# Brain-specific gene expression by immortalized microglial cell-mediated gene transfer in the mammalian brain

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**Abstract The intra-arterial injection of immortalized microglia transfected with the lacZ gene, resulted in the expression of β-galactosidase in the rat brain at 48 h and the activity of β-galactosidase was detected for up to 3 weeks post-injection. More than 30-fold higher activity of β-galactosidase was detected in the brain than in the liver, lung or spleen at 48 h post-injection. This method allows us to easily deliver the gene of interest to the brain without influencing other organs. Our brain-targeting gene delivery system can facilitate gene therapy of several brain disorders, including brain tumor, metabolic disorders, and degenerative disorders, as well as investigation into the roles of particular genes in brain function and development.**



*Key words:* Microglia; Microglial cell line; Gene transfer; Brain-specific; Drug delivery system

## 1. Introduction

The ability to manipulate the expression of genes within the mammalian brain provide unique opportunities to study and to potentially treat neurologic disorders. The introduction of certain genes specifically in brain has been done with viral vectors or cells carrying DNA. However, these methods require surgery. Recently, we found that primary isolated microglia specifically entered the brain from blood flow when the cells were injected intra-arterially [1]. Since intra-arterially injected microglia were labeled with fluorescent dye microparticles by their phagocytic activity, this system could apply to a brain-specific delivery for medicines, or other bioactive materials, such as proteins or genes.

Microglia, macrophage-like cells in the brain, are multifunctional cells; they play important roles in the development, differentiation and maintenance of neural cells via their phagocytic activity and production of enzymes, cytokines and trophic factors [2]. Although activated microglia show similar phenotypes to macrophages in isolated conditions, they appear to display phenotypes differing from macrophages in vivo and in vitro [3–11]. An example of phenotypic difference between both types of cells is the affinity and migrating activity of isolated microglia for the brain [1].

In this paper, we investigated the possibility that microglia can deliver the gene of interest to brain without any effects to other organs, by injection of microglia transfected β-galactosidase gene expression vector to a vertebral artery of the rats.

## 2. Materials and methods

### 2.1. Cell cultures

Primary mixed glial cell cultures were prepared in Eagle's MEM supplemented with 10% fetal calf serum, 5 μg/ml bovine insulin, and 0.2% glucose from neonatal Fisher rats as described previously [6,12]. Medium was changed every 3 days. Microglia were isolated on the 14th day by mechanical agitation and purified using non-coated plastic dishes (Falcon 1001, Becton-Dickinson Japan, Tokyo, Japan) as described previously [6]. The purity of microglia was more than 99% as determined by immunostaining with FITC-labeled IgG (used in 1:100 dilution, Cappel, West Chester, PA). Ra2 cells, immortalized microglial clone cells, were maintained in the culture medium described above containing 1 ng/ml recombinant mouse GM-CSF (Genzyme, Cambridge, MA). Ra2 cells stop to proliferate without GM-CSF in vitro, and there was not observed any tumorigenic activity in brain parenchyma. Procedures for establishment of mouse and rat microglial cell lines were described in a manuscript submitted elsewhere.

### 2.2. Gene transfection

Twenty four hours prior to transfection, purified microglia and Ra2 cells were seeded at $5\times10^6$ cells in a 10-cm plastic dish (Falcon 3003, Becton-Dickinson Japan, Tokyo, Japan). Fifteen microgram of pEF1-Geo, an expression vector of lacZ-neo$^r$ fusion gene under control of human EF1 promoter (a kind gift from Dr. Y. Kadokawa, Fujita Health University) in 0.36 ml of HEPES-buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4) was carefully mixed with 0.14 ml of DOTAP transfection reagent (Boehringer Mannheim Japan, Tokyo, Japan) by gentle pipetting and stood for 15 min at room temperature. DNA-DOTAP transfection solution was mixed with 6.5 ml fresh culture medium, then added to the cells in 10-cm dish. After incubation for 16 h cells were washed twice with 5 ml culture medium, then incubated another 48 h.

