A novel method of intracranial injection via the postglenoid foramen for brain tumor mouse models

Laboratory investigation

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Object. Mouse models have been widely used in developing therapies for human brain tumors. However, surgical techniques such as bone drilling and skin suturing to create brain tumors in adult mice are still complicated. The aim of this study was to establish a simple and accurate method for intracranial injection of cells or other materials into mice.

Methods. The authors performed micro CT scans and skull dissection to assess the anatomical characteristics of the mouse postglenoid foramen. They then used xenograft and genetically engineered mouse models to evaluate a novel technique of percutaneous intracranial injection via the postglenoid foramen. They injected green fluorescent protein–labeled U87MG cells or virus-producing cells into adult mouse brains via the postglenoid foramen and identified the location of the created tumors by using bioluminescence imaging and histological analysis.

Results. The postglenoid foramen was found to be a well-conserved anatomical structure that allows percutaneous injection into the cerebrum, cerebellum, brainstem, and basal cistern in mice. The mean (± SD) time for the postglenoid foramen injection technique was 88 ± 15 seconds. The incidence of in-target tumor formation in the xenograft model ranged from 80% to 100%, depending on the target site. High-grade gliomas were successfully developed by postglenoid foramen injection in the adult genetically engineered mouse using virus-mediated platelet-derived growth factor B gene transfer. There were no procedure-related complications.

Conclusions. The postglenoid foramen can be used as a needle entry site into the brain of the adult mouse. Postglenoid foramen injection is a less invasive, safe, precise, and rapid method of implanting cells into the adult mouse brain. This method can be applied to both orthotopic xenograft and genetically engineered mouse models and may have further applications in mice for the development of therapies for human brain tumors.

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Key Words • brain tumor • intracranial injection • mouse model • postglenoid foramen • oncology

MALIGNANT brain tumors, including brain metastases, are among the most common and devastating human cancers. Among brain tumors, high-grade gliomas are the most common primary malignant tumors. Thus far, treatment regimens for these tumors have had only a modest effect despite recent advances in surgery, radiotherapy, and chemotherapy. One of the reasons for resistance to treatment in high-grade gliomas is their heterogeneity, and several strategies for classifying these tumors have been reported on the basis of molecular abnormalities. In addition, high-grade gliomas in adults have been distinguished from those occurring in childhood on the basis of their biological characteristics. Thus, there is an urgent need to identify molecular subtypes of these tumors and to develop corresponding therapies.

Mouse models ranging from basic xenograft tumors to complex genetically engineered models have been essential in the development of various therapies for brain tumors. Development of orthotopic xenograft models and virus-mediated genetically engineered mouse models of brain tumors requires basic surgical techniques, including skin incision, bone drilling, and skin suturing, for injecting tumor cells into the brains of adult mice. These techniques, however, are invasive and complicated, necessitating the use of specialized instruments and resulting in excessive stress for the animals. Simpler, less invasive methods that have a high degree of accuracy should be developed to improve this process for the laboratory animals, as well as the researchers.

Abbreviations used in this paper: GFP = green fluorescent protein; PDGF = platelet-derived growth factor; RCAS = replication-competent avian leukosis virus splice acceptor.
The postglenoid foramen is a natural cavity on the lateral side of the skull, and it represents one of the main venous foramina; it is also known as the false jugular foramen. This cavity occurs in rodents, armadillos, members of the Canidae family, and many ungulates, but the size and shape vary among the primates. In humans, the postglenoid foramen is rarely found and is usually very small when detected. In rats, however, it occurs as a single and relatively large foramen on the rostral area of the opening of the external acoustic meatus; morphological variability is not usually detected. The anatomical locations of these foramina are easily identified on both sides of the head by observing the head surface. In the present study, we describe a novel method of intracranial injection via the postglenoid foramen, which we used as an entrance to the adult mouse brain. Postglenoid foramen injection enabled percutaneous access to 4 major sites of brain tumors commonly found in the central nervous system: the cerebrum, cerebellum, brainstem, and basal cistern. We have shown that postglenoid foramen injection is an accurate and rapid method for both orthotopic xenograft and genetically engineered mouse models using imaging and histological analyses. To our knowledge, this is the first report describing a physiological access route to the adult mouse brain for percutaneous injection.

