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Transformation of resident microglia into phagocytic cells in the transected and cycloheximide-treated rat facial nucleus

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ABSTRACT

We analyzed the ability of microglia to transform into phagocytic cells in the rat facial nucleus by administering the protein synthesis inhibitor cycloheximide at the transected facial nerve. Immunoblotting revealed that a phagocytic marker, the cluster of differentiation 68 (CD68) protein, was induced in the transected and cycloheximide-treated facial nucleus on day 5 post-insult, but not significantly induced in either the normal facial nucleus or simply transected facial nucleus. The time-course experiment indicated that the levels of CD68 protein peaked 5-7 days after transection and cycloheximide-administration. Avidin-biotin peroxidase complex staining revealed that the CD68-expressing cells appeared at the transected and cycloheximide-treated facial nucleus, and were located in the vicinity of motoneuron cell bodies. Fluorescent double-staining revealed that the CD68-expressing cells were almost positive for ionized Ca^{2+}-binding adapter molecule 1 (a microglial marker). Collectively, these results demonstrated that the resident microglia can transform into phagocytic cells in response to injury and cycloheximide-treatment of motoneurons in the facial nucleus.

KEYWORDS: facial nucleus, axotomy, motoneurons, cycloheximide, phagocytes

ABBREVIATIONS

ABC, avidin-biotin peroxidase complex; CD68, cluster of differentiation 68; CH, cycloheximide; ChAT, choline acetyltransferase; HRP, horseradish peroxidase; Iba1, ionized Ca^{2+} binding adapter molecule 1; m2MAchR, m2 muscarinic acetylcholine receptor; VAchT, vesicular acetylcholine transporter.

INTRODUCTION

Although microglia have traditionally been regarded as “macrophages” in the brain [1-3], they do not always act as macrophages. Indeed, in the normal adult nervous system, resident microglia occur in a ramified form and do not express macrophage features [4, 5]. However, in the presence of dead or dying parenchymal neurons, the microglia are generally thought to transform into macrophages and serve as phagocytes to remove the dead cells [2, 3, 6]. In fact, microglia-derived phagocytes have been definitively observed in a model of lethal motoneuron injury in which virulent ricin was applied to facial nerves [7-9]. In such cases, the motoneurons are thought to undergo toxic damage and release “find me” and “eat me” signals [6] to which resident microglia respond and change into phagocytes. However, it has not been clearly determined whether a toxin-based model of lethal motoneuron injury is sufficient in itself to transform microglia into phagocytic cells. Alternatively, metabolic inhibitors may be expected to induce similar effects. To examine these factors, we administered the protein synthesis inhibitor cycloheximide (CH) at the injury site of transected rat facial nerves and analyzed the transformation of resident microglia into phagocytic cells in the ipsilateral facial nucleus.

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MATERIALS AND METHODS

Reagents and antibodies

Cycloheximide (CH) was obtained from MERK Biosciences (Tokyo). Ethanol was supplied by Kanto Chemicals (Tokyo). Anti-cluster of differentiation 68 (CD68), anti-choline acetyltransferase (ChAT) antibody, anti-vesicular acetylcholine transporter (VAchT) and anti-m2 muscarinic acetylcholine receptor (m2MAchR) antibody were purchased from Millipore (Bedford, MA). Anti-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against ionized Ca^{2+} binding adapter molecule 1 (Iba1) was purchased from Wako Pure Chemical Industries (Osaka).

Horseradish peroxidase (HRP)-conjugated anti-mouse IgG, HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-goat IgG were purchased from Santa Cruz Biotechnology. The Vectastain avidin-biotin peroxidase complex (ABC) kit was purchased from Vector Laboratories (Burlingame, CA). Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 568-conjugated anti-rabbit IgG were obtained from Invitrogen (Carlsbad, CA).

Animals and the operation procedure

Male Wister rats (8 weeks old) were obtained from Clea Japan (Tokyo) and kept on a 12-h daylight cycle with food and water. Animal experiments were carried out in accordance with the guidelines laid down by the NIH regarding the care and use of animals, and approved by the ethics committee of Soka University.

Right facial nerves of adult rats were transected at the stylomastoid foramen under diethylether anesthesia, as reported previously [10, 11]. A piece of gel foam (2 mm^3; Pfizer, Berlin) soaked in 15 μL of 0.25 M CH/ethanol solution was then placed at the transection site. As a control, gel foam soaked in 15 μL of ethanol (vehicle) was placed at the cut nerve. The rats were reared for 1, 3, 5, 7 or 14 days and decapitated under anesthesia. The whole brains were removed, frozen on dry ice, and stored at -80°C until use.

