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14. ABSTRACT Due to delays in obtaining regulatory approval from both our own IACUC and the DOD, we were unable to begin our study until very recently. Despite the delays, we have made progress in establishing the cell lines necessary for the study to proceed, characterization of the cell lines, and further developing and establishing surgical methods for sciatic implantation and convection enhanced delivery within the tumors. Despite the delays, we are confident that we will be able to complete the proposed work within the time-frame initially proposed, with an accelerated schedule					
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Convection enhanced delivery (CED) in an animal model of malignant peripheral nerve sheath tumors and plexiform neurofibromas.

PI: Kaleb Yohay

Introduction

Neurofibromatosis Type 1 (NF1) is a common autosomal dominant neurocutaneous disorder that results in a predisposition to the development of benign and malignant tumors. Plexiform neurofibromas (PN) are complex, often large tumors that usually involve multiple nerves and may cause significant morbidity. Complete surgical resection is rarely feasible. PNs may undergo malignant transformation to become Malignant Peripheral Nerve Sheath Tumors (MPNST). MPNSTs are aggressive, often fatal sarcomas and are the leading cause of death in adults with NF. Currently there are no available effective medical therapies for PNs or MPNST. EGFR is not normally expressed by Schwann cells, however, EGFR expression has been demonstrated in association with the development of benign and malignant peripheral nerve sheath tumors, making this receptor a potential therapeutic target. Effective *in vivo* delivery of traditional chemotherapy and newer molecularly targeted therapeutics to tumors of the nervous system remains a significant obstacle. Systemic delivery is restricted by systemic toxicity, non-targeted distribution and subtherapeutic tumor levels of potentially curative agents. Interstitial infusion, also referred to as convection-enhanced delivery (CED), is a mode of local drug delivery which relies on a pressure-dependent gradient to accomplish uniform infusate dispersion and high drug concentrations directly in tumor or tissue while avoiding systemic exposure and hence dose-limiting toxicity.

In the current study we have proposed that CED may be used as a safe, reliable and effective means of delivery of therapeutic agents in animal models of plexiform neurofibromas and malignant peripheral nerve sheath tumors.

Specific Aim 1: To use an animal model to determine the distribution of macromolecules delivered to intraneural PNs and MPNST via CED.

Design: Orthotopic xenograft models of sciatic intraneural NF1 MPNST and PNs in scid mice as described by Perrin et al. (2007a, 2007b) will be used. Six to eight weeks after implantation, the location and extent of tumor development within the sciatic nerve will be assessed by MRI. The sciatic nerve tumor will be surgically exposed and a silica infusion catheter will be placed within the tumor. HRP-labeled albumin or gadolinium-albumin will be infused. Several infusion rates and volumes will be assessed. Volume of distribution within the tumor and sciatic nerve will be assessed with immunohistochemistry and *in vivo* MRI.

Specific Aim 2: To determine the efficacy CED of the epidermal growth factor receptor (EGFR) inhibitor erlotinib in animal models of intraneural PNs and MPNST.

Design: Orthotopic xenograft models of sciatic intraneural NF1 MPNST and PNs in scid mice will be used. EGFR positive cell lines will be used for implantation. Six to eight weeks after implantation, the location and extent of tumor development within the sciatic nerve will be assessed by MRI. Erlotinib or vehicle will be infused into the tumor via CED using the infusion parameters optimized from the results of Specific Aim 1. Tumor growth and angiogenesis will be monitored by *in vivo* contrast enhanced MRI at several time points after infusion and tumor growth will be compared in erlotinib versus vehicle treated animals. Phosphorylated Akt and EGFR will be measured in tumor samples to assess for biologic activity of the erlotinib.

Body

Unfortunately, due to delays in obtaining regulatory approval from both our own IACUC and the DOD, we were unable to begin our study until very recently. Despite the delays, we have made progress in establishing the cell lines necessary for the study to proceed, characterization of the cell lines, and further developing and establishing surgical methods for sciatic implantation and convection enhanced delivery within the tumors. Despite the delays, we are confident that we will be able to complete the proposed work within the time-frame initially proposed, with an accelerated schedule.

