Original Article

SDF1/CXCR4 contributes to neural regeneration in hemiplegic mice with a monkey ES-cell-derived neural graft

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We induced neural cells by treating cynomolgus monkey embryonic stem cells with retinoic acid. The retinoic acid-treated cells had elongated axons and expressed βIII tubulin, neurofilament middle chain (NFM) and Islet1 *in vitro*, suggesting their differentiation into motoneurons. The monkey ES derived neural cells were transplanted to hemiplegic mice with experimental brain injury. Injured mice with the neural cell graft gradually recovered motor function, whereas injured mice with vehicle (PBS) injection and injured mice with undifferentiated monkey ES cell graft remained hemiplegic. After transplantation into hemiplegic mice, the neural cells that had grafted into the periventricular area migrated and located near the corpus callosum by day 7. The neural cells distributed over the injured cortex at day 21. The cells expressed CXCR4, a receptor for chemokine SDF1. In a microchemotaxis assay, the neural cells responded to SDF1, and AMD3100, an antagonist of CXCR4, abrogated their migration. The injured cortex initially produced SDF1, and the graft expressed CXCR4 in the brain. SDF1 accelerated NCAM mRNA expression in the neural cells *in vitro*. The neural cells distributed over the cortex expressed L1CAM, NCAM, and N-Cadherin extensively after reaching the injured cortex. Administration of AMD3100 forced the graft to stay at the injection site. Thus, chemokine, chemokine receptor, and neural cell adhesion molecules seem to be involved in the regeneration of neural networks and functional recovery of hemiplegic mice.

Rec.10/2/2009, Acc.11/30/2009, pp193-205

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Key words hemiplegia, neural differentiation, nonhuman primate embryonic stem cells, SDF1, CXCR4

Introduction

Neural cell transplantation has received broad attention as a new strategy for treating neurodegenerative diseases and brain damage[1]. Cell sources for neural cell transplantation are rather limited. Embryonic stem (ES) cells have been shown to differentiate into neurons, holding out the hope of reversing neurodegeneration associated with many brain disorders[2-5].

We reported previously that retinoic acid (RA) treatment induced predominantly neurons, especially motoneurons and small numbers of glial cells from mouse and monkey ES cells[6-9]. We have reported functional recovery of hemiplegic mice by transplantation of neural cells derived from mouse and primate ES cells[6-9]. For functional repair, grafted neural progenitors must migrate to the regions of brain injury, differentiate into cells with the correct phenotype, and integrate appropriately with the regenerating neuronal circuits[1,10]. However, how the regeneration process proceeds is poorly understood. It has been demonstrated that neural progenitors transplanted into the brain migrate toward either localized or diffuse areas of brain damage[2,11,12]. These observations suggest that factors associated with damaged areas of the brain can direct the migration of progenitors.

Chemokines play fundamental roles in the control of leukocyte development and migration[13,14], and an important part in the development of CNS[13,14]. Stromal cell-derived factor-1 (SDF1) and its unique receptor, chemokine (CXC motif) receptor 4 (CXCR4), are important in this regard[15]. Mice that lack either CXCR4 receptor or SDF1 show abnormal development of the CNS[16-18]. In both cases, it appears that SDF1 acts as an important chemoattractant for neural cells[16,17,19,20].

Here, we demonstrate that CXCR4 is expressed on migrating neural stem/progenitor cells derived from RA treated monkey ES cells and that glial cells in the damaged brain produce SDF1 that acts as a chemoattractant for these cells. Disruption of CXCR4 signaling by AMD3100, an antagonist of the binding of SDF1 to CXCR4[21,22], produces inhibition of migration activity of the grafted neural stem/progenitor cells. Brief treatment of the neural stem/progenitor cells with SDF1 induced NCAM expression, which plays an important role in axonal regeneration. Our study suggested an important role for chemokine signaling in the regeneration of the damaged brain in the case of monkey ES-cell-derived neural stem/progenitor cell transplantation.

