximates the milieu of the original tumor. The model described in this report is practical and can readily be applied in the laboratory setting by nonsurgical trained researchers.

This study demonstrates that a gene construct delivered via ILP can result in transduction of cells in the perfused extremity and to the best of our knowledge is the first such report dem-
onstrating this novel gene delivery approach. These results suggest that ILP might be useful for delivery of therapeutic genetic constructs to extremity tumors, thereby enhancing tumor control. Perfusion with angiogenesis inhibitors, tumor suppressor gene constructs, blocking peptides, or other modifiers of the malignant phenotype may enhance response to traditional therapies, if not lead to direct tumor cytoreduction. Preclinical studies are presently being conducted to explore these possibilities and may ultimately prove beneficial to patients burdened by soft-tissue sarcoma of the extremities.

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