Immunoblotting

The contralateral and ipsilateral facial nuclei were carefully cut out from the frozen brainstem [12]. The cut facial nuclei were solubilized with non-reducing sample buffer and centrifuged at 100,000 g for 30 min. The supernatant of each facial nucleus was recovered as tissue extract and subjected to immunoblotting for the detection of CD68 (1:100), Iba1 (1:1000), actin (1:1000), ChAT (1:1000), VAchT (1:1000) and m2MAchR (1:1000). The staining methods have been reported previously [12].

Immunohistochemistry

The brainstem was cut into 20-μm-thick sections with a cryostat (Leica CM1510; Leica Biosystems, Nussloch, Germany) at the level of the facial nuclei. These sections were fixed in 3.7% paraformaldehyde in phosphate buffered solution and treated sequentially with 50%, 100% and 50% acetone for 2, 3, and 2 min, respectively. The sections were then blocked with a blocking solution containing 2% skim milk.

For the ABC staining method, the cryosections were incubated with anti-CD68 antibody (1:100) at 4 °C overnight and then with biotinylated secondary antibody (1:200) for 1 h. The antigens on the sections were finally detected by adding 0.001% H_2O_2 and 0.08 mM diaminobenzidine as reported previously [12].

For dual fluorescent staining, the cryosections were first incubated with anti-CD68 antibody (1:100) for 16 h and then with anti-Iba1 antibody (1:100) for 16 h at 4 °C. Subsequently, these sections were incubated with Alexa Fluor 488-conjugated anti-mouse IgG (1:100) and Alexa Fluor 568-conjugated anti-rabbit IgG (1:200) for 3 h at room temperature. The stained sections were dehydrated and mounted, and observed under biological microscope or fluorescent microscope.

Statistical analysis

The densities of CD68 protein bands in the immunoblotting were measured by densitometry using ImageJ software (NIH, Bethesda, MD) and expressed as the means ± SDs of three separate experiments. The value relative to that in the transected and CH-administered facial nucleus (defined as 1.0) was assessed via Student's *t*-test. P values less than 0.01 were considered to be significant.

RESULTS

Effect of cycloheximide administration to the facial nerve

To induce metabolic damage in the motoneurons injured by transection, a protein synthesis inhibitor CH [13] was administered to the transected rat facial nerve, and then the emergence of phagocytic cells was examined in the ipsilateral facial nucleus by monitoring the levels of CD68 protein, a lysosomal membrane protein [14]. Immunoblotting showed that the CD68 protein was not expressed either in the normal facial nucleus (Fig. 1A, L and R in case 1, L in cases 2-3) or in the transected facial nucleus (Fig. 1A, R_{O+E} in case 2) on day 5 post-insult. In contrast, the CD68 protein was unambiguously detected in the transected and CH-treated facial nucleus on day 5 post-insult (Fig. 1A, R_{O+C} in case 3). Iba1 increased in the transected facial nucleus (Fig. 1A, R_{O+E} in case 2) and in the transected and CH-treated nucleus (Fig. 1A, R_{O+C} in case 3), suggesting that the microglia were activated and/or proliferating in the ipsilateral nucleus in both rats.

The CD68 protein was induced in the three transected and CH-treated facial nuclei along with similar expressions of actin (Fig. 1B, left; R_{O+C}). The quantification of the results of immunoblotting indicated that the CD68 protein was significantly induced in the ipsilateral nucleus (R_{O+C}) on day 5 post-insult and CH-administration (Fig. 1B, right). These results suggested that the resident microglia express phagocytic properties in response to CH treatment of transection-injured motoneurons.

In case 3, we investigated the changes in injured and CH-treated motoneurons. The levels of ChAT, VAchT and m2MAchR as functional markers of motoneurons were remarkably down-regulated in the ipsilateral nucleus (Fig. 1C). There was no significant difference in the levels of actin between the contralateral and ipsilateral facial nuclei (Fig. 1C). These results indicated that facial motoneurons down-regulate their function, probably due to degeneration.