Materials and Methods

1) Induction of neural differentiation of cynomolgus monkey ES cells by RA

The cynomolgus monkey ES cell line CMK 6.4-6 (Passage

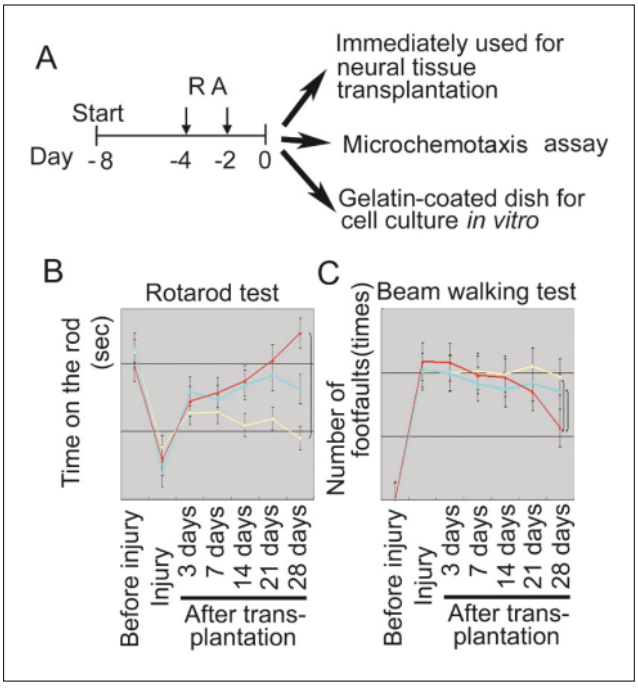

Fig.1 Transplantation of the cynomolgus monkey ES-cell-derived neural cells brought about recovery of motor function in mice with experimental brain injury

(A): Schedule for induction of neural stem/progenitor cells from cynomolgus monkey ES cells and their transplantation. Undifferentiated ES cells (day -8) were cultured in a floating fashion for 4 days (this culture terminated at day -4). RA (1 μM) was added twice. RA treated cells (1 x 10^5) at day 0 were transplanted into the periventricular region underneath the damaged motor cortex. Immunosuppressants were given to the recipients from the day of transplantation to that of sacrifice. RT-PCR was conducted using mRNAs from the RA treated cells on day 0. The motor cortex of normal mice was damaged at day -7. The mice were transplanted with either the graft cells or vehicle at day 0. Motor function was evaluated by beam walking and rotarod tests. In the experiments, RA treated neural cells, undifferentiated ES cells, or vehicle (PBS) were injected. Data shown are the mean ± SD at each point after injection.

(B): The scores of the rotarod test were significantly improved in the neural cell transplanted mice (n=18) as compared with vehicle treated mice (n=18) (* in the rotarod test; day 21-day 28; $p<0.05$).

(C) The numbers of footfaults in the beam walking test were significantly decreased in mice transplanted with the neural cells (n=18) as compared with vehicle (PBS) treated mice (n=18) after day 21 (* in the beam walking test; day 21-day 28, $p<0.01$). We have conducted the experiments three times and representative results are presented. Yellow, vehicle injection (n=18); Red, neural cell transplantation (n=18); Blue, undifferentiated ES cell transplantation (n=18).

number 53-99, normal karyotype) was used in this study[9,23]. In the transplantation experiments, both unlabeled and GFP labeled ES cells were used[9,23,24].

We induced neural differentiation of the ES cells in the presence of all-*trans* RA (Sigma, Tokyo, Japan)[9]. In brief, the ES cells in the maintenance culture were recovered and cultured in DMEM/F12 supplemented with 20% knockout serum replacement (day -8). Four days later, floating cell aggregates, embryoid bodies (EB), were transferred to fresh petri dishes to which 1 μM RA was added twice at day -4 and day -2. For transplantation, the EB was recovered at day 0 (totally cultured for 8 days *in vitro*) and a single cell suspension of the EB was immediately used as a graft (Fig.1A)[9]. It contained feeder cells less than 0.01%.