Methods

This study was approved by the Nagoya University Animal Ethics Committee.

Twenty BALB/c Slec-nu/nu mice (purchased from Chubu Kagaku Shizai Co., Ltd.) were used for the orthotopic xenograft model, and 12 Gtv-a mice expressing the TVA receptor (the receptor for avian leukosis virus) from the glioblastoma acidic protein promoter were obtained from the US National Cancer Institute for use in the genetically engineered mouse model. Gtv-a mice have a mixed genetic background: C57BL6, 129, Balb/C, and FVB/N. One BALB/c Cr Slc mouse (Chubu Kagaku Shizai Co., Ltd.) was used to obtain micro CT images. In addition, skull specimens were dissected from 9 mice that had been euthanized with CO2 inhalation after they were used in another study.

Computed Tomography Scan and Preparation of Mouse Skull Specimens

One 4-week-old BALB/c Cr Slc mouse was anesthetized with an intraperitoneal injection of 45 mg/kg sodium pentobarbital (Dainippon Sumitomo Pharma Co., Ltd.) and examined with micro CT. The mouse was euthanized by CO2 gas inhalation after imaging. The micro CT images were obtained using an R_mCT2 micro CT system (Rigaku Corp.). The x-ray power was 90 kV, 160 μA, with an exposure time of 17 seconds. The images were reconstructed and analyzed using R_mCT2 software (Rigaku Corp.). For measuring angles from the CT scans, the reference planes and axis were defined as follows: horizontal reference plane, the plane that passes through both postglenoid foramina and the frontonasal suture; coronal reference plane, the plane that passes through both postglenoid foramina and is perpendicular to the horizontal plane; and anteroposterior axis, the reference axis that passes through the right postglenoid foramen and is perpendicular to the coronal plane.

Mouse skull specimens were obtained from 9 dead mice as described above. Skin, muscle, and brain tissues were surgically removed from the skulls, and the remaining soft tissue was further allowed to decompose in water. The skulls were then washed and dried.

Cell Culture and Transfection of Retroviral Vectors

Human glioblastoma cell line U87MG (American Type Culture Collection), human embryonic kidney cell line GP2-293 (Clontech Laboratories, Inc.), and chicken embryo fibroblast line DF-1 (American Type Culture Collection) were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin and grown at 37°C in a humidified 5% CO2 atmosphere. A retrovirus expressing GFP was generated using the Retro-X Universal Packaging System (Clontech). GP2-293 cells were transfected with pRetroQ-AcGFP-C1 along with a pVSVG plasmid (Clontech). After 48 hours, cell-free viral supernatant was obtained and stored at -80°C. U87MG cells were transfected with the retroviral vectors encoding GFP using the recombinant fibronectin fragment FN-CH 296 (RetroNectin; Takara Bio) according to the manufacturer’s protocol. The replication-competent avian leukosis virus splice acceptor (RCAS)/tv-a system used here has been described previously. The RCAS-PDGFB plasmid, which contains full-length, wild-type human PDGFB, was a gift from Eric C. Holland (Memorial Sloan–Kettering Cancer Center) and was transfected into DF-1 cells using Lipopectamine 2000 (Invitrogen), as recommended in the manufacturer’s protocol.

Generation of Tumor-Bearing Mice

The mice were anesthetized with an intraperitoneal injection of 45 mg/kg sodium pentobarbital (Dainippon Sumitomo Pharma Co., Ltd.) before all postglenoid foramen injections. After induction of anesthesia, the mice were placed on a 1-mm grid sheet, and the appropriate angle and depth were determined for accurate needle insertion. A disposable pipette tip (D1000ST Diamond Precision Tips; Gilson, Inc.) and a 26-gauge, 10-μl glass syringe (calibrated 701N syringe, Hamilton Company) were used for the injection. In the orthotopic xenograft model, 20 four-week-old BALB/c Slec-nu/nu mice were administered intracranial injections of 5 μl of a solution containing of 2.5 × 106 freshly dissociated GFP-labeled U87MG cells via the postglenoid foramen. In the genetically engineered mouse model, twelve 4-week-old Gtv-a mice were administered intracranial injections of approximately 1.5 μl containing 1 × 106 DF-1 cells producing the RCAS-PDGF retrovirus via the same injection site. Mice were monitored daily for signs of complications or symptoms of tumor development. Symptomatic mice were killed with CO2 and examined histologically.