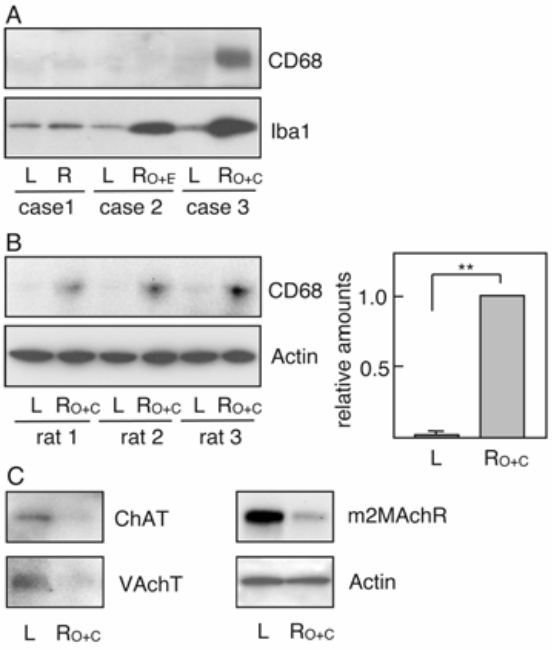

Fig. 1. Alterations in the transected and CH-treated facial nucleus.
A. Induction of CD68: Three rats were used in this experiment. The first rat was not subjected to treatment of either the left (L) or right (R) facial nerves and served as a control (case 1). In the second rat (case 2), the right facial nerve was transected and a piece of gel foam soaked in ethanol (vehicle) was applied (R_{O+E}), while the left facial nerve was untreated (L). The right facial nerve of the third rat (case 3) was transected, and a gel foam soaked in CH/ethanol was applied at the site of injury (R_{O+C}), while the left one was untreated (L). Five days later, these rats were decapitated, and each facial nucleus in each rat was removed. The facial nucleus samples in three cases were immunoblotted for CD68 and Iba1. **B.** Reproducibility of CH effects: The experiment with case 3 in A was carried out by using three rats. Five days later, each facial nucleus of each rat was simultaneously immunoblotted for the detection of CD68 and actin (left panel). The density of the CD68 protein bands was determined by densitometry and quantified. Data are expressed as values relative to that of the transected and CH-administered nucleus (defined as 1.0). The results are means ± SDs (**P < 0.01; right panel). **C.** Alterations in motoneurons: The samples of case 3 in A were immunoblotted for the detection of ChAT, VAchT, m2MAchR, and actin. The results shown are representative.

Time course of the CD68 protein induction

We next investigated the time course of CD68 protein induction in the transected and CH-treated facial nucleus. The CD68 protein was not detected on day 3, after transection and CH-treatment (Fig. 2A). The levels of CD68 appeared to reach a maximum at 5-7 days, and thereafter decreased (Fig. 2A). In the same samples, Iba1 levels were

enhanced in the transected and CH-treated nucleus (R_{O+C}) during the period of 5-14 days (Fig. 2A). The actin levels were almost the same in both nuclei throughout the period of 1-14 days (Fig. 2A).

To confirm the maximum point of CD68 induction, the time course of the changes in protein levels was statistically analyzed. The results indicated that the CD68 levels peaked 5-7 days after injury and CH-treatment (Fig. 2B). There was no significant difference between the level on day 5 and that on day 7.

Immunohistochemical observation of CD68

To identify the cells inducing CD68 protein in the facial nucleus, the brainstem on day 5 post-insult and CH-treatment was immunohistochemically investigated using anti-CD68 antibody. The ABC-staining images indicated that the CD68 protein was hardly expressed in the left nucleus of the control (Fig. 3A, L), but was expressed to a significant degree in the ipsilateral nucleus (Fig. 3A, R_{O+C}), as expected from the results of Fig. 1 and Fig. 2. In the magnified photo, the CD68-expressing cells appear as globular/oval shapes (Fig. 3B). Characteristically, some motoneuron cell bodies appeared to be enclosed by such CD68-expressing cells.

The dual fluorescent staining revealed that the CD68-expressing cells were almost Iba1-positive (Fig. 3C, D), indicating that the phagocytic cells came from the resident microglia in the ipsilateral nucleus.

