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Safety and feasibility of convection-enhanced delivery of nimustine hydrochloride co-infused with free gadolinium for real-time monitoring in the primate brain

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Objectives: Convection-enhanced delivery (CED) has been developed as an effective drug-delivery strategy for brain tumors. Ideally, direct visualization of the tissue distribution of drugs infused by CED would assure successful delivery of therapeutic agents to the brain tumor while minimizing exposure of the normal brain tissue. We previously showed the anti-tumor efficacy of nimustine hydrochloride (ACNU) delivered via CED against a rodent intracranial xenografted tumor model. Here, we developed a method to monitor the drug distribution using a non-human primate brain.

Methods: CED of a mixture of ACNU with gadodiamide was performed using three non-human primates under real-time magnetic resonance imaging monitoring. Animals were clinically observed for any toxicity after infusion. Two months later, their brains were subjected to histological examination for the evaluation of local toxicity. Another one animal was euthanized immediately after CED of a mixture of ACNU, gadodiamide, and Evans blue dye to evaluate the concordance between ACNU and gadodiamide distributions. The harvested brain was cut into blocks and the ACNU content was measured.

Results and discussion: Real-time magnetic resonance imaging monitoring of co-infused gadodiamide confirmed the success of the infusion maneuver. In the monkey that also received Evans blue, the distribution of Evans blue was similar to that of gadodiamide and paralleled the measured ACNU content, suggesting concordance between ACNU and gadodiamide distributions. Histological examination revealed minimum tissue damage with the infusion of ACNU at 1 mg/ml, determined as a safe dose in our previous rodent study. CED of ACNU can be co-administered with gadodiamide to ensure successful infusion and monitor the distribution volume.

Keywords: Convection-enhanced delivery, Magnetic resonance imaging, Primate, Chemotherapy, Central nervous system

Introduction

Although the recent advent of temozolomide has improved the survival of patients with high-grade gliomas, the efficacy of systemic chemotherapy is still unsatisfactory.[1] The blood–brain barrier (BBB), although compromised to some extent in tumor tissue, can limit the effective distribution of systemically administered agents.[2] Therefore, local drug delivery methods that bypass the BBB are attractive alternative methods to further improve the efficacy of chemotherapy against high-grade gliomas.[3] Several investigators have recently demonstrated the efficacy of convection-enhanced delivery (CED) as an alternative therapeutic strategy for treating focal central nervous system (CNS) diseases such as brain tumors.[4–6] This local infusion technique, utilizing bulk flow, enables the delivery of large and small molecules to clinically significant volumes of targeted tissues, offering an improved volume of distribution (Vd) compared to simple diffusion. CED of therapeutic agents bypasses the BBB and leads to a high concentration of the agents within the injection site and a larger distribution of agents within the target site.[7] We have been working to develop effective chemotherapy with CED of nimustine hydrochloride for the treatment of high-grade gliomas. Using a rodent brain tumor model, we previously demonstrated the efficacy of CED of nimustine hydrochloride.[8,9]

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To further develop this CED-based strategy toward clinical application, we evaluated the safety and feasibility of performing real-time magnetic resonance imaging (MRI) monitoring of the drug distribution by co-infusing nimustine hydrochloride (ACNU) with gadolinium during CED infusion in the brains of non-human primates. Mixtures of ACNU with a gadolinium (Gd) chelate — gadodiamide (Gd-DTPA-BMA; gadolinium-diethylenetriaminepentaacetic-acid-bis-methylamide) — were imaged in real-time during CED to assess the distribution. Animals were carefully observed after the infusion, and histological examinations were performed 2 months after infusion to address the safety of this strategy in the primate CNS.

