Video Article

An *In Vivo* Murine Sciatic Nerve Model of Perineural Invasion

Sylvie Deborde*[1], Yasong Yu*[1], Andrea Marcadis[1], Chun-Hao Chen[1], Ning Fan[2], Richard L. Bakst[3], Richard J. Wong[1]

[1]Department of Surgery, Memorial Sloan Kettering Cancer Center

[2]Molecular Cytology Core Facility, Memorial Sloan Kettering Cancer Center

[3]Department of Radiation Oncology, Mount Sinai Hospital

*These authors contributed equally

Correspondence to: Sylvie Deborde at debordes@mskcc.org

URL: https://www.jove.com/video/56857
DOI: doi:10.3791/56857

Keywords: Cancer Research, Issue 134, Perineural Invasion, sciatic nerve, cancer, *in vivo*, tumor, PNI, mouse, pancreatic adenocarcinoma

Date Published: 4/23/2018

Citation: Deborde, S., Yu, Y., Marcadis, A., Chen, C.H., Fan, N., Bakst, R.L., Wong, R.J. An *In Vivo* Murine Sciatic Nerve Model of Perineural Invasion. *J. Vis. Exp.* (134), e56857, doi:10.3791/56857 (2018).

Abstract

Cancer cells invade nerves through a process termed perineural invasion (PNI), in which cancer cells proliferate and migrate in the nerve microenvironment. This type of invasion is exhibited by a variety of cancer types, and very frequently is found in pancreatic cancer. The microscopic size of nerve fibers within mouse pancreas renders the study of PNI difficult in orthotopic murine models. Here, we describe a heterotopic *in vivo* model of PNI, where we inject syngeneic pancreatic cancer cell line Panc02-H7 into the murine sciatic nerve. In this model, sciatic nerves of anesthetized mice are exposed and injected with cancer cells. The cancer cells invade in the nerves proximally toward the spinal cord from the point of injection. The invaded sciatic nerves are then extracted and processed with OCT for frozen sectioning. H&E and immunofluorescence staining of these sections allow quantification of both the degree of invasion and changes in protein expression. This model can be applied to a variety of studies on PNI given its versatility. Using mice with different genetic modifications and/or different types of cancer cells allows for investigation of the cellular and molecular mechanisms of PNI and for different cancer types. Furthermore, the effects of therapeutic agents on nerve invasion can be studied by applying treatment to these mice.

Video Link

The video component of this article can be found at https://www.jove.com/video/56857/

Introduction

Nerves form a specific tumor microenvironment that stimulates cancer growth and migration[1,2,3]. Perineural invasion (PNI) is the process through which cancer cells invade in and around the nerves. It may be considered as a unique route of metastasis since cancer invasion extends away from the sites of origin along nerves. PNI is found in several cancer types including pancreatic, prostate, head & neck, salivary, cervical, and colorectal cancers with an incidence ranging from 22% to 100%[1,2]. PNI is associated with pain and correlates with poor prognosis and worse survival rates[1,2].

Developing models of perineural invasion is essential to elucidate the cellular and molecular mechanisms of this process, and to test candidate therapeutic agents to decrease PNI. *In vitro* methods of studying interactions between cancers and nerves include the co-culture of cancer cells with nerve explants[4], with dorsal root ganglions[5,6,7], or with specific cells from the nerve microenvironment such as Schwann cells[7]. *In vivo* approaches, however, are more physiologically relevant, include the use of cancer mouse models in which cancer has been induced or transplanted and have the advantage of accounting for the entire nerve microenvironment. In orthotopic models of pancreatic or prostate cancer, PNI has been reported[8,9,10] and the incidence of PNI may be recorded, but because of the small size of the nerves in those organs, it is difficult to see the entire nerve and therefore to quantify the extent of PNI. The model we describe here is an *in vivo* model of PNI in which cancer cells are injected into the sciatic nerve of mice through a simple surgical procedure[11]. The heterotopic transplant invades within the nerve toward the spinal cord. The length of the nerve invasion from the site of injection to the spinal cord may be measured, as well as the volume of the cancer within the nerve. Importantly, the invaded nerve can also be collected for a variety of assays including microscopic, and molecular analyses. A variety of cancer cells can be tested, and the host mice that have been genetically modified or treated with specific compounds may be used as well. This powerful assay allows for the cancer cells and the host microenvironment to be modified for investigation into the mechanisms of PNI.

Protocol

All of the procedures with animal subjects were approved by the Institutional Animal Care and Use Committee at Memorial Sloan Kettering Cancer Center.

