Published in final edited form as:

J Surg Res. 2013 December ; 185(2): . doi:10.1016/j.jss.2013.06.051.

Murine Portal Vein Catheterization to Analyze Liver Directed Therapies

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Abstract

Background—Small interfering RNA (siRNA) provides a highly selective method to target mutated pathways; however, its use is complicated by specific delivery to tumor cells. The aim of this study were to: i) develop a novel murine model of portal vein catheterization (PVC) for the chronic delivery of therapeutic agents to liver metastases, ii) determine the benefits of local delivery of siRNA to liver metastases, and iii) determine the utility of epithelial cell adhesion molecule (EpCAM) as a selective target for siRNA delivery to colorectal cancer (CRC) metastases.

Materials and Methods—i) PVC was performed through midline laparotomy in 2 mo-old Balb/C mice. ii) Portal venous flow distribution and catheter patency were evaluated using fluorescently-labeled microspheres. Metastatic studies were performed by splenic injection of CT26 murine colon cancer cells; uptake of DY-547-labeled siRNA was assessed by IVIS imaging and delivery to metastases confirmed using fluorescent microscopy. iii) EpCAM expression was evaluated using IHC staining of human tissue microarrays.

Results—i) Successful PVC was confirmed by saline injection and ultrasound. ii) Fluorescent imaging of microspheres confirmed excellent distribution and catheter patency. Portal venous injection of DY547-labeled siRNA demonstrated a high level of fluorescence throughout the liver with siRNA also identified within the liver metastases. iii) All primary CRCs and liver metastases stained strongly for EpCAM with no expression in normal hepatocytes.

Conclusions—Liver-directed therapy provides selective delivery of siRNA to CRC metastases. EpCAM expression in CRC, but not normal liver, may further selectively target hepatic metastases of epithelial origin.

Keywords

Portal vein; siRNA; colorectal cancer; liver metastasis; catheterization; animal model

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Disclosures: The authors have no conflicts of interest regarding publication of this manuscript.

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Introduction

Molecular targeted therapies have the ability to more selectively affect cancer cells compared to their normal counterparts and may offer a benefit over traditional chemotherapy. This is often accomplished through the use of small molecule inhibitors; however, off-target effects still occur and may result in associated toxicity. The use of small interfering RNA (siRNA) offers an alternative to small molecule inhibitors. siRNA is a 21-23 nucleotide RNA sequence capable of silencing gene expression by binding to and destroying complementary RNA strands (1). The highly selective and specific nature of siRNA can result in decreased toxicity. Additionally, it is also able to knockdown targets that are currently undruggable, such as the commonly mutated *KRAS*(2). Despite the many benefits of siRNA, difficulties related to *in vivo* delivery and rapid clearance from the circulation have limited its clinical application (1, 3). Increased stability through modifications of the siRNA structure and enhanced delivery secondary to advances in nanotechnology have improved systemic delivery; however, effective *in vivo* treatment remains difficult (1, 4).

Local delivery strategies may provide a method to further improve *in vivo* delivery. Local delivery techniques currently exist in the clinical setting and have the potential to enhance drug administration preferentially to diseased tissue. Transarterial chemoembolization (TACE) is one such example. In this technique, the branch of the hepatic artery that supplies a tumor is catheterized in order to directly infuse cytotoxic chemotherapy to the tumor and to perform particle embolization of the tumor's blood supply. The use of this local delivery technique has been linked with a potential survival advantage in controlled clinical trials (5, 6). Isolated limb perfusion for the treatment of in transit metastatic melanoma is yet another example. This strategy allows for the use of higher concentrations of therapeutic agents while minimizing exposure to the remainder of the body (7). Despite the clinical use of multiple local delivery techniques, a good murine model to study liver directed therapies does not currently exist.