Materials and Methods

Experimental subjects

The protocol was reviewed and approved by the Institutional Animal Care and Use Committees at the Tohoku University Graduate School of Medicine. Four adult male cynomolgus monkeys (*Macaca fasicularis*, 3–4 kg) were individually housed in stainless steel cages. Each animal room was maintained under a 12-hour light/dark cycle, and the room temperature ranged between 20 and 25°C. The Purina primate diet was provided on a daily basis in amounts appropriate for the size and age of the animals. This diet was supplemented with fruit or vegetables daily. Also, small bits of fruit, cereal, or other treats were provided as part of the environmental enrichment program. Tap water was available *ad libitum* for each animal through an automatic watering device or an attached water bottle. Prior to assignment to the study, all imported animals underwent at least a 31-day quarantine period; this was only a period of observation. Using three monkeys, infusion of ACNU was performed in three hemispheres and infusion of an ACNU/Gd-DTPA-BMA mixture was performed in three hemispheres and three brainstems. These animals were euthanized two months after infusion. Using the other one animal, the infusion of ACNU/Gd-DTPA-BMA/Evans blue mixture was performed. This animal was euthanized immediately after infusion to measure the ACNU content.

CED

Three adult primates (*Macaca fascicularis*) secured in a stereotactic frame underwent CED of a saline solution of ACNU (1.0 mg/ml, 300 μl; Sankyo Co., Ltd, Tokyo, Japan) or saline solution of ACNU (1.0 mg/ml, 300 μl) containing Gd-DTPA-BMA (1 mmol/l; Amersham Health, Princeton, NJ, USA) to the corona radiata in the frontal lobe or brain stem. For infusion into corona radiata, saline solution of ACNU was infused into right hemisphere and saline solution of ACNU containing Gd-DTPA-BMA into left hemisphere. The infusion system consisted of a reflux-free, step-design infusion cannula connected to a loading line (containing ACNU solution) and an olive oil infusion line.[10] For the present study, we manufactured infusion cannula placing silica tubing, which was connected to a loading line, inside the outer catheter of 22 gauge intravenous catheter (SURFLO; Termo, Tokyo, Japan). By protruding the tip of silica for 5 mm from the tip of outer catheter of 22 gauge intravenous catheter, we made a step design at the tip of infusion cannula. A 1-ml syringe (filled with oil) mounted onto a micro-infusion pump (BeeHive; Bioanalytical Systems, West Lafayette, IN, USA) regulated the flow of fluid through the system. The infusion cannula was secured in place with methylmethacrylate. The infusion rate was initiated from 0.2 μl/minute, and was increased to 0.5, 0.8, 1.0, 1.5, and 2.0 μl/minute with every 10-minute interval. Finally, the rate reached 3.0 μl/minute after 70 minutes, and was maintained at 3.0 μl/minute until the 300 μl was infused. MR images were obtained every 20–40 minutes during infusion for a total imaging time of about 140 minutes for the 300 μl infusion. After the infusion, we removed the cathers together with methylmethacrylate and sutured the wounds.

MRI acquisition

During the MRI, animals were sedated using sodium pentobarbital. T1-weighted images of the primates' brains were acquired using a 0.3 T Toshiba Opart scanner (Toshiba Co., Tokyo, Japan). Prior to inserting infusion catheters, baseline images were taken: repetition time/echo time/flip angle=24 milliseconds/10 milliseconds/30°, number of excitations=6, matrix=144 × 192, slice thickness=0 mm. Coordinates for infusion including site of burr hole, entry point, and trajectory were calculated from these baseline images. Once the catheters were inserted and the infusion commenced, T1- and T2-weighted images with the following conditions were taken consecutively throughout the infusion: repetition time/echo time/flip angle=4000 milliseconds/240 milliseconds/90°, number of excitations=3, matrix=208 × 208, slice thickness=0 mm. The scan time ranged from 23 minutes 54 seconds to 25 minutes 40 seconds.

Volume quantification from MR images

The volumes of Gd-DTPA-BMA distributions within each brain region at each time point were quantified from the final set of images acquired during infusion. In total, data for three infusions in the corona radiata and three infusions in the brain stem were acquired. We calculated the Vd as the Vd from MRI containing at least 10% of the total increase in signal intensity due to the addition of contrast agents.[11] Regions of interest were automatically generated during the segmentation to allow for visual inspection and

editing for accuracy. Open-source OsiriX imaging software (http://www.osirix-viewer.com) was used for this calculation. Algorithm used was threshold (lower/upper bounds). Data were checked and confirmed by multiple observers (SS, RS, and YY).