(1)RT-PCR

The total RNA extraction and cDNA synthesis methods have been reported previously[9]. Because sequence data of cynomolgus monkey mRNAs of the following markers were not available, we designed primers according to the human sequences reported previously[9]. PCR primers of human chemokine receptors including CXCR4, CCR2a, CX3CR1, CCR8, CXCR3, CCR3, CXCR1, CXCR2, and CCR1 were purchased from Maxim Biotech. Inc. (South San Francisco, CA, USA). In the kits, positive control DNA was provided by the manufacturer. Diethylpyrocarbonate (DEPC)-treated water served as a negative control.

2) Experimental brain injury by cryogenic injury and transplantation

Female C57BL/6 mice (6-8 weeks old) were used as transplant recipients[6-9]. This study was approved by the Institutional Review Board of Animal Experiments and all subsequent procedures were conducted along with the Institutional and National Institutes of Health guidelines. Anesthesia with sevoflurane was used. Mice were placed in a stereotaxic frame (Narishige, Tokyo, Japan). The burr hole mark was made in the left parietal bone at the midpoint between the coronal and lambdoid sutures and 3.0 mm lateral to the sagittal suture. A metal probe chilled with liquid nitrogen was applied to the surface of the intact burr hole marks by force of 100 g for 30 sec four times.

When mice were exposed to cold injury three times or less (compression of the metal probe treated with liquid nitrogen for 30 seconds three times or less), development of motor deficits varied considerably among the mice; 20 % of the mice showed spontaneous recovery and the remaining 80 % developed persistent hemiplegia. However, when mice were given cryogenic injury four times, almost all the mice showed complete hemiplegia; less than 1 % showed spontaneous recovery at day 2 and less than 5 % showed spontaneous recovery at day 5 after the injury (please see below). Thus, we can reliably reproduce hemiplegia by administering cold injury four times in each mouse[25-27].

Two and 5 days later, the mice were tested their motor function, and those that showed good functional recovery were excluded from the study cohort, because of insufficient cryoinjury. At day 7 after the cryogenic injury, the mice were randomly separated into two groups; neural cell recipients and control recipients. 5 μl of cell suspension (1.0×10^5 cells) or vehicle (PBS) were implanted into the ipsilateral motor cortex through center of the burr hole and -2.0 mm ventral to the dura with a 5 μl hamilton syringe attached to a 26s gauge needle. Because our previous experiments demonstrated that the transplantation of neural cells to the injured cortex was not appropriate for survival of the grafted cells, they were injected to the periventricular area underneath the injured cortex of left hemisphere[6]. In some experiments, dead graft cells were used as control. The RA treated EB at day 8 was fixed with 3.7 % formaldehyde for 10 min, washed extensively with PBS, and 1.0×10^5 dead graft cells were transplanted. The mice did not show functional recovery[6,9]. When we used undifferentiated mouse ES cells as a graft, the mice did not show their functional recovery[6,9]. Nonetheless we included undifferentiated cynomolgus monkey ES cells as a graft (RA-cell: n=11, undifferentiated ES cell: n=6, PBS: n=8). The cellular suspension was infused in 1 μl increment over 2 min, 2 min for the final injection pressure to equilibrate before slowly withdrawing the injection needle. In preliminary experiments, we found that transplantation of cynomolgus monkey ES derived cells to the mice was unsuccessful without immunosuppression. Therefore, we used 10 μg/g cyclosporine (subcutaneous injection) and 0.2 μg/g dexamethazone (intraperitoneal injection) one hour before the transplantation. From the next day of the transplantation, 10 μg/g cyclosporine was given each day until the mouse was sacrificed. The control mice were also treated with immunosuppressants as mice with transplantation to avoid the direct influence of these reagents on neural protection.

To elucidate the possible role of SDF1/CXCR4 signaling in grafted cell migration, the mice was treated with subcutaneous administration of 1.25 μg/g AMD3100 (Sigma), a CXCR4 antagonist, twice a day[21,22].