Brain Sectioning, H & E Staining, and Fluorescence Imaging

Entire brains were dissected en bloc and fixed in 10%
buffered formalin (Wako Pure Chemical Industries) for 24 hours. Four sections were obtained from each brain and embedded in paraffin using a Tissue-Tek rotary tissue processor (Sakura), and 5-μm sections were cut with a Leica microtome (Leica Microsystems). The sections were stained with H & E and examined with a BZ-9000 microscope (Keyence Corp.). The brains implanted with GFP-labeled U87MG cells were visualized with a fluorescence imaging system (IVIS Spectrum, Caliper Life Sciences).

Results

Micro CT and skull dissections were performed in the initial part of this study to assess the anatomical characteristics of the mouse postglenoid foramen and the potential use of this foramen as a needle insertion site. The head CT scan clearly showed the location and morphology of the foramina (Fig. 1A–D). In the dissected skulls, the postglenoid foramina were always located on the rostral areas of the opening of the external acoustic meatus without significant morphological variety. Representative examples are shown in Fig. 1E and F. These anatomical findings convinced us of the technical feasibility of percutaneous injection into the mouse brain via the postglenoid foramen.

We then selected 4 major sites—the cerebrum, cerebellum, brainstem, and basal cistern (which represents the subarachnoid space in front of the pons)—as targets for brain tumor development through implantation via postglenoid foramen injection. The approach side is generally determined according to the target region and the operator’s dominant hand, and we selected a right-side approach for this study. We calculated needle trajectories based on 3D CT images and measured the insertion depths. The trajectories were projected on horizontal and sagittal planes, and insertion angles were measured relative to the anteroposterior axis in each plane. We assessed the appropriateness of these trajectory planes using mouse skull specimens (Fig. 2A and B). The appropriate angles and depths to each target site that we used in this study are listed in Fig. 2C.

The needle insertion point for postglenoid foramen injection is just rostral to the opening of the external acoustic meatus, which is visible beneath thin, hairless skin (Fig. 3A). Retracting the auricle forward made this point more visible and prevented bleeding and CSF leakage after needle puncture. A 26-gauge, 10-μl glass syringe was covered with a disposable pipette tip to adjust the optimal depth of insertion (Fig. 3B). Prior to the application of the technique in live mice, we performed an experiment of percutaneous penetration with steel needles ranging from 20- to 30-gauge through the posterior glenoid foramina using C57BL/6 Cr Slc mouse cadavers, and found that all needles were inserted into the brain smoothly. We then passed a nylon suture through a 23-gauge hollow needle between the left and right posterior glenoid foramina to confirm the needle tract. The nylon suture ran beneath the ventral border zone between the cerebral hemispheres and brainstem, piercing right above the initial segments of both trigeminal nerves in the basal cistern (Fig. 3C).

**Fig. 1.** Anatomical assessment of the mouse postglenoid foramen. **A:** Three-dimensional CT reconstruction of the head of a 4-week-old BALB/c Cr Slc mouse showing the location and morphology of the external acoustic meatus (black arrow) and postglenoid foramen (red arrow). **B:** CT image in the coronal reference plane demonstrating the left and right postglenoid foramina (red arrows). **C:** Midsagittal section showing the horizontal reference plane (yellow line). **D:** CT image in the horizontal reference plane showing the left and right postglenoid foramina (red arrows). **E** and **F:** Photographs showing lateral (E) and inferior (F) views of the skull of a C57BL/6 Cr Slc mouse. The red arrows indicate the postglenoid foramina; the black arrows, the external acoustic meatuses. The mandible was removed. Bar = 5 mm.