### 2.3. Fluorescent dye staining

To recognize the exogenous cells within the brain, the cells were tagged with a lipid-soluble, fluorescent dye, PKH26 [13,14] (Zynaxis Cell Science, Inc., Malvern, PA). PKH26 stained microglia efficiently; the purified microglia were stained with intensity at least two orders higher than the purified astrocytes. The cells in a 10-cm plastic dish were incubated with PKH26 staining solution containing 10 mM of PKH26, 50% Diluent B (a phagocytic cell-labeling solution, Zynaxis, Malvern, PA), and 50% culture medium for 15 min, then washed three times in 10 ml serum-containing medium. PKH26-stained cells were harvested using a rubber policeman in 2 ml of ice-cold phosphate-buffered saline (pH 7.2), washed with 5 ml ice-cold phosphate-buffered saline (pH 7.2) by centrifugation three times, then $2\times10^6$ cells were injected into a male Fisher rat intra-arterially.

### 2.4. Intra-arterial injection

Male Fisher rats weighing about 200 g were anesthetized with 42 mg/kg sodium pentobarbital by i.p. injection. Cells were injected into

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the recipient's subclavian artery. At 2 h, 48 h, 9, 16 and 23 days following injection, rats were treated with about 200 ml isotonic saline by intra-aortic perfusion, then the brain, liver, spleen and lung were isolated, frozen in liquid nitrogen, and embedded in O.C.T. compound (Tissue Tek, Elkhart, IN). Sections (8 μm) were cut with a cryostat microtome, transferred to slide glass and dried. Fluorescently labeled cells were photographed under a fluorescence microscope (IX70-FLA, Olympus, Tokyo, Japan).

*2.5. Detection of β-galactosidase in tissues*

The cells expressing β-galactosidase were identified following incubation with X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside, Nakalai Tesque, Osaka, Japan). Tissue sections were fixed for 15 min in a solution containing 1% glutaraldehyde, 0.1 M sodium phosphate buffer (pH 7.0), and 1 mM $MgCl_2$, and then incubated with a solution containing 0.2% X-gal, 10 mM sodium phosphate buffer (pH 7.0), 150 mM NaCl, 1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6$, and 3.3 mM $K_3Fe(CN)_6$. Following incubation for 6–12 h at 37°C, the staining solution was removed, then sections were washed three times by 0.1 M sodium phosphate buffer (pH 7.0), mounted with 70% glycerol, and photographed.

*2.6. Measurement of β-galactosidase activity*

The activity of β-galactosidase derived from cells expressing lacZ gene in tissue section was determined by Galacto-Light chemiluminescent assay kit (Tropix, Bedford, MA). Tissue sections were lysed in 0.2 ml lysis solution containing 0.1 M potassium phosphate (pH 7.8), 0.2% Triton X-100 and 1 mM DTT. The lysate was centrifuged for 2 min at 13 000 rpm, and the supernatant was collected. Aliquot 20 μl of supernatant was reacted with Galacton chemiluminescent substrate (100-fold diluted) in 0.2 ml of reaction buffer containing 0.1 M sodium phosphate buffer (pH 8.0) and 1 mM $MgCl_2$. After 60 min incubation, 0.3 ml accelerator was added and mixed quickly. After 5 s delay following addition, chemiluminescence was measured by Lumat LB9507 chemiluminometer (Berthold Japan, Tokyo, Japan).

## 3. Results and discussion

At 48 h after the fluorescent-labeled purified microglia were injected intra-arterially, many fluorescent cells were observed in the brain section of the rat (Fig. 1A). A higher magnification shows that a large portion of the fluorescent cells were in the brain parenchyma including cerebral cortex, hippocampal region, brainstem and cerebellum, apart from blood capillaries and that some of these fluorescent cells had large processes (Fig. 1B). A small portion of the fluorescent cells were observed in the brain capillaries, attached on the capillary wall. Two hours following the injection, migration of microglia into the brain parenchyma was observed (Fig. 1C). Exogenous fluorescently labeled microglia were observed to adhere to a vessel in the brain. Some microglia crossed the vessel and entered the parenchyma. Similar results were obtained with Ra2 cells (data not shown). When a frozen brain section were stained with X-gal as a substrate for exogenously introduced β-galactosidase, many lacZ-positive cells were observed in the brain from the rats at 48 h after Ra2 which were transfected with a lacZ gene expression vector were injected intra-arterially (Fig. 1D). Similar results were obtained with purified microglia (data not shown). Therefore, intra-arterially injected microglia and Ra2 cells can migrate to the brain and can express the genes transfected in vitro and translate them into biologically active proteins in the brain.