3) Motor function analyses: Beam Walking test

The beam walking test allows the assessment of refined forelimb and hindlimb locomotor activity, and is used to assess the

recovery of hemiplegic model of brain injury[28-31]. The animal was trained to walk along a narrow wooden beam 6 mm wide and 120 mm in length, suspended 300 mm above a 100 mm soft pad, and the number of footfaults for contralateral (right) hindlimb recorded over 50 steps. Foot faults were directly observed in accordance with gait disturbance and were counted. Normal mice grasped the beam completely on every foot, and the foot fault was defined as that without complete grasp of the beam. A basal level of competence at this test was established before injury with acceptance level of < 5 faults per 50 steps.

4) Rotarod test

The rotarod test allows the assessment of refined motor function and coordination, and is used to assess the recovery of hemiplegic model of brain injury[29-33]. The rotarod unit (Muromachi, Tokyo, Japan) consists of a rotating rod of 3.5 cm diameter and individual compartment for each mouse. Infrared beams were used to detect when a mouse has fallen onto the grid beneath the rotarod. Before brain injury, mice have trained on the rolling rod and a basal level of competence at this test was established with acceptance level for > 200 sec at the speed of 20 rpm. After injury, the mice were placed on the rod, and then run on the rolling rod at 30 rpm for the maximum of 300 sec. The system logs the total time running on the rod, as well as the time of the fall and all experimental set up parameters are recorded. The mice were given 5 min interval for helping to reduce stress and fatigue. Each animal received at least two consecutive trials, the longest time on the rod being used for analysis.

5) Immunofluorescence staining

Immunofluorescence staining was conducted as reported previously[6-9]. In brief, for all immunofluorescence procedures, adjacent sections served as negative controls and were processed using identical procedures, except for incubation without the primary antibody in each case. The sections were incubated overnight with appropriate primary antibodies, then with biotinylated second antibody (DakoCytomation, Kyoto, Japan), and finally with Alexa488-conjugated streptavidin (Molecular Probes, Eugene, OR, USA) and Cy3-conjugated streptavidin (Jackson Immuno Research, West Grove, PA, USA).

The primary antibodies included anti-human NFM (Chemicon), anti-mouse GFAP (Dako), and anti-human nuclear protein (Chemicon), anti- human L1CAM (BD Pharmingen, San Diego, CA, USA), anti- human N-Cadherin (NCAD)(QED Bioscience, San Diego, CA, USA), anti-human NCAM (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse SDF1 (R & D systems, Minneapolis, MN, USA), goat anti- human CXCR4 (Novus Biological, Littleton, CO, USA), anti-βIII tubulin (Promega, Madison, WI), anti-Islet1 (Hybridoma bank, Iowa City, IA) antibody. When the primary antibody was a mouse monoclonal antibody, an M.O.M.TM Kit was used. Fluorescence was recorded with a confocal laser microscope (Carl Zeiss, Jena, Germany). Appropriate control antibodies were included in all experiments. Anti-human nuclear protein antibody specifically reacts with cynomolgus monkey cells but not with mouse cells. In some experiments, we used immunohistochemical staining as previously reported[9].

We counted 100 cells each of 5 different high power filds (totally 500 cells), and caliculated percent positive cells of the relevant immune- staining.

6) Microchemotaxis assay and chemotaxis

To assess the chemotaxis in response to SDF1 (R & D systems), we cultured monkey ES-cell-derived neural cells within a microchemotaxis assay. The assay contained lower and upper chambers separated by a 5 μm polycarbonate membrane (Whatman, Tokyo, Japan) that allowed for the generation of a chemotactic gradient and the observation of cell migration in the context of the gradient. Neural cells were dissociated and then placed into the lower chamber. Using a small needle, media alone or media containing SDF1 (1 ng/ml, 10 ng/ml, or 100 ng/ml; the three different concentrations of SDF1 gave almost similar results in the assays, thus, results obtained at 10 ng/ml were presented in Fig.2E) or other chemokines was gently dispersed into the upper chamber, making sure that no bubbles were formed. The cells were then cultured in an incubator set at 37℃ for 90 min to allow cells to migrate and adhere to the membrane. Subsequently, the membrane was washed extensively and the cells adhering to the membrane were stained with Diff-quick solution. The membrane was placed in an upright microscope and cell counts were made using the 20 x objective lens.