1. Preparation of the Cancer Cells

1. Harvest sub-confluent Panc02-H7 cells with 0.25% trypsin for 5 min at 37 °C. Collect the cells in a 15 mL centrifuge tube.
   NOTE: The cells are grown in T-225 flask, which contains about 12 x 10^6 cells per flask at 80% confluency and 4 mL trypsin/ flask is used.
2. Centrifuge the cells at x 900 g at 4°C for 5 min. Wash the cells by resuspending the cell pellet in 1 mL of PBS (by pipetting up and down twice), and transfer the suspension to a 1.5 mL microcentrifuge tube on ice.
3. Centrifuge the cells again at x 900 g at 4°C for 5 min, and discard the supernatant without disturbing the pellet. Keep the pelleted cells on ice until injection.

2. Preparation of the Mice and Surgery

Note: 8-week-old, male and female C57BL/6J mice are used in this study. The surgery conditions follows the IACUC rules of our institution. The instruments are sterilized, the surgical working surface is disinfected, the animal is disinfected and the surgeon wears sterile gloves.

1. On the day before the surgery, anesthetize the mouse using 2% isoflurane and then remove the fur along the length of femur on the dorsal side with either a thin razor or a chemical hair removal agent.
2. On the day of the surgery, anesthetize the mice using 2% isofluorane in an induction chamber. Confirm the anesthetization by a toe pinch stimulus and a lack of response.
3. Apply vet ointment on eyes to prevent dryness under anesthesia.
4. Place the anesthetized mouse on its ventral side, and gently secure each limb with hypoallergenic tape to create mild tension in the limb to be injected. Anesthesia is maintained using isoflurane delivered via a precision vaporizer and nose cone.
5. Clean the injection site with Betadine, then again with 70% alcohol. Repeat this process two more times. Make sure that no loose hair remains on the surgical field.
6. Make a 1 cm incision with small scissors about 2 mm below and parallel to the femur. Retract the skin with forceps laterally to expose the muscles underneath.
7. The sciatic nerve runs deep to the gluteus maximus and biceps femoris muscles. Separate these two muscles along a fascial plane with small scissors and expose the sciatic nerve underneath. Free the nerve from the surrounding muscles using blunt dissection.
8. Draw 3 µL of cancer cells (about 50,000 cells) from the pellet into a 10 µL syringe.
   NOTE: Alternatively, draw 3 µL of PBS as a control.
9. Place a small metal spatula underneath the nerve at the point of injection for support. Under visualization with a dissecting microscope, insert the needle into the nerve against the metal base, keeping the needle as parallel to the nerve as possible upon insertion. Be careful as not to puncture through the back of the nerve. Minimize the handling of nerve as much as possible throughout this process.
10. Slowly inject into the nerve over 5 s. A formation of a bulb in the injection area indicates a good injection. Then leave the needle in place for 3 s before removing the needle gently. Keeping the needle in place for 3 s minimizes backflow of the cells out of the nerve.
    NOTE: Injection can be performed toward the distal nerve or the spinal cord. It is important to remain consistent within a set of experiments. If the cells spill outside, the animal should not be included in the analysis of the experiment. With experience, these events are very rare.
11. Return the nerve to its original position. Cover the nerve with the overlying muscles. Treat the mice with proper analgesia, and then close the skin with 5-0 Nylon sutures.
12. Place the mouse alone in a clean cage for observation during recovery, until it fully awakens from anesthesia.
    NOTE: It takes 5-15 min for the animal to regain full consciousness. The animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency. The animal is not returned to the company of other animals until fully recovered. Thereafter, evaluate recovery at least once every 24 h for 72 h.

3. Sciatic Nerve Extraction

1. On postop day #7, euthanize the mice with CO_2. Place the mouse on its ventral side, and stabilize the distal limbs using pins.
2. Remove the skin on the dorsal side of the injected limb and torso.
3. Using blunt dissection, expose the sciatic nerve deep to the muscles.
4. The nerve courses between the ilium and the sacrum. To have access to the nerve at the spinal cord region, separate the two bones. First insert closed scissors in the narrow area where the sciatic nerve is located and then open the scissors while holding the mouse. Be delicate and maintain the integrity of the sciatic nerve during the dissection.
5. Carefully dissect the sciatic nerve distally to the end of the femur, and proximally to the spinal cord.
6. NOTE: Invaded nerves are extremely fragile and prone to breaking under tension or forceful handling.
7. Harvest the nerve by first cutting its distal end. Carefully lift the nerve while freeing it from adjacent tissue. Cut the nerve at the proximal end, as close as possible to its exit from the spinal column.
8. Record then gross length of invasion using a Vernier caliper. This macroscopic estimation is only indicative.