The specificity of Ra2 migration was determined by measuring β-galactosidase activity in the brain and in other tissues. Using a highly sensitive detection method for β-galactosidase activity with a chemiluminescent substrate we could detect

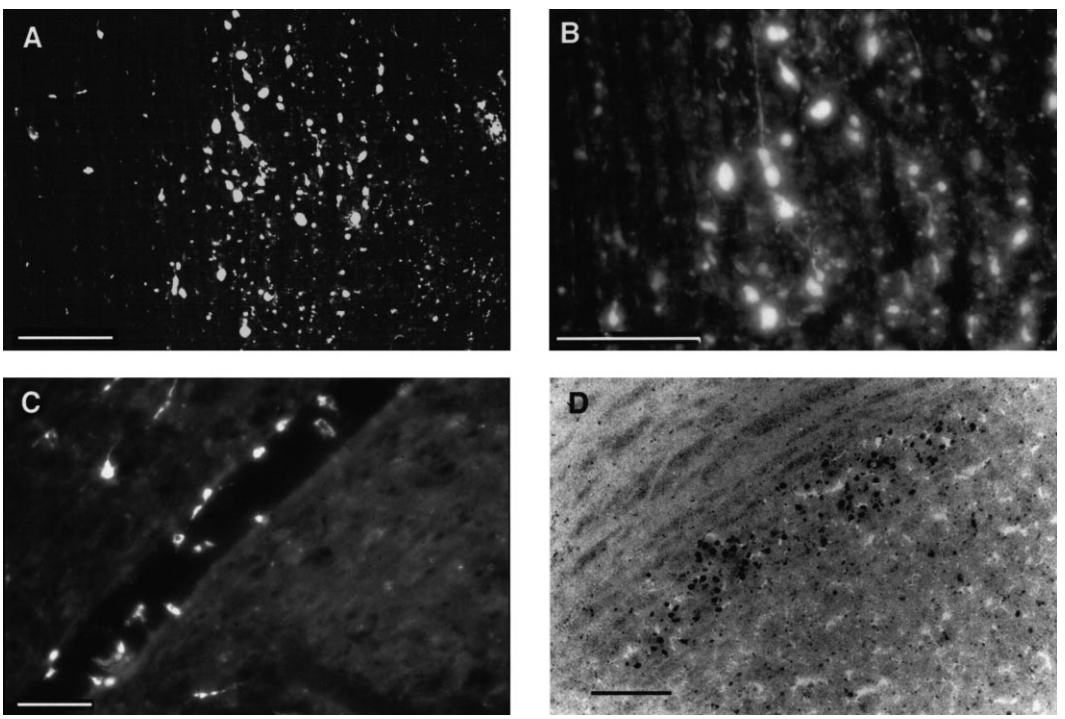


Fig. 1. A, B: Existence of fluorescently labeled microglia in the cerebellar parenchyma of the brain section from the rat at 48 h following intra-arterial injection with purified microglia. B indicates the higher magnification of the same field. C: Exogenous microglia that were fluorescently labeled were observed to adhere to a vessel in the brain section from the rat at 2 h following intra-arterial injection. Some microglia have crossed the vessel and have entered the parenchyma. D: Existence of lacZ-positive microglia in the cerebellar parenchyma of the brain section from the rat at 48 h following intra-arterial injection with Ra2 transfected with a lacZ gene expression vector stained by X-gal as a substrate for β-galactosidase. Bars indicate 100 μm in A and D, and 50 μm in B and C.

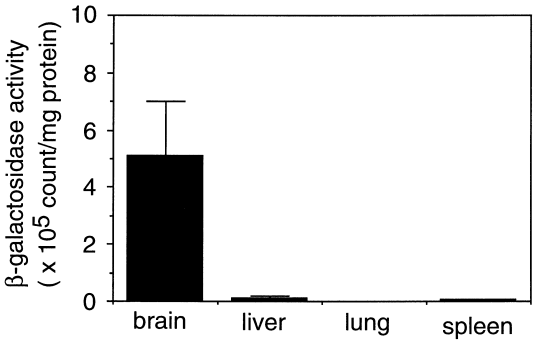


Fig. 2. Distribution of the activity of β-galactosidase derived from Ra2 cells expressing lacZ gene in tissue sections. The activity was determined using sections from brain, liver, lung and spleen isolated from the rats at 48 h following intra-arterial injection with $2\times10^6$ Ra2 cells. $n=3$.