The use of targeting molecules has also been explored as a method to enhance therapeutic efficacy. Mutated cancer cells often abnormally express cell surface receptors (8). For example, the upregulation of folate and transferrin receptors has been previously identified in multiple cancer types (9). Epithelial cell adhesion molecule (EpCAM) has emerged as an attractive target as well, and its over-expression has been identified in colon, stomach, pancreas, and prostate cancers (10, 11). In colorectal cancer (CRC), the second leading cause of cancer-related deaths in the US and a common source of hepatic metastases, EpCAM over-expression has been demonstrated in up to 98% of specimens, with a much more limited expression in normal epithelium (10, 12). Despite the encouraging data for EpCAM, its applicability to CRC hepatic metastases remains to be defined. Therefore the aims of our study were to: i) develop a novel murine model of portal vein catheterization (PVC) for the chronic delivery of therapeutic agents to liver metastases, ii) determine the benefits of local delivery of siRNA to liver metastases, and iii) determine the utility of EpCAM as a selective target for siRNA delivery to CRC metastases.

Materials and Methods

Materials

McCoy's 5A medium and Hank's balanced salt solution (HBS) were obtained from Mediatech (Herndon, VA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). The stereo microscope was obtained from Meiji Techno (Santa Clara, CA). Polyurethane catheters (1.2F) and miniature tubing injection ports were acquired from Instech Laboratories (Plymouth Meeting, PA). Histoacryl blue bioadhesive

glue was purchased from Progressive Medical International (Vista, CA). The Vevo 2100 high-frequency ultrasound (US) system was obtained from Visual Sonics Inc. (Toronto, Canada). Green fluorescently-labeled polystyrene microspheres (10 μ M) were purchased from Life Technologies (Grand Island, NY). DY547-fluorescently labeled siRNA and DOTAP liposomal transfection reagent were acquired from Dharmacon (Lafayette, CO). Tissue microarrays were obtained from Accurate Chemical and Scientific Corp (Westbury, NY). Balb/C mice were acquired from Taconic (Hudson, NY).

PVC, distribution studies, siRNA preparation, and model for liver metastases

All animal studies were performed in accordance with the ethical standards of the division of laboratory animal research at our institution. The procedure was performed using a stereo microscope under 6 $\times$ magnification. Balb/C mice (2-month-old) were anesthetized and a midline laparotomy was performed. A small incision was then made at the base of the neck and a 1.2F polyurethane catheter with a miniature tubing injection port was tunneled in a subcutaneous plane to the abdominal cavity, passing through the abdominal wall approximately 1 cm lateral to the midline incision. The portal vein was subsequently isolated and a 2-0 silk suture placed around the vessel with tension applied for proximal control of blood flow. A venotomy was performed using a 27 gauge needle followed by insertion of the catheter into the portal vein. Histoacryl glue (Progressive Medical International, Vista, CA) was applied to secure the catheter and for hemostasis. The catheter was flushed with injectable saline and blanching of the liver was used to confirm appropriate location and function of the catheter at the time of placement. The abdomen was closed with a 4-0 monocril suture and the skin closed with clips.

For portal venous distribution studies, fluorescently-labeled polystyrene microspheres 10 μ M in 200 μ L) were injected via the portal venous catheter followed by flushing of the catheter with 100 μ L of injectable saline. Imaging was performed 15 min after injection using a green-fluorescent filtered lens. Fluorescent siRNA based studies were performed using DY547-fluorescently labeled siRNA prepared with DOTAP liposomal transfection reagent in HBS incubated for 15 min. siRNA (50 μ g) in 300 μ L of volume was used for injections.

The murine colon cancer cell line, CT26, was used for the metastatic model. Cells were cultured in McCoy's 5A medium supplemented with 10% FBS. Cell cultures were maintained with a mixture of air and 5% CO₂ at 37°C. CT26 cells were trypsinized and re-suspended in phosphate buffered saline (PBS). Two million cells in 100 μ L of volume were injected into the spleen of Balb/C mice and metastases were allowed to establish over a 12d period.

In vivo imaging

Imaging was performed one week following PVC. Mice were anesthetized and monitors placed for heart rate and temperature. Hair removal cream was applied to the abdomen followed by US transmission gel. B-mode imaging was performed using a Vevo 2100 high-frequency US system.