Observation and histological examination

Animals were observed daily for medical or neurological difficulties following infusion and weekly for weight and general health. Observation included their appearance, motor weakness, and daily activity. All primates were euthanized 2 months after ACNU infusion, and their brains were removed, fixed, and subjected to paraffin sectioning. The sections (5 μm) were stained with hematoxylin and eosin.

Comparison of Gd-DTPA-BMA and ACNU distributions

One adult primate (*Macaca fascicularis*) secured in a stereotactic frame underwent CED of a saline solution of ACNU (5.0 mg/ml, 300 μl) containing Gd-DTPA-BMA (1 mmol/l; Amersham Health) and 4 mM Evans blue. Four infusions were performed in total. The coordinates for infusions were 10 mm bilaterally from the midline, 10 mm anterior or 5 mm posterior from the line connecting both ear bar, and 12 mm deep. Immediately after infusion, a T1-weighted MR image was obtained. After MRI, the infused brain was harvested and sliced in the coronal section parallel to the infusion cannula. Using the distribution of Evans blue as a guide, the brain was cut into approximately 5-mm squares to measure the ACNU content.

Measurement of ACNU content

After adding 300 μl of Milli-Q water, brain blocks were homogenized using an ultrasonic homogenizer. The brain homogenate was sent to Mitsubishi Chemical Medience Corporation (Tokyo, Japan) to measure the ACNU content. The method for ACNU measurement was as follows. Methanol (90 μl) was added to 10 μl of brain homogenate and centrifuged at 10 000 rev/minute for 10 minutes. After centrifugation, 50 μl of supernatant was taken and added to 50 μl of Milli-Q water. This solution was transferred to a high-performance liquid chromatography (HPLC) autosampler vial. HPLC was performed using a C18 3-μm column (2.0 × 100 mm; column temperature: 40°C; flow rate: 0.2 ml/minute; injection volume: 1.0 μl) with gradient elution from 10 mM ammonium acetate/acetonitrile to water/2-propanol/acetonitrile over 5 minutes. A hybrid triple quadrupole-ion trap mass spectrometer (model QTRAP 5500; AB Sciex, Concold, Canada) was used for mass spectrometry. The general setting used was selected reaction monitoring, with an electrospray ionization interface, a temperature of 700°C, and a scan time of 300 milliseconds. The lower limit of detection for the HPLC-MS/MS was 0.5 ng/ml for the brain homogenate.

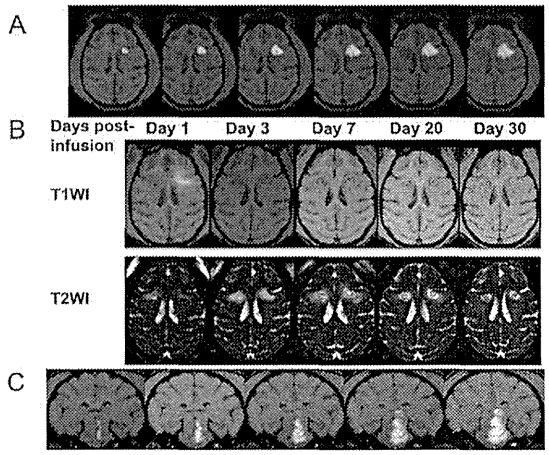

Figure 1 Nimustine hydrochloride (ACNU) solution or ACNU solution mixed with gadodiamide (Gd-DTPA-BMA) was infused by convection-enhanced delivery (CED) at a volume of 300 μl into the corona radiata of the right or left hemispheres, respectively, in three non-human primates. T1-weighted MR images were obtained every 25 minutes during infusion (A). Representative images are shown. The T1-weighted (B, upper) and T2-weighted MR images (B, lower) were acquired 1, 3, 7, 20, and 30 days post-infusion. ACNU solution mixed with gadodiamide was also infused by CED into the brain stem of three non-human primates. Coronal T1-weighted MR images, acquired every 25 minutes during infusion (C).