4. Nerve Processing and Quantification

1. Embed the dissected nerve in OCT compound. Make sure to place nerves longitudinally and as flat on the bottom of the mold as possible.
2. Indicate on the cassette the proximal and distal side of the nerve by marking the letter P and D.
3. Place embedded nerves on top of dry ice, if they are not sectioned immediately. Sample could be preserved at -80 °C for several weeks.
4. Section samples using a Cryostat microtome at 5 µm thickness and place sections on glass slides. If possible, fit 2 nerve sections per slide. Indicate proximal side of the nerve.
5. Stain slides using H&E staining[12].
6. Digitally scan stained nerve sections with a slide-scanner that provides high-resolution digital data.

7. Using imaging software, quantify the length of invasion by clicking the measure distance button, area of invasion, or other desired parameters. For a good estimation of the length of invasion, use multiple sections (2 to 4) of the same nerve.

Representative Results

This method describes the surgical implantation of pancreatic cancer cells into the murine sciatic nerve to create an *in vivo* model of quantifiable nerve invasion. **Figure 1** illustrates the anatomical location of the sciatic nerve and the site of injection. **Figure 2** shows the two sciatic nerves of a nude mouse. A PBS injected nerve (left) may be compared to a nerve injected with MiaPaCa-2 cancer cells (right). The nerve injected with cancer cells is infiltrated by tumor, and appears both thicker and darker than the nerve injected with PBS.

Figure 3 shows the quantifiable differences of sciatic nerve invasion by pancreatic cancer cell Panc02H7 in wild type and NCAM KO mice. NCAM KO mice are mice in which the adhesion molecule neural-cell adhesion molecule 1 is deficient[13]. Length and area of invasion have been quantified using imaging software. Invasion was found significantly reduced in NCAM KO mice[7].

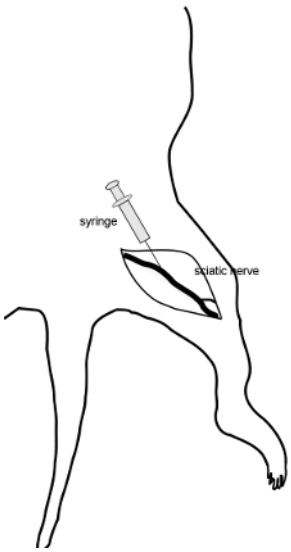

Figure 1: The mouse sciatic nerve and the site of injection. Please click here to view a larger version of this figure.

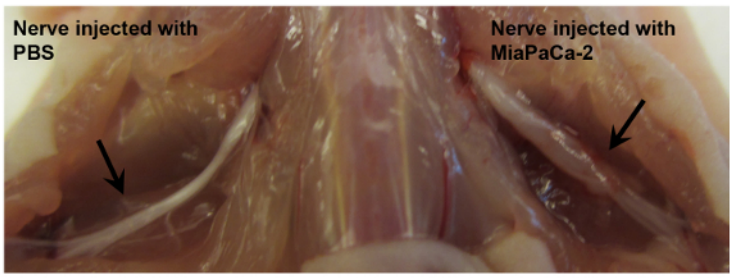

Figure 2: The two dissected sciatic nerves of a mouse. A PBS injected nerve (left) and a nerve injected with MiaPaCa-2 cancer cells (right) are shown. Black arrows show the site of injection. Please click here to view a larger version of this figure.