The liver, lung, and kidneys were collected 15 min after injection with fluorescent siRNA. Fluorescent imaging was performed with an excitation filter of 535 nm and an emission filter of 580 nm. Measurements were performed using region of interest (ROI) analysis. Specimens were fixed in formaldehyde and frozen in optimal cutting temperature (OCT) compound. Sections were stained with DAPI for visualization of the nuclei.

Immunohistochemistry (IHC)

Tissue microarrays of normal colon, normal liver, primary CRCs, and liver metastases were blocked in avidin, biotin, and 5% normal goat serum and incubated overnight at 4°C with monoclonal antibody against EpCAM. After 3 washes with TBS-T, the sections were incubated for 30 min with secondary antibody labeled with peroxidase. Specimens were then washed 3 more times followed by addition of DAB substrate for 30 min. All sections were counterstained with hematoxylin and observed by light microscopy.

Results

PVC is technically feasible, provides access to a wide distribution of the liver, and maintains patency at 2 weeks

PVC was performed as described above. Positioning within the portal vein was initially evaluated by direct visualization under 6× magnification at the time of the procedure (Fig. 1A, left). Following placement, 100 µL of saline was used to flush the catheter. Appropriate catheter location and function were confirmed by directly visualizing blanching of the liver. Incorrect placement was most commonly the result of through and through injury of the portal vein, which was usually identified by significant bleeding or filling of the abdominal cavity with saline solution upon flushing. Right upper quadrant US imaging was also performed in a subset of mice (n=2); B-mode imaging confirmed the presence of the catheters within the portal vein at one week (Fig. 1A, right).

We next determined whether the portal vein would provide widespread hepatic delivery following catheter based injection. Portal vein catheters were again placed as previously described (n=2 mice). Shortly after placement, 200 µL of fluorescently-labeled polystyrene microspheres (10 µM) were injected via the catheter followed by 100 µL of injectable saline flush. Imaging under a green-fluorescent filtered lens showed widespread distribution throughout the majority of the liver (Fig. 1B, left). We next determined if catheter patency and portal vein distribution were maintained long-term. Two weeks after catheterization, fluorescent microspheres were injected as detailed above (n=2 mice). Imaging using a green-fluorescent filtered lens again showed widespread distribution within the liver, confirming both continued catheter and portal venous system patency (Fig. 1B, right).

Portal vein injection enhances delivery of siRNA to the liver and reduces renal clearance as compared to systemic delivery

We next determined if portal vein injection improved delivery of siRNA relative to systemic administration. Mice (n=1 mouse per group) were injected with a total volume of 300 µL containing either vehicle control (DOTAP and HBS delivered via tail vein injection), 50 µg of DY547-labeled siRNA in DOTAP/HBS via tail vein injection, or 50 µg of DY547-labeled siRNA in DOTAP/HBS via portal vein injection. The liver, lung, and kidney were excised 15 min after injection and imaged using IVIS (Fig. 2A, B). Quantification of fluorescent signal using ROI was used to compare the concentration of siRNA within the organs. There was an approximately 60% increase in signal intensity within the liver following portal vein injection as compared to tail vein injection. Furthermore, signal intensity in the kidney was approximately four times greater with tail vein injection compared with injection into the portal vein. Together, these data indicate increased uptake of siRNA within the liver and decreased renal clearance following portal vein delivery compared to systemically administered siRNA.

Local delivery of siRNA via portal vein injection results in delivery to metastatic disease

After demonstrating improved delivery with portal vein injection relative to systemic administration, we next determined if portal vein injection results in siRNA delivery to

metastatic colon cancer. CT26 cells (2×10^6) suspended in 100 μ L of PBS were injected into the spleen of 2 month-old Balb/C mice. Liver metastases were allowed to establish for 12 d. Mice (n=2) were then injected via the portal vein with 300 μ L of either vehicle control or 50 μ g of DY547-labeled siRNA in DOTAP/HBS. The liver, lungs, and kidneys were excised and imaged using IVIS (Fig. 3A). A strong uptake of fluorescently-labeled siRNA was noted throughout the liver and a low level of expression identified within the kidney, consistent with the findings in the previous uptake model shown in Fig. 2. Moreover, this fluorescent signal was noted not just within normal tissue, but also within areas of metastases. Liver sections of the tissue were also analyzed using fluorescent microscopy (Fig. 3B). Fluorescent signal was identified within metastatic deposits, indicating delivery of siRNA to metastases.