Results

MRI monitoring during and after CED

In order to test the feasibility of CED of ACNU with MRI monitoring in the non-human primate brain, ACNU solution or ACNU solution mixed with gadolinium was infused by CED at a volume of 300 μl into the corona radiata of the right and left hemispheres, respectively, in three non-human primates. The T1- and T2-weighted MR images were acquired during infusion and 1, 3, 7, 20, and 30 days post-infusion (Fig. 1). During CED, T1-weighted MR images, acquired every 25 minutes, showed a high-intensity signal only in the left hemisphere that received a mixture of ACNU with gadolinium (Fig. 1A). The high-intensity signal could be detected after 1 day of infusion; however, it disappeared afterwards, indicating the wash-out of the co-infused gadolinium (Fig. 1B). T2-weighted images showed a high-intensity signal in the right hemisphere but low intensity in the left hemisphere. The high-intensity signal detected in the right hemisphere persisted until 30 days post-infusion. The low-intensity signal in the left hemisphere gradually changed to a high intensity from 1 day post-infusion. As this change was concordant with wash-out of the gadolinium signal, the low-intensity signal immediately after infusion in the

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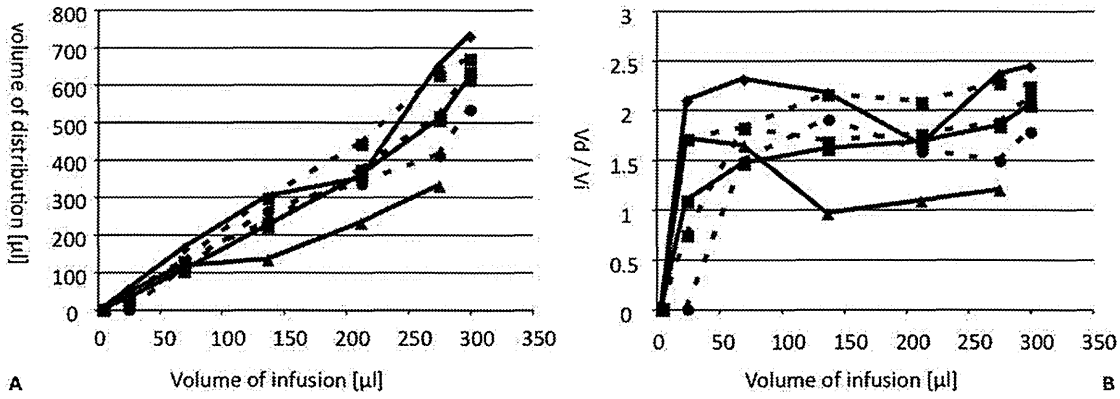


Figure 2 The volume of distribution (Vd) defined as the Vd from magnetic resonance imaging containing at least 10% of the total increase in signal intensity due to the addition of contrast agents was plotted against the volume of infusion (Vi, A). Each solid line indicates the infusion results on each infusion into white matter, and each dotted line indicates the infusion results on brain stem infusion. The line demonstrates the success of convection-enhanced delivery, as the Vd rose with an increasing Vi. The Vd/Vi ratio plotted against the Vi demonstrated almost the same Vd/Vi during the increase in Vi (B).

left hemisphere may have been caused by the negative signal of gadolinium. Afterward, the high-intensity signal was observed until 30 days post-infusion, gradually decreasing in size. In this study, ACNU solution mixed with gadiamide was also infused by CED into the brain stem of three non-human primates. Coronal T1-weighted MR images, acquired every 25 minutes during infusion, are shown in Fig. 1C.