**Fig. 2.** Trajectory plans for each of the target sites in the horizontal (A) and sagittal (B) planes. Green arrows (a) indicate cerebrum injection; blue arrows (b), cerebellum injection; red arrows (c), brainstem injection; and yellow arrows (d), basal cistern injection in front of the pons. **C:** The angle and depth of needle insertion from the postglenoid foramen to the target sites. Angles are given in the horizontal plane (HP) and sagittal plane (SP). *At a right angle to the sagittal plane.
Postglenoid foramen injection in mice

After confirming the technical feasibility and anatomical relationship of posterior glenoid foramen injection, we administered the injection to live mice. The syringe was filled with cell suspension, the needle tip was positioned on the insertion point at an optimal angle, and the needle was gently inserted percutaneously into the brain of the anesthetized mouse. The solution was injected over 5–30 seconds, depending on the volume. The needle was then drawn out slowly over several seconds. While withdrawing the needle, minimal finger pressure was applied to stop bleeding at the puncture site, and this method of applying pressure was continued for another 30 seconds after withdrawal of the needle.

Using the posterior glenoid foramen injection technique, we selectively injected GFP-labeled U87MG cells into each target site in 4-week-old BALB/c Slc-nu/nu mice. Despite the fine vasculature around the rodent mandibular joint,\(^1\) bleeding from the needle entrance was easily stopped. In our experience of 20 procedures, the mean time required for the posterior glenoid foramen injection technique was 88 ± 15 seconds (± SD), excluding the period required for anesthesia. There were no procedure-related complications such as hemorrhage, CSF leakage, or infection. All mice displayed symptoms of tumor development, including seizure, ataxia, and lethargy, and were killed within 3 weeks. The location of the tumor was evaluated by fluorescence imaging and H & E staining. Representative samples of tumor formation at each target site are shown in Fig. 4. The incidence of in-target tumor formation was 100% in the cerebrum and basal cistern groups, but 80% in the cerebellum and brainstem groups, as shown in Table 1. In 1 mouse in the cerebellum injection group, the tumor developed in the cerebrum, and in 1 mouse in the brainstem group the tumor developed in the basal cistern.

To expand the application of postglenoid foramen injection, we then tried to create high-grade gliomas using a RCAS/tv-a system. We applied the same technique in 12 Gtv-a mice, injecting 1.5 μl of RCAS-PDGFB–producing DF-1 cells into the brain. Histologically confirmed high-grade gliomas were successfully induced by postglenoid foramen injection in 6 of these 12 mice. Four of these lesions were glioblastomas, with pseudopalisading and microvascular proliferation as shown in Fig. 4E–G.

Discussion

In this study, we demonstrated the utility of the postglenoid foramen as an entrance to the adult mouse brain. There were no complications from the injection procedure, and the overall incidence of in-target tumor formation was more than 80%, indicating the safety and accuracy of the technique. Notably, postglenoid foramen injection provides access to the skull base and adjacent area with minimal brain damage from needle penetration. Considering that metastatic tumors and high-grade gliomas frequently disseminate within the subarachnoid space,\(^5,11,18\) safe access to the basal cistern may be valuable for the establishment of models of disseminated tumors.

To evaluate the accuracy and reproducibility of postglenoid foramen injection, we selected 4 target sites for injection in the orthotopic xenograft model, and a high incidence of in-target tumor formation was observed in all 4 groups. Among these sites, the cerebral hemisphere is the most commonly used tumor location in many mouse models, and it is also the most suitable target for postglenoid foramen injection because of its proximity to the injection site. In contrast, 1 mouse in each of the cerebellum and brainstem groups developed a tumor outside the target area, suggesting that these sites are relatively difficult to inject due to their small size and the blockage of the ipsilateral petrous bone. The petrous bone is located between the postglenoid foramen and posterior fossa, and it blocks access to the lower part of the brainstem and cerebellum. This limitation must be borne in mind and the trajectory should be carefully planned when determining posterior fossa targets. Development of a simplified stereotactic system for postglenoid foramen injection is a potential solution to the need for improved injection accuracy for distant and small targets.