EpCAM is highly expressed in colon cancer but not in normal hepatocytes

After confirming the benefit of local delivery techniques for administration of siRNA, we next determined if EpCAM would provide a selective target to further enhance specificity. Tissue microarrays of 89 normal colon samples, 129 primary CRCs, 4 liver metastases, and a normal liver specimen were analyzed using IHC (Fig. 4). One hundred percent of primary CRCs and liver metastases stained strongly for EpCAM. In contrast, staining was absent in normal hepatocytes, while only minimal staining of biliary radicals was identified.

Discussion

In this study, we evaluated targeting strategies to optimize siRNA delivery to metastatic CRC. First, we showed the technical feasibility of a novel murine PVC technique for the study of liver-directed therapies. Second, we demonstrate that PVC provides a method for repeated delivery of therapeutic agents and has the potential to provide an advantage in siRNA delivery over systemic administration. Third, we demonstrate the ability to deliver siRNA via the portal vein to hepatic CRC metastases. Finally, we demonstrate the potential of EpCAM as a targeting molecule due to its overwhelming presence in primary and metastatic colon cancer with a general absence in normal hepatocytes.

CRC remains the second leading cause of cancer-related deaths in the United States and most commonly metastasizes to the liver (13, 14). Despite efforts to improve outcomes, survival in advanced disease remains poor (14). Local, adjunctive techniques, such as TACE, have demonstrated some benefit; however, no good animal model exists to study therapies that may further derive advantage from directed delivery techniques. Mice provide an ideal animal model due to the rapid breeding and relatively low cost compared to other organisms (15). Although there are many benefits to a murine model, the diminutive anatomy of mice makes catheterization of the hepatic artery virtually unachievable. Conversely, the portal vein offers a relevant alternative to the hepatic artery. The portal vein is responsible for 75% of blood flow in humans (16). In our study, we use fluorescent microspheres to demonstrate that mice also have a similarly widespread distribution of portal venous circulation, indicating that therapies administered via the portal vein are able to be delivered to hepatic metastases. Furthermore, we show that despite the small size of the portal vein, successful placement of portal venous catheters is achievable with the use of an operating microscope and can be confirmed with the use of small animal ultrasound. Injection of microspheres 2 weeks post operatively confirms the continued patency of the catheter, indicating the ability to use this technique for the chronic delivery of therapeutic agents.

In addition to improvements in delivery techniques, the development of molecular targeted therapies has allowed for specific targeting of abnormal pathways in cancer and has shown a great deal of promise in cancer treatment. The use of siRNA to target these mutated

pathways is even more encouraging due to its high specificity and efficacy, even compared to small molecule inhibitors (1). The barriers to its use in the clinical setting are largely related to problems with delivery. In order to have an effect, siRNA must be delivered to the organ of interest and enter the desired cell while evading degradation by endogenous nucleases and clearance by the kidney (3). In this study, we show that hepatic delivery via PVC has the potential to increase the concentration of siRNA delivered to the liver and may diminish renal clearance compared to systemically delivered siRNA via tail vein injection. This finding may be due to the high concentration of siRNA directly administered to the liver prior to any systemic clearance or degradation. Diminishing the total amount of siRNA that arrives in the systemic circulation would potentially be associated with decreased adverse effects. Furthermore, the proximity of siRNA to cancer cells, as is shown by the presence of siRNA not only in normal tissue but also within metastatic disease, supports the therapeutic benefit of siRNA administered using hepatic catheterization.