COLOUR FIGURE

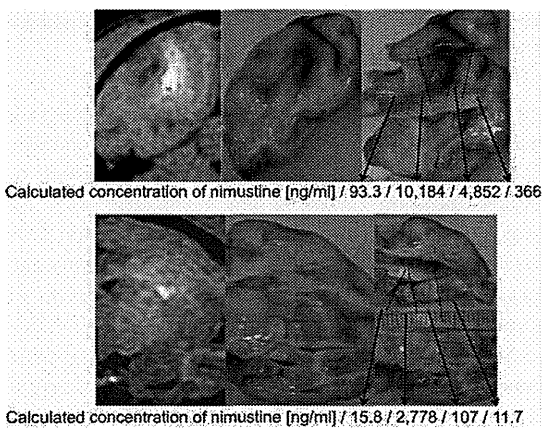


Figure 3 One adult primate (*Macaca fascicularis*) underwent convection-enhanced delivery of a saline solution of nimustine hydrochloride (ACNU) (5.0 mg/ml, 300 μl) containing gadodiamide (Gd-DTPA-BMA; 1 mmol/l; Amersham Health, Princeton, NJ, USA) and Evans blue. Immediately after infusion, a T1-weighted MR image was obtained (left). After MRI, the infused brain was harvested and sliced in the coronal section parallel to the infusion cannula (middle). Using the distribution of Evans blue as a guide, the brain was cut into 5-mm squares to measure the ACNU content (right). Each brain block was added to 300 μl of Milli-Q water, homogenized, and subjected to quantification. Measured ACNU concentrations are expressed as the concentration of the homogenate. Data from two infusion sites are demonstrated.

Infusion of gadolinium to monitor the quality of CED

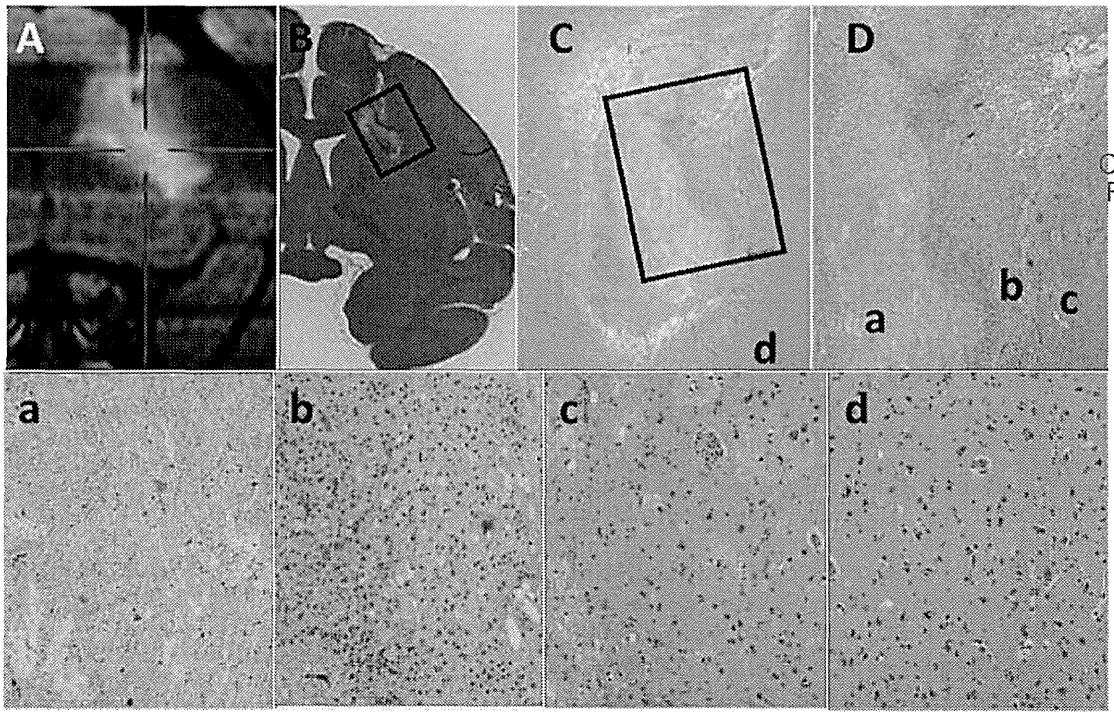
The detection of co-infused gadolinium enabled us to monitor the quality of CED. Real-time monitoring detected the widening of the infused area on MR images. The graph was obtained by plotting the Vd against the volume of infusion (Vi, Fig. 2A). The line demonstrated the success of CED as the Vd rose with an increasing Vi. The Vd/Vi ratio plotted against the Vi showed almost the same Vd/Vi during the increase in Vi (Fig. 2B). This suggested the success of CED because, if there is leakage, Vd/Vi may diminish during infusion.