β-galactosidase activity in a frozen section with about 8 μm thick of brain and other tissues. β-galactosidase activity in tissues derived from Ra2 cells at 48 h following intra-arterial injection was highest in the brain; over 30-fold than that in the liver and spleen and was not detected in the lung sections (Fig. 2). Therefore, we concluded that most of the injected Ra2 cells migrated to the brain. These results are consistent with our previous observation which shows that highly purified microglia from rat mixed brain cultures migrated specifically into the brain, but were rarely found in the liver [1]. On the other hand, purified macrophages migrated to liver but were not found in the brain of normal rats [15]. The data indicate that microglia have a characteristic differing from macrophages; the former have a specific affinity and migrating activity to the brain.

The stability of gene expression in brain was determined by measuring β-galactosidase activity in brain sections at 2, 9, 16 and 23 days after intra-arterial Ra2 injection. β-galactosidase activity in the brain sections was highest at 2 days and later gradually decreased (Fig. 3A). At 23 days the β-galactosidase activity was about a half of that at 2 days; product of the transferred gene was still active. Twenty three days after the injection, the fluorescent Ra2 was still present in the brain sections in a similar number to that of day 2, although the fluorescence intensity of Ra2 was much weaker than that of the day 2 brain section (Fig. 3B). Therefore, decrease of β-galactosidase activity was seemed to be due to decrease of the expression of lacZ gene in Ra2 cells because it was transiently transfected. This means that if Ra2 which expressed the gene permanently was injected, the genes of interest can be expressed in the brain for more than 20 days.

Many types of methods and techniques for in vivo gene transfer have been developed, and some of them have already been applied in clinical trials. The retroviral system, the most widely accepted gene transfer method to date, can achieve highly efficient integration, providing the potential for permanent gene expression. However, the system has some major disadvantages such as the typically low titer, instability of the viral vector obtained, and the requirement for target cell division for integration and expression [16]. The adenoviral system can provide more efficient gene transfer and stability of virus, however, the difficulties in control of target cells and of re-administrations necessitated by the strong antigenicity of the virus are serious problems [17]. The in vivo electroporation has been demonstrated in highly efficient gene transfer into the brain [18]. The systems using adeno-associated viruses and HIV-based vectors as potential vectors are also available for gene therapy. But all of these methods require a major surgical procedure to transfer cells carrying genes, or to insert a stainless steel electrode when they were attempted to apply for brain disorders.

We found that an intra-arterially injected microglial cell line migrated specifically into the brain. Using this cell line we established a technique to carry and express a recombinant gene specifically into the brain, and not to other tissues, using only a cannulation in the vertebral artery. With our method, no major surgical procedure is required. Since microglia are normally negative for MHC class I and II antigens in vivo, this brain-targeting gene delivery system can facilitate gene therapy of several brain disorders, including brain tumor, metabolic disorders, and degenerative disorders, as well as investigations into the roles of particular genes in brain function and development.

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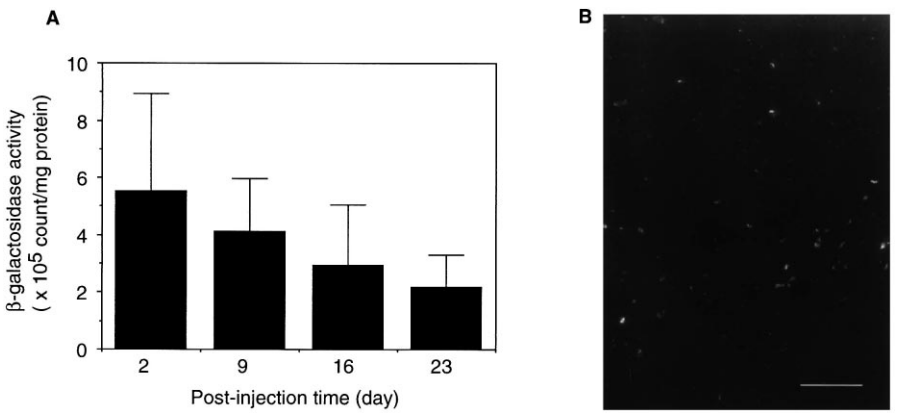


Fig. 3. A: Time-dependent change of the activity of β-galactosidase derived from Ra2 cells expressing lacZ gene in brain sections. The activity was determined using sections from brains isolated from the rats at 2, 9, 16 and 23 days following intra-arterial injection with $2\times10^6$ Ra2 cells. $n=3$. B: Existence of fluorescently labeled Ra2 cells in the cerebellar parenchyma of the brain from the rat at 23 days following intra-arterial injection with $2\times10^6$ Ra2 cells. Bars indicate 100 μm.

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