Key Research Accomplishments

Establishment of MPNST cell line:

Tumor tissue was collected from an adult MPNST patient with NF1 in 1x Hanks Balanced Salt Solution (HBSS) containing 0.6% glucose. The tissue was digested in HBSS containing 12% papain and 10 µg/ml DNase with gentle shaking at 37°C for 1 hour. After 1 hour, fetal bovine serum (FBS) was added to the solution to a final concentration of 10% to abrogate digestion. The tissue was gently triturated, and the resultant cell suspension was filtered through a sterile 70µm cell strainer (BD Biosciences). After being spun down in a tabletop centrifuge for 5 minutes at 1,200rpm, the cells were resuspended in RPMI-1640 medium containing 4mg/L glutamine and 10% FBS. After repeating the process once, the cells were plated at a density of 1,000-1,500cell/cm² in cell culture dishes in the same medium. The cells are passaged to the same starting density when they reach 80-90% confluence. The culture has been maintained for more than 10 passages.

Characterization of the status of signaling molecules of MPNST and PN cells:

The cells were grown on poly-D-lysine-coated coverslips. After fixation in 2% paraformaldehyde at room temperature for 10 minutes, the cells were blocked and permeabilized with 5% normal goat serum and 0.3% Triton X-100 in phosphate-buffered saline (PBS), pH 7.4, at room temperature for 1 hour. The cells were incubated with primary antibodies raised in rabbit diluted in the block solution at manufacture recommended concentration overnight at 4°C. The cells were then washed in PBS 3 times, 2-3 minutes each time, and incubated with goat anti-rabbit secondary antibodies (labeled with either Alexa Fluor 488 or Alexa Fluor 555) at room temperature for 1 hour. After washing off secondary antibodies in PBS (2-3 minutes x 3 times), the coverslips were mounted face down in Vectashield on glass slides with DAPI as a counterstain for cell nuclei.

So far the following molecules have been characterized for epithelial growth factor receptor (EGFR), AKT and phosphorylated AKT. We are in the process of characterizing the cells with regard to their phosphorylated EGFR status.

Determination of the surgical procedures:

Utilizing mouse cadavers, we have refined our techniques for tumor cell implantation and drug delivery using convection-enhanced delivery (CED).

For tumor cell implantation, the following procedure will be used::

After proper anesthesia is achieved, the hindquarter is shaved and then prepared with povidone-iodine swabs or gauzes. 70% alcohol swab or gauze is used to remove the remaining povidone-iodine from the skin. ~100µL 1% lidocaine is injected along the incision line to ensure adequate analgesia.

A 15mm dorsal longitudinal skin incision is made slightly to the left of midline over the pelvic girdle. A faint separation of muscle groups can be visualized slightly caudal and ventral to the left hip joint. A 10 mm incision is made on the fascia along the separation of the muscle groups and deepened by blunt dissection. The sciatic nerve can be visualized running parallel to the length of the femur. An injection site is chosen slightly proximal to the sciatic trifurcation and the nerve is secured with a small vessel loop at this site. A few drops of lidocaine are dropped on the nerve. A 34 Gauge fused silica (WPI) cannula is inserted at a sharp angle (~10°) along the nerve to a depth of ~3mm (tip ~0.5mm under the nerve sheath). 5×10⁵ malignant peripheral nerve sheath tumor (MPNST) cells in 5µL of medium are slowly injected using a 10µL precision syringe. After completion of the injection, the cannula is left in place for ~5min then slowly withdrawn. Gentle pressure may be applied through a cotton swab at the injection site to reduce backflow. The muscle groups are then approximated, and the skin incision is closed with 3-0 nylon sutures in a continuous subcuticular fashion.

For drug delivery using CED, the following procedure will be used:

After proper anesthesia is achieved, the hindquarter is shaved and then prepared with povidone-iodine swabs or gauzes. 70% alcohol swab or gauze is used to remove the remaining povidone-iodine from the skin. ~100µL 1% lidocaine is injected along the incision line to ensure adequate analgesia.