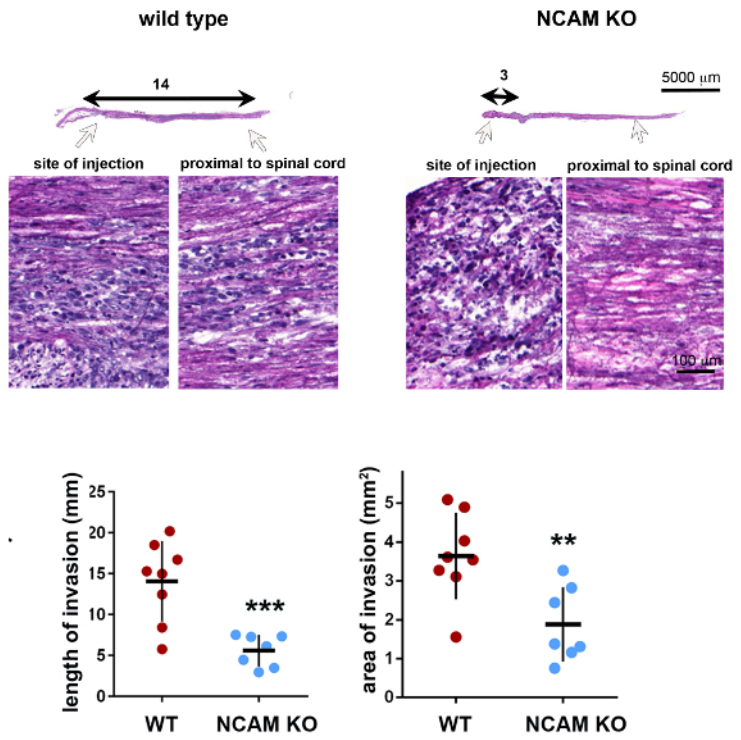

Figure 3: H&E Longitudinal sections of sciatic nerves injected with Panc02H7 cancer cells in wild type and NCAM KO mice. White arrows indicate the site of injection and proximal side of spinal cord. The black arrows indicate the length of invasion with corresponding values (mm). Scale bar: 5 mm (top images) and 0.2 mm (bottom images). Quantification of nerve invasion, as measured by length and area of invasion. Data represent mean ± SD. n = 8 (NCAM1 WT), n = 7 (NCAM1 KO), **P < 0.005, ***P < 0.0005 using t test. (Results from Deborde *et al.*, 2016[7]). Please click here to view a larger version of this figure.

Discussion

In this protocol we describe an *in vivo* murine model of perineural invasion that allows for the quantification of sciatic nerve invasion by pancreatic cancer cells. This model enables the study of molecular mechanisms of nerve invasion. Successful experiments using this technique require a careful approach to three critical steps in the process: 1) the injection of cancer cells (steps 2.7, 2.8), 2) the extraction of invaded nerves (step 3.4), and 3) processing of harvested nerves (step 4.1).

The injection should be done with great caution to avoid puncturing through the back wall of the nerve, which leads to a loss of cancer cells. Once the needle is in place, the injection should be done at a slow and steady pace over 5 s to prevent the hydraulic force from propelling the cancer cells too far forward from the point of injection. Leave the needle in the nerve after injection for an additional 3 s will minimize back flow and cancer cell leakage. During sciatic nerve extraction, dissection must be very gentle as the invaded sciatic nerve is very fragile. Cancer invasion disrupts the normal cellular organization of the sciatic nerve. Even though invaded nerves appear thicken in diameter compared to non-injected or non-invaded nerves, they are more prone to breaking with even minor stretching and manipulation. During embedding of the excised nerve in OCT for processing, it is very important to insure that the nerve is laid completely flat onto the mold. This step allows for the sectioning of the sample to capture the entire length of the nerve for proper analysis. Solid tumor on the nerve often needs to be trimmed to prevent the nerve segment proximal to the tumor from raising the nerve off the bottom of the mold. The entire length of the nerve must be laid completely flat against the mold.

Many modifications can be considered while using this protocol. When a small metal spatula is not available to be placed under the sciatic nerve, the surface of a pair of wide forceps can be used instead. A forceps may be used to hold the needle in place after insertion, although the downsides of this approach are a diminished view of the injection and potential to crush the nerve if excessive force is used. Another way is to use a fine forceps to lift the nerve, then opening the forceps to stretch the nerves. This method is easier to execute and provides both tension and stabilization during injection, but may damage the nerve with excessive stretching of the nerve. The injection can be done in either a proximal or distal direction, but we have found that in both cases, the direction of the cancer invasion is typically towards the spinal cord. Therefore, we prefer injecting distally to further minimize hydraulic force that may push the cancer cells towards the spinal cord, and confound later measures of length of invasion. We advise that it is important to be consistent in the choice of direction of injection within a set of experiments.

Although we present a model of syngeneic grafting, a similar technique can be used for xenografts human cancer cells into immunodeficient mice, with only slight variations. Xenografts in nude mice may required longer periods of cancer invasion to yield similar lengths of nerve invasion as in syngeneic models. Different cancer cell lines may also be used. PNI is not only prevalent in the cancer of the pancreas, but also of the head

and neck, prostate, and skin cancers. Our lab has successfully injected nude mice in the sciatic nerve with human pancreatic cancer cell lines MiaPaCa-2 and Panc1[7,14,15].