Comparison of Gd-DTPA-BMA and ACNU distributions

The distribution of Gd-DTPA-BMA detected by MRI was similar to that of Evans blue in the brain slices (Fig. 3). This suggests the similar distribution of Gd-DTPA-BMA and Evans blue. The ACNU content measured in the brain blocks was parallel to the distribution of Evans blue. Data from a representative brain slice are demonstrated in Fig. 3. These findings suggest the similarity of distribution between Gd-DTPA-BMA and ACNU. Findings were similar in all four infusion sites. Therefore, distribution of Gd-DTPA-BMA detected by MRI likely reflects the distribution of ACNU.

Evaluation of toxicity

To confirm the safety of this strategy, histological examination of monkey brains was performed 2 months after CED of ACNU mixed with Gd-DTPA-BMA. Infusion volumes were 300 μl. Robust distributions of Gd were observed at each infusion site in the T1-weighted MR images obtained immediately after infusion (Fig. 4A). The animals were euthanized 2 months after infusion. The animals developed no abnormal symptoms during the observation period.



COLOUR FIGURE

Figure 4 The local toxicity of the infused nimustine hydrochloride (ACNU) and gadodiamide (Gd-DTPA-BMA) mixture was evaluated 2 months after detecting the robust distribution of gadodiamide under magnetic resonance imaging (A). The animals were euthanized 2 months after the infusion and processed for histological examination. Hematoxylin and eosin staining showed some tissue damage at the cannula tract; however, the tissue damage was limited to the area adjacent to the cannula (B). Findings were similar among the animals tested. (C) Higher magnification of the squared area of B; original magnification ×20. (D) Higher magnification of the squared area of B; original magnification ×40. (a–d) correspond to the indicated regions in C and D; original magnification ×200. Necrotic tissue can be noted at the needle tract (a) surrounded by a region containing foamy cells and reactive astrocytes (b). No clear tissue damage was found at (c) and (d).

Postmortem hematoxylin and eosin staining showed some tissue damage at the cannula tract (Fig. 4). However, the tissue damage was limited to the area adjacent to the cannula. Throughout the study, there were no adverse clinical effects observed in any of the animals at any time points following CED of ACNU and Gd mixture.

Discussion

ACNU was most often used in Japan via intravenous injection before the era of temozolomide as a counterpart of carmustine (BCNU) in the USA. The major obstacle for successful chemotherapy with intravenously delivered nitrosoureas is systemic side effects: myelosuppression.[12] Recently, the contribution of O(6)-methylguanine-DNA methyltransferase (MGMT) to chemoresistance to nitrosoureas has led to a focus on the possibility of using nitrosoureas and agents that deplete MGMT activity.[13] However, this strategy was again hindered by severe systemic toxicity.[14] On the other hand, the local delivery of nitrosourea is emerging as a successful strategy.[15] Gliadel wafers, BCNU-containing polymers that are deposited in the resection cavity during surgery and release BCNU over a certain period of time, were proven to be effective in the management of high-grade gliomas.[15] The known disadvantage of BCNU polymers is that the distribution of BCNU ultimately depends on simple diffusion of just a few millimeters from the deposited sites.[16] In consideration of the fact that if we could achieve the robust local distribution of nitrosoureas, we may be able to achieve better efficacy, we conducted a study and verified the efficacy of ACNU delivered via CED in a rodent intracranial xenografted model.[8] The ACNU used in this study has several advantages when compared to BCNU. The ACNU is both a lipid- and water-soluble compound. In contrast to BCNU that is highly lipophilic, ACNU can be dissolved in water to form an aqueous solution. This characteristic of ACNU favors its use in CED studies.[17] As previously demonstrated, the CED distribution of hydrophilic agents is better than that of lipophilic agents because lipophilic agents have a higher affinity for the surrounding tissues.[17]