A 15mm dorsal longitudinal skin incision is made slightly to the left of midline over the pelvic girdle. A faint separation of muscle groups can be visualized slightly caudal and ventral to the left hip joint. A 10 mm incision is made on the fascia along the separation of the muscle groups and deepened by blunt dissection. The sciatic nerve can be visualized running parallel to the length of the femur. An injection site is chosen slightly proximal to the sciatic trifurcation and the nerve is secured with a small vessel loop at this site. A few drops of lidocaine are dropped on the nerve. A 34 Gauge fused silica (WPI) cannula is inserted at a sharp angle along the nerve into the center of the tumor. The drug of choice will be loaded in a precision 10µL syringe and infused at 0.1µL/min into the nerve sheath tumor driven by a WPI ultramicro pump. After completion of the infusion, the cannula is left in place for ~5min then slowly withdrawn. Gentle pressure may be applied through a cotton swab at the injection site to reduce backflow. The muscle groups are then approximated, and the skin incision is closed with 3-0 nylon sutures in a continuous subcuticular fashion.

Reportable Outcomes

None

Conclusions

Despite delays in initiating our work due to difficulty in obtaining regulatory approval, we now have all necessary approvals and have successfully obtained and characterized the cell lines to be used and have refined our surgical technique for tumor cell implantation for sciatic xenografts and drug delivery using convection-enhanced delivery (CED). Despite delays we plan to complete our project in the time-frame initially proposed. We have updated our time-line as documented below:

Specific Aim #1: To use an animal model to determine the distribution of macromolecules delivered to intraneural PNs and MPNST via CED.

1. Task 1: Establishment of Xenografts (present-December 2011)
 - 1.1. EGFR characterization of MPNST and PN cell lines (completed)
 - 1.2. PN implantation in sciatic nerve (October-November 2011) (animals used=12)
 - 1.3. MPNST implantation in sciatic nerve (October- November 2011) (animals used=12)
 - 1.4. MRI tumor confirmation (November-December 2011)
2. Task 2: CED of HRP-albumin (November 2011-February 2012)
 - 2.1. HRP-Albumin Infusion and harvesting of sciatic nerves (November 2011-February 2012)
 - 2.2. Gd-Albumin Infusion and *in vivo* MRI (November 2011-February 2012)
3. Task 3: Assessment of distribution (January 2012-April 2012)
 - 3.1. Immunohistochemistry and data analysis (January 2012-April 2012))
 - 3.2. MRI data analysis (January 2012-April 2012)

Specific Aim #2: To determine the efficacy CED of the epidermal growth factor receptor (EGFR) inhibitor erlotinib in animal models of intraneural PNs and MPNST.

4. Task 4: Establishment of Xenografts (January-May 2012)
 - 4.1. PN implantation in sciatic nerve (January-March 2012) (animals used=20)
 - 4.2. MPNST implantation in sciatic nerve (January-March 2012)) (animals used=20)

4.3. MRI tumor confirmation (February-May 2012)

5. Task 5: CED of erlotinib (February-May 2012)

5.1. Infusion of erlotinib in PN and MPNST (March-May 2012)

6. Task 6: Assessment of efficacy (March-September 2012)

6.1. In vivo MRI #1 (March-June 2012)

6.2. In vivo MRI #2 (April-July 2012)

6.3. Sciatic nerve harvesting (May-August 2012)

6.4. MRI data analysis (June-August 2012)

6.5. Histologic preparation, Westerns for phosphor Akt and EGFR and analysis (June-August 2012)

6.6. Data analysis and manuscript Preparation (August-September 2012)

References:

Perrin GQ, Fishbein L, Thomson SA, et al., Plexiform-like neurofibromas develop in the mouse by intraneural xenograft of an NF1 tumor-derived Schwann cell line. J Neurosci Res, 2007. 85(6): p. 1347-1357.

Perrin GQ, Li H, Fishbein L, et al., An orthotopic xenograft model of intraneural NF1 MPNST suggests a potential association between steroid hormones and tumor cell proliferation. Lab Invest, 2007. 87(11): p. 1092-1102.