The ability to specifically deliver a therapy to a cancer cell while sparing normal surrounding tissue would also be of great clinical benefit. This is particularly true in the case of siRNA, where intracellular incorporation of siRNA is necessary in order to have an effect. One method to accomplish this task is through binding of cell surface molecules. Cancer cells demonstrate a modified expression of cell surface molecules (8). In order to be effective as a targeting molecule for siRNA delivery, the cell surface marker needs to be expressed in the tumor cells with minimal expression in normal tissue. CRC is one of the most common causes of hepatic metastases making targeting of this tumor a high priority. In the case of CRC, EpCAM provides an encouraging cell surface target due to its high expression in CRC tissue. Additionally, the internalization of this molecule would provide a mechanism for the delivery of siRNA into cancer cells. In this study, we show that EpCAM is highly expressed in both primary and metastatic CRCs and, in fact, was identified in 100% of specimens tested. Furthermore, we show that there is only minimal expression in biliary radicals and almost no expression in normal hepatocytes. Together, these data provide strong support for the use of EpCAM as a targeting molecule for the delivery of siRNA therapy.

In summary, our data indicate that PVC in mice is a feasible method for the chronic delivery of therapeutic agents and offers a novel model for the study of liver-directed therapies. Liver-directed administration of siRNA has the potential to increase delivery to hepatic metastases, while minimizing undesired accumulation in other tissues, diminishing nuclease degradation within the circulation, and decreasing the rate of clearance by the kidneys. Additionally, the high expression of EpCAM within metastatic CRC along with its relative absence in normal hepatocytes offers a promising mechanism to further target hepatic metastasis. Our current study provides an important proof of concept that chronic portal vein catheterization in mice can be achieved with delivery of agents to liver metastases; however, we recognize that the study is somewhat limited by the relatively small sample size due to the technical difficulty and the time required to perform an individual procedure. Despite the small sample sizes, the consistency of results between the metastatic and non-metastatic studies demonstrates the reproducibility of the results. Collectively, targeted delivery combined with the already specific effects of siRNA, provide a highly selective therapeutic strategy for the treatment of CRC metastasis.

Acknowledgments

We would like to thank Catherine Anthony for manuscript preparation.

Financial Support: This work is supported by grant P20CA153043 (GI SPORE) from the National Institutes of Health.

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Figure 1. Confirmation of portal vein catheter placement, portal venous distribution, and patency at 2 weeks

(A, left) Saline (100 μ L) was injected through the portal vein catheter at the time of placement. Appropriate catheter location and function were confirmed by directly visualizing blanching of the liver. Closed arrow = portal vein. Open arrow = portal vein catheter. (A, right) In a subset of mice, B-mode ultrasound imaging was used to confirm appropriate catheter location within the portal vein one wk after initial placement. (B, left) Green fluorescently-labeled polystyrene microspheres (10 μ M) were injected (200 μ L) via the portal venous catheter and the catheter flushed with 100 μ L of injectable saline flush. Imaging was performed using a green-fluorescent filtered lens. (B, right) Microspheres were again injected at 2 wks as described above.

Figure 2. Portal vein injection enhances siRNA delivery to the liver while reducing renal clearance

(A) Mice were injected with a total of 300 μ L of volume comprised of either vehicle control (DOTAP and HBS) delivered via tail vein (**top**), 50 μ g of DY547-labeled siRNA in DOTAP/HBS via tail vein injection (**middle**), or 50 μ g of DY547-labeled siRNA in DOTAP/HBS via portal vein injection (**bottom**). The liver, lung, and kidney were collected 15 min post injection and the fluorescent signal quantified using region of interest (ROI).

(B) Graphic representation of ROI measurements.

Figure 3. Portal venous delivery of siRNA results in high concentrations within the liver
(A) Splenic injections of CT26 cells (2×10^6) were performed in Balb/C mice and metastatic disease established over a 12 d period. The mice were then given either vehicle control (**top**) or DY547-labeled siRNA (**bottom**) via the portal vein. The liver, lungs and kidneys were collected after 15 min and imaged using IVIS. **(B)** Liver specimens were collected and imaged by fluorescent microscopy following injection of DY547-labeled siRNA. Open arrow = siRNA within metastasis.

Figure 4. EpCAM is highly expressed in colon cancer but not in normal hepatocytes

The presence of EpCAM was evaluated using IHC staining of microarrays containing 89 normal colon samples, 129 primary CRCs, 4 liver metastases, and a normal liver specimen. All primary CRCs and liver metastases stained strongly for EpCAM. Staining was absent in normal hepatocytes and only minimal staining was present in biliary radicals.