7) Statistical analysis

Continuous variables subjected to repeated measurements over a period of time (Beam Walking and Rotarod tests) were analyzed using a repeated measurements analysis of variance (ANOVA) followed by Tukey's pairwise comparison at each time point[6-9].

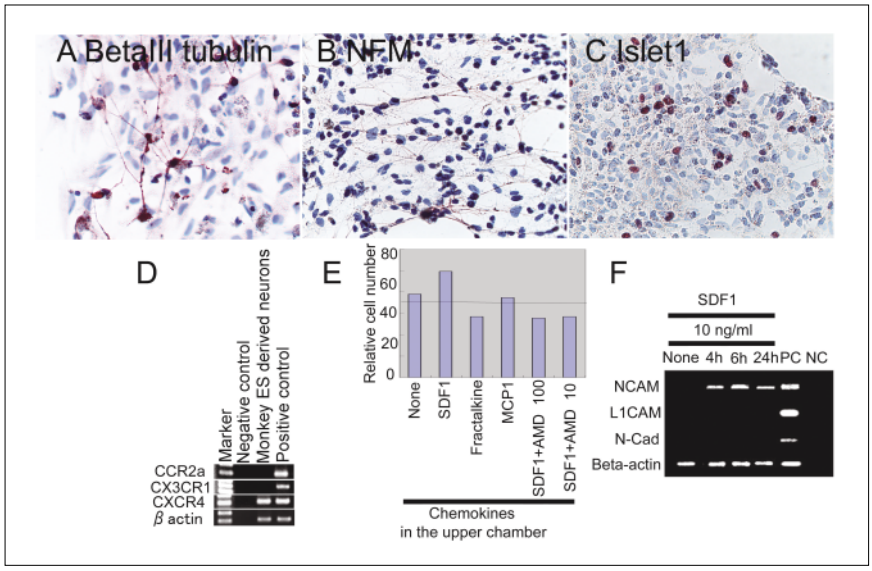

Fig.2 Expressions of neural cell antigens and chemokine receptors in neural stem/progenitor cells derived from RA treated cynomolgus monkey ES cells and their *in vitro* migration in response to chemokines in a microchemotaxis assay

(A,B,C): The cells were cultured on the 8 well chamber slides. After fixation, the cells were stained with the indicated antibody. Brown color indicates immunopositive cells and blue color represents counter staining for nuclei with hematoxyline

(A): Some of the RA treated cells derived from cynomolgus monkey ES cells at day 8 were βIII tubulin positive with axon like processes. x 400.

(B): The cells at day 14 had axons expressing NFM. x 400.

(C): Nuclei of the cells at day 14 were Islet1 positive.

(D): mRNA expression of several chemokine receptors in the RA treated cells was determined by RT-PCR. The RA treated neural cells derived from ES cells at day 0 were used as the starting material. The RA treated cells at day 0 (cultured for 4 days without RA, followed by 4 days culture with RA) that was used for transplantation expressed CXCR4.

(E): SDF1 is a chemoattractant for neural cells migrating in the microchemotaxis assay. The RA treated neural cells derived from cynomolgus monkey ES cells at day 0 (1 x 10^4 cells) were cultured in the lower chamber. To the upper chambers, several chemokines (10 ng/ml) and inhibitors were introduced. After 90 min culture at 37 ℃, the membrane separating the upper and lower chambers was recovered and washed extensively. The membrane was stained with Diff-quick solution, and the cells that migrated and adhered to the membrane were counted under the microscope. AMD3100 (100 ng/ml and 10 ng/ml), a specific inhibitor of CXCR4, inhibited migration of the neural cells efficiently.

(F): Induction of neural cell adhesion molecule expression by neural stem/progenitor cells after a brief treatment with SDF1 *in vitro*. The RA treated cells derived from ES cells at day 0 (1 x 10^5 cells) were cultured with SDF1 10 ng/ml. After the culture, the cells were recovered and mRNA expressions were studied by RT-PCR. NCAM mRNA was detected after as little as 4 hours of culture.