The development of an accurate and effective monitoring system for CED is of considerable interest. Gd-DTPA encapsulated in liposomes or conjugated to albumin have been developed thus far to achieve this aim. Real-time monitoring using

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Gd-DTPA encapsulated in liposomes co-infused with liposomal drugs enabled the real-time detection of the distribution of a liposomal drug.[18,19] Gd-DTPA conjugated albumin could be used to monitor the distribution of protein-based agents including chimeric proteins.[20] Other agents such as radiolabeled albumin,[21] antibody-conjugated iron oxide nanoparticles,[22] and a dual CT-MR dendrimer contrast agent[23] have also been developed for the visualization of drug delivery. As previously demonstrated, the surface properties of the infusates markedly affect the Vd after CED.[17] Therefore, every single agent used for CED should be labeled to monitor their exact distribution. However, in contrast, the labeling of each compound for clinical use is not an easy task. In 2007, Murad *et al.* demonstrated the CED of gemcitabine mixed with Gd-DTPA.[24] In 2010, Ding *et al.* demonstrated the use of Gd-DTPA, as a surrogate tracer, co-infused with recombinant immunotoxin for drug distribution monitoring.[25] In 2010, Heiss *et al.* reported a CED-based strategy to deliver muscimol for epilepsy patients with MR monitoring by mixing muscimol and Gd-DTPA.[26] As Gd-DTPA is a highly water-soluble compound, the distribution of Gd-DTPA may mimic the distribution of agents co-infused if the agent is sufficiently water-soluble. In this study, we infused a mixture of ACNU and Gd-DTPA-BMA. The distribution of Gd-DTPA-BMA reflected the measured ACNU content in brain tissue, suggesting the possibility of real-time monitoring using MRI. It is true that this study has inherent limitations as the paralleism of the diffusion of ACNU, Gd-DTPA-BMA, and Evans blue was tested by indirect methodology. Diffusibility and/or clearance rate can be different among each infusates. Moreover, it was only tested in the normal brain parenchyma, which may not reflect the clinical situation infusing into brain tumor. In addition, distribution can be different depending on the anatomical location of the infusion. However, even if there existed a slight difference in the distribution of Gd-DTPA-BMA and ACNU, detecting the distribution of Gd-DTPA-BMA at least ensured the quality of the CED maneuver. Since many failures of CED occur due to the loss of continuous pressure caused by leakage of the infusates by reflux or by draining into ventricles or sulci, evaluating the Vd/Vi ratio over time will give us information about the quality of CED methods. If the Vd/Vi ratio remains stable, it confirms the success of CED. As many agents used in clinics cannot easily be detected on images, this strategy may provide an alternative monitoring method for the CNS delivery of chemotherapeutics.

Local toxicity always hinders the local application of chemotherapeutic agents. Phase I/II trial of the stereotactic injection of DTI-015 (BCNU in 100% ethanol) into recurrent malignant gliomas was carried out, with notable safety.[5] The ACNU used in this study is a counterpart of BCNU, and dissolves in aqueous solution. We observed local tissue degeneration only adjacent to the needle tract, as demonstrated in Fig. 4. Moreover, ACNU is an agent that can be used for intrathecal chemotherapy.[27] Considering that the dose used for interparenchymal infusion in this study was much lower than that used for intrathecal perfusion studies, intratumoral infusion should be safer. As this study evaluated the histological profile obtained after the infusion of a mixture of ACNU and Gd-DTPA-BMA, the toxicity of locally applied Gd-DTPA-BMA is already included in the analysis. Although the number of monkeys (n=3) used in this study was limited, infusion to multiple brain regions resulted in no severe toxicity.

In summary, we have developed and evaluated a strategy for CED infusion of ACNU into the brain of non-human primates. This primate study verified the ability of CED to successfully and safely distribute ACNU. The procedure could be accurately performed while monitoring co-infused Gd-DTPA-BMA with MRI. Since CED of chemotherapeutic agents is emerging as a promising strategy to overcome this devastating disease, clinical application of this present strategy is planned.

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