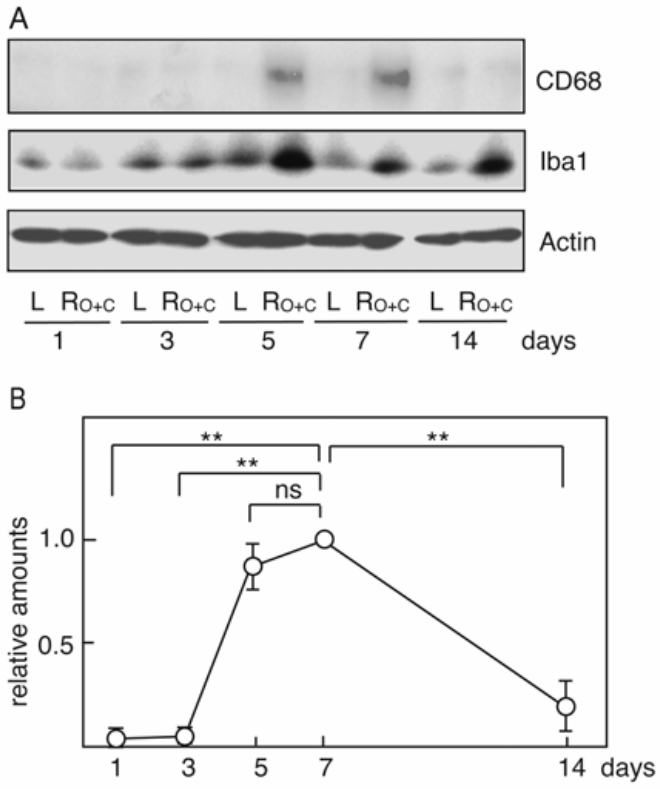

Fig. 2. Time course of the CD68 induction.
A. Induction of CD68: Five rats whose right facial nerves were transected and treated with CH at the cut sites were decapitated on day 1, 3, 5, 7 and 14 post-operation and CH-administration, respectively. The control facial nucleus (L) and CH-treated facial nucleus (R_{O+C}) of each rat were analyzed for CD68, Iba1 and actin by immunoblotting. The results shown are typical. **B.** Statistical analysis of CD68: The intensity of CD68 bands of R_{O+C} in A was quantified by a densitometer, and the results are shown as the means ± SDs from three separate experiments. The value was normalized against the value of R_{O+C} on day 7 (defined as 1.0). **P < 0.01; ns: not significant.