Results

1) Improved motor functions of hemiplegic mice with experimental brain injury after transplantation of the neural cells derived from cynomolgus monkey ES cells

To confirm our previous findings, we studied again whether the neural cells induced from cynomolgus monkey ES cells were functionally relevant for use as a graft[9]. We confirmed again the recovery of motor functions of the transplanted mice by beam walking and rotarod tests (Fig.1B,C). There was spontaneous recovery in beam walking test over time in the PBS treated group. It was probably due to the training and learning by repeated tests. Thus, the training and learning effect may be seen in the groups with undifferentiated monkey ES cell graft and with neural cell graft. However, the statistical difference was significant between

the PBS treated group and the group with neural cell graft. The mice transplanted with undifferentiated monkey ES cells frequently developed tumors in the injection site. Thus, further study of the mice was given up.

2) Expression of chemokine receptors in neural cells derived from RA treated cynomolgus monkey ES cells

We previously found that RA was most suitable for inducing differentiation of monkey ES cells into neurons, especially motoneurons, with high cell viability *in vitro*[9]. The grafted cell preparation contained cells expressing nestin, which shared characteristics with neural stem/progenitor cells[9]. The cell preparation contained cells having axon like processes (Fig.2A). The cells expressed βIII tubulin, NFM and Islet1 (Fig.2B,C). The percentage of βIII tubulin positive neurons was 35% 8 days after cell culture and NFM and Islet1 positive neurons was 65% and 50 % 14 days after cell culture (data shown were a representative of three independent experiments).

We previously found directional migration of neural cells derived from RA treated cynomolgus monkey ES cells grafted into the damaged brain[9]. To elucidate the migratory mechanisms of neural cells in the presence of the brain damage, we first attempted to characterize chemokine receptor expression in the neural cells *in vitro*. In our preliminary experiments with mouse ES-cell-derived neural cells treated with RA that were treated in exactly the same manner as with monkey ES cells, we examined mRNA expression of mouse chemokine receptors including CCR1-11, CXCR1-6, CX3CR1, and XCR1 by using RT-PCR. We only found expression of CXCR4 mRNA in the mouse ES-cell-derived neural cells reproducibly. SDF1/CXCR4 is important for early neural cell migration in fetal brain development[15]. The other chemokine receptors tested so far were not expressed in mouse ES-cell-derived neural cells by RT-PCR (data not shown).

Then, we studied the mRNA expression levels of CXCR4, CCR2a, CX3CR1, CCR8, CXCR3, CCR3, CXCR1, CXCR2, and CCR1 in monkey ES-cell-derived neural cells. We found that monkey ES-cell-derived neural progenitors expressed CXCR4 mRNA (Fig.2D). Other chemokine receptors tested so far were found to be negative (Fig.2D and data not shown). Undifferentiated monkey ES cells did not express CXCR4. Thus, the neural cells derived from RA-treated monkey ES cells cultured for 8 days used as the neural cell graft expressed CXCR4.

SDF1 induced migration of the neural stem/progenitor cells *in vitro*

We next addressed the possibility that SDF1 acts as a chemoattractant migratory cue for monkey ES-cell-derived neural cells using a microchemotaxis assay. This assay allowed us to assess the migration of cells in response to a gradient of a chemoattractant. We found that monkey ES-cell-derived neural cells migrated toward a source of SDF1. The cells did not respond to a number of other chemokines including CTACK (cutaneous T cell-attracting chemokine), Monocyte chemotactic protein 1 (MCP1), RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted), Fractalkine, or Macrophage inflammatory protein 1. The addition of a selective CXCR4 antagonist, AMD3100 (10 ng/ml and 100 ng/ml), resulted in the inhibition of migration of the neural cells in the assay (Fig.2E and data not shown).

To address whether SDF1/CXCR4 receptors were functioning in other activities of monkey ES-cell-derived neural cells, we performed RT-PCR studies on cells cultured with SDF1 (Fig.2F). In response to SDF1, the neural stem/progenitor cells started to express neural cell adhesion molecule (NCAM) mRNA after as little as 4 hours of culture. L1CAM mRNA and N-Cadherin mRNA expression was not detected in these culture conditions.