Possible adverse events include wound dehiscence, as the mice occasionally chew their stitches resulting in opening of the wound. In that case, the wound would need to be re-stitched. We check the wound daily for the first 3 post-operative days. The mice may also walk with a slight limp postoperatively, likely due to pain from both the surgical procedure as well as invasion of their sciatic nerve with cancer. This is usually not severe enough to require pain medication.

This model has limitations. Some amount of stretching and pinching of the nerve during the procedure might create damages in the nerve. In addition, it is difficult to control the exact number of cells going into the nerve. Furthermore, this model is limited to the study of cells that have already invaded nerves. It is not a model to study the interaction of nerves with cancer cells located in the primary tumor. The main disadvantage of using the sciatic nerves versus nerves of the gastrointestinal tract is that the sciatic nerve is not a metastatic site of pancreatic cancer. The difference in the composition and the nature of the nerve fibers could influence the invasion. However, the accessibility and very small size of the pancreatic nerves would not allow such a study.

Our key suggestions for the beginners would be to remove the hair in a wide area around the planned incision sites so that the landmarks can be easily identified and to make sure to have a steady position before beginning to inject. After many injections, the performer should be able to get 100% of animals with nerve invasion.

This model allows for the study the interactions between cancers and nerves in an animal model. It can be applied to a wide range of oncologic studies by using genetically modified mice, cancer cells, or both. Pharmacological treatments against tumor invasion along nerves can also be tested by comparing length and area of invasion between treatment and control groups. These different possible applications make this model an extremely versatile and powerful tool for studying PNI.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors acknowledge the technical services provided by the molecular cytology facility and the animal facility of Memorial Sloan Kettering Cancer Center. This work was supported by NIH grants CA157686 (to R.J. Wong) and P30 CA008748 (Memorial Sloan Kettering Cancer Center support grant).

References

1. Liebig, C., Ayala, G., Wilks, J. A., Berger, D. H., & Albo, D. Perineural invasion in cancer. *Cancer.* **115** (15), 3379-3391 (2009).
2. Bapat, A. A., Hostetter, G., Hoff, Von, D. D., & Han, H. Perineural invasion and associated pain in pancreatic cancer. *Nat Rev Cancer.* **11** (10), 695-707 (2011).
3. Deborde, S., & Wong, R. J. How Schwann cells facilitate cancer progression in nerves. *Cell Mol Life Sci.* **341** (177-186), 1236361-16 (2017).
4. Abiatari, I., *et al.* Consensus transcriptome signature of perineural invasion in pancreatic carcinoma. *Mol Cancer Ther.* **8** (6), 1494-1504 (2009).
5. Ayala, G. E., *et al.* In vitro dorsal root ganglia and human prostate cell line interaction: redefining perineural invasion in prostate cancer. *Prostate* **49** (3), 213-223 (2001).
6. Gil, Z., Cavel, O., *et al.* Paracrine regulation of pancreatic cancer cell invasion by peripheral nerves. *J Natl Cancer Inst.* **102** (2), 107-118 (2010).
7. Deborde, S., T., *et al.* Schwann cells induce cancer cell dispersion and invasion. *J Clin Invest.* **126** (4), 1538-1554 (2016).
8. Pour, P. M., Egami, H., & Takiyama, Y. Patterns of growth and metastases of induced pancreatic cancer in relation to the prognosis and its clinical implications. *Gastroenterology.* **100** (2), 529-536 (1991).
9. Eibl, G., & Reber, H. A. A xenograft nude mouse model for perineural invasion and recurrence in pancreatic cancer. *Pancreas.* **31** (3), 258-262 (2005).
10. Stopczynski, R. E., *et al.* Neuroplastic changes occur early in the development of pancreatic ductal adenocarcinoma. *Cancer Res.* **74** (6), 1718-1727 (2014).
11. Gil, Z., *et al.* Nerve-sparing therapy with oncolytic herpes virus for cancers with neural invasion. *Clin Cancer Res.* **13** (21), 6479-6485 (2007).
12. Cardiff, R. D., Miller, C. H., & Munn, R. J. Manual hematoxylin and eosin staining of mouse tissue sections. *Cold Spring Harb Protoc.* **2014** (6), 655-658 (2014).
13. Cremer, H., *et al.* Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. *Nature* **367** (6462), 455-459 (1994).
14. He, S., *et al.* The chemokine (CCL2-CCR2) signaling axis mediates perineural invasion. *Mol Cancer Res.* **13** (2), 380-390 (2015).
15. He, S., *et al.* GFRα1 released by nerves enhances cancer cell perineural invasion through GDNF-RET signaling. *P Natl Acad Sci USA*, 201402944 (2014).