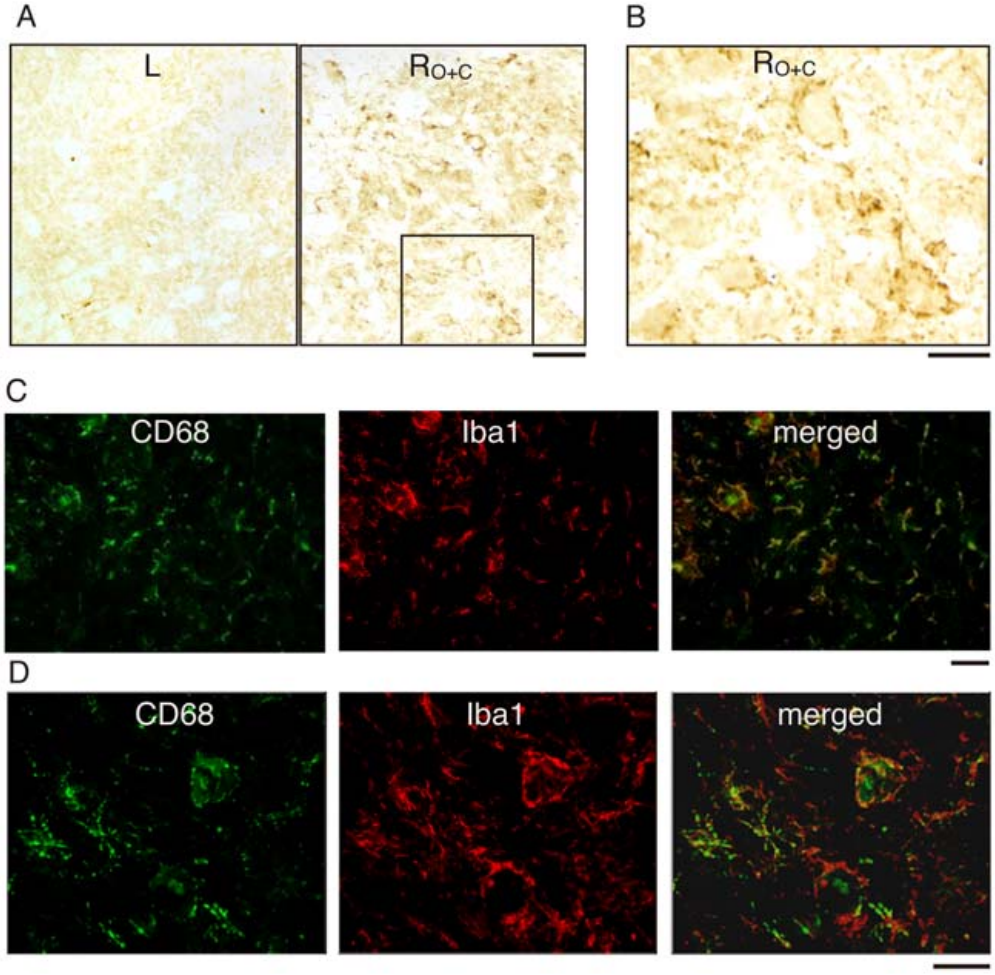

Fig. 3. Immunohistochemistry for CD68.
A. CD68 staining by the ABC method: Cryosections of brainstem obtained on day 5 post-operation and CH-administration were stained for CD68 by the ABC method, as described in the Materials and Methods section. An untreated left facial nucleus (L), and transected and CH-treated nucleus (R_{O+C}) are shown. Scale bar = 100 μm. **B.** Magnified image of R_{O+C}: A square in R_{O+C} (in A) was magnified. Scale bar = 50 μm. **C and D:** CD68 staining by the double-fluorescent method. The same brainstem sections as in panel B were dually stained with anti-CD68 antibody (green) and anti-Iba1 antibody (red) as described in the Materials and Methods section. Scale bar = 50 μm.

DISCUSSION

The adult rat facial nerve injury model is considered to be advantageous for analyzing the intercellular interactions between injured neurons and surrounding glial cells, since the nerve injury is carried out far from the nucleus where the motoneuron cell bodies exist, and the injury does not affect the normal blood brain barrier [5, 11]. Thus, this system was applied for analyzing the transformation of microglia to phagocytic cells in the present study.

To prepare a model in which motoneurons suffer from metabolic stress, the antibiotic CH was selected. CH can inhibit protein synthesis, specifically in the translocation step in eukaryotes [13]. Like ricin, this reagent is known to cause DNA fragmentation in T blasts [15]. Furthermore, CH promotes the apoptosis of leukemic HL-60 cells *in vitro* [16] and hepatocytes in rats [17] and enhances TRAIL/Apo-2L-dependent apoptosis of glioma cells [18]. Thus, these properties of CH were expected to damage motoneurons in the ipsilateral facial nucleus.

The phagocytic ability was monitored by measuring the expression of the CD68 protein. Since CD68 is a highly glycosylated membrane protein and belongs to a family of lysosomal glycoprotein/plasma membrane shuttling proteins that play a role in lysosomal trafficking and/or endocytosis [14, 19], its expression indicates the formation of phagosomes/lysosomes essential for degrading waste materials and endocytosed materials in the cells. Using an antibody against CD68, we have previously shown that phagocytic microglia clear away dead motoneurons in the transected neonatal rat facial nucleus [11]. Thus, the CD68 protein can be considered an appropriate phagocytic marker *in vivo*.

In this study, we showed that CD68-expressing cells emerge in the transected and CH-treated facial nucleus (Figs. 1-3). CH administration was crucial for inducing CD68 protein, while nerve-cutting alone was not sufficient for the induction, which is in good agreement with a previous report [4]. CH was found to induce motoneuronal damage by arresting the protein synthesis.

A possible explanation for the activities observed in the ipsilateral facial nucleus is as follows. When CH was added to cut nerves at the stylomastoid foramen, it would have been transported by reverse axonal transport to the motoneuronal cell bodies, where it would act as an inhibitor of protein synthesis. This inhibition would severely stress the motoneurons, in some cases causing them to undergo degeneration. The degenerating cells would then release specific signals known as “find me” and “eat me” signals [6]. After sensing the “find me” and “eat me” signals by means of various receptors, the resident microglia would transform into phagocytic cells, inducing the production of CD68 protein after 5-7 days (Fig. 2A). Then, after serving as phagocytic cells, the microglia would gradually return to acting as non-phagocytic cells by the end of 14 days.

Generally, chemokines, including fractalkine [20] and nucleotides such as uridine diphosphate (UDP) [21] are classified as “find me” signals, while phosphatidylserine is considered the main “eat me” signal [22-24]. These molecules could be released from the injured and CH-stressed motoneurons. ATP/ADP [25] are also released from injured neurons and act as possible stimulators of microglia. Thus, all these neuron-derived molecules might be associated with the transformation of microglia to phagocytic cells in the facial nucleus.

CONCLUSION

In conclusion, the resident microglia in the rat facial nucleus were found to have the ability to transform to phagocytic cells in response to CH-stressed motoneurons. This also indicates that, as long as the facial nucleus is not injured and/or stressed, the resident microglia will conceal their phagocytic ability.

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CONFLICT OF INTEREST STATEMENT

None of the authors has any conflict of interest to declare.

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