There have been few reports of complications following conventional stereotactic implantation of cells into mouse brain. Ragel et al.\(^17\) reported operative mortality rates of 7%–11% in a skull base meningioma model us-

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**TABLE 1: Incidence of tumor formation by target site in 20 mice**

<table>
<thead>
<tr>
<th>Target Site</th>
<th>No. of In-Target Tumors (%)</th>
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<tr>
<td>cerebrum</td>
<td>5/5 (100)</td>
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<tr>
<td>cerebellum</td>
<td>4/5 (80)</td>
</tr>
<tr>
<td>brain stem</td>
<td>4/5 (80)</td>
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<tr>
<td>skull base</td>
<td>5/5 (100)</td>
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\(^1\) Source: J Neurosurg / Volume 116 / March 2012
ing conventional stereotactic methods. We also have had experience with xenograft glioma models using conventional stereotactic systems; in our hands, procedure-related mortality was estimated as approximately 5%–10% with the conventional stereotactic method. Head fixation with a head frame poses the danger of suffocation of the mouse, and this was the main cause of operative death in our series. In the current study, however, we did not observe any complications of postglenoid foramen injection. Thus, we concluded that this technique is safer.

Postglenoid foramen injection has several advantages over the conventional implantation technique. First, the duration of the procedure is short (mean time of 88 ± 15 seconds). Although there have been few reports assessing the time required for conventional implantation of cells into mouse brain, the reported duration has ranged from 8 to 25 minutes.12,22 Thus, the procedural time for postglenoid foramen injection is less than one-fifth that required by the conventional implantation technique. Second, postglenoid foramen injection requires only a simple injection, while the conventional method requires surgical skills. Third, postglenoid foramen injection can be performed with a syringe, while the conventional method requires specialized instruments for fixing and drilling the mouse cranium. These advantages of postglenoid foramen injection may be valuable, especially for researchers who are unfamiliar with surgical procedures or who need a large-scale preclinical trial. In the RCAS/tv-a system, RCAS-producing cells were initially injected into neonatal mouse brains, which allowed direct injection via the thin, soft cranium. However, a recent report has shown that the RCAS/tv-a system can be successfully used in adult mice to create high-grade gliomas.3 The need for simpler and safer injection techniques is increasing. The postglenoid foramen injection technique can also be applied for other purposes, such as administration of therapeutic products, insertion of electrodes, and sample collection. In vivo electroporation is another

Fig. 4. Representative histological images of orthotopic xenograft and spontaneous tumor formation. A–D: Fluorescence images and corresponding H & E-stained sections of the implanted tumors are shown. The implanted U87MG tumor cells labeled with GFP developed in each injection site in the cerebrum (A), cerebellum (B), brainstem (C), and basal cistern (D). The emitted green light from the GFP was captured by a charge-coupled device camera. After fluorescence imaging, the brains were processed for H & E staining. The tumor margins are demarcated by black dotted lines; the trigeminal nerves are indicated by black arrows. Scale bar = 1 mm. E–G: Histology of a PDGFβ-driven glioblastoma in a Gtv-a mouse. The low-magnification image of an H & E-stained section (E) shows a diffuse infiltrating tumor in the cerebrum (arrowheads). The high-magnification images (F and G) show pseudopalisading necrosis (arrows) and microvascular proliferation (arrowheads) recapitulating the histological features of human glioblastoma. Original magnification × 40 (E), × 100 (F), and × 200 (G).
promising application of postglenoid foramen injection. Moreover, this technique can be applied to other animals with postglenoid foramina, after confirming the species-specific anatomy.

Conclusions

The postglenoid foramen can be used as a needle entry site into the adult mouse brain and can provide percutaneous access to the cerebrum, cerebellum, brainstem, and basal cistern. Postglenoid foramen injection is a less invasive, safe, rapid, and accurate method of injecting cells into the adult mouse brain, with several advantages over the conventional implantation technique. This method can be applied not only to orthotopic xenograft models but also to genetically engineered mouse models, both of which have been widely used in developing therapies for human brain tumors. Further application of postglenoid foramen injection is expected as an experimental procedure in mouse models of human central nervous system diseases.

Disclosure

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Author contributions to the study and manuscript preparation include the following. Conception and design: Iwami, Momota. Acquisition of data: Iwami, Momota, Natsume, Kinjo, Nagatani. Analysis and interpretation of data: Wakabayashi, Iwami, Momota. Drafting the article: Iwami, Momota. Critically revising the article: Natsume, Kinjo, Nagatani, Wakabayashi. Reviewed final version of the manuscript and approved it for submission: all authors. Administrative/technical/material support: Natsume, Kinjo, Nagatani.

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