1) Oriented migration of the ES-cell-derived neural cells in experimentally injured mouse brain

We conducted a histological analysis of injured brain tissue following cell transplantation. The mouse brains were fixed and recovered at various time points after transplantation. We found cell aggregates in the periventricular region, which corresponded to the transplantation site, 3 days after transplantation by HE (Hematoxline-Eosin) staining (Fig.3A). The control group of PBS injected mice did not show such cell aggregation in the brain tissue (data not shown).

We then analyzed the localization of the grafted cells by confocal laser microscopy. We stained brain sections with anti-NFM antibody in red, and confocal images indicated that the GFP (green fluorescence protein) positive neural cells were clustered and the grafted cells did not express NFM clearly at day 3. At day 7, the neural cells were located close to the corpus callosum and were easily distinguished from the recipient cells by their deep blue color in HE staining (Fig.3B). Confocal images confirmed the findings. At day 14, the majority of grafted cells reached the injured cortex and distributed widely over the injured cortex. The neural cells had moderately elongated axons at day 14 (Fig.3C). At day 21, the grafted neural cells were distributed widely but they also had a tendency to accumulate predominantly near the damaged region. Immunostaining disclosed that percentages of NFM positive (red) cells over GFP positive cells

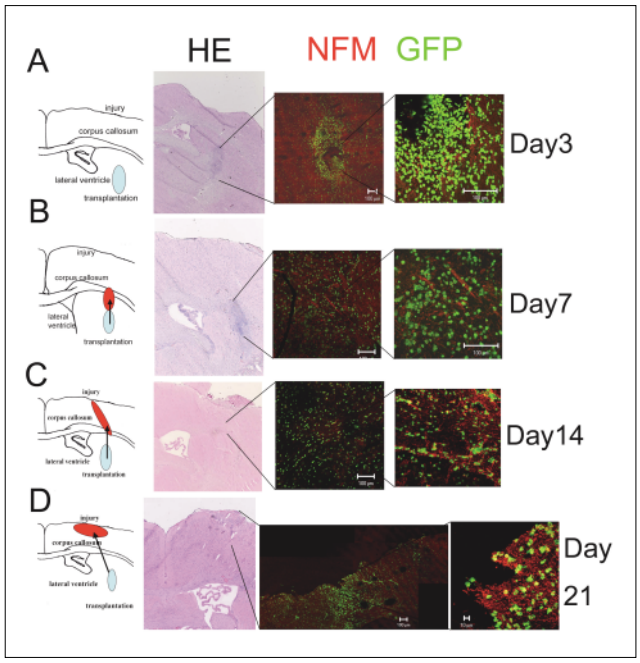

Fig.3 Migration of the monkey ES-cell-derived neural cells grafted into the periventricular area toward the damaged brain cortex of hemiplegic mice

(A): The mouse brain was cryo-injured at day -7 for use as a model of brain damage. The motor cortex of the left hemisphere was damaged extensively, but the depth of the cryogenic injury was restricted to the cortex and reached the upper surface of the corpus callosum. A single cell suspension of monkey ES-cell-derived neural cells at day 0 was used as a tissue graft. The cells were injected into the periventricular area of the left hemisphere (underneath the lesion) on day 0. Schematic representation of HE staining shows the location. The blue area in the schema indicates the position of graft cell deposition at the beginning. The red area indicates the position of graft cell migration at the indicated time point. Three days after transplantation, the brain was recovered for HE staining. Confocal images were further processed for the assessment of cell migration. The immunostaining for neurofilament middle chain (NFM)(lower and higher magnifications) is shown in red. The grafted cells are shown by GFP (green), which enabled us to identify the grafted cells. The grafted cells were located close to the injection site at day 3. The cells lacked NFM expression.

(B): The monkey ES-cell-derived neural cells formed a cell cluster under the corpus callosum at day 7. Some of the cells located in the corpus callosum

(C): At day 14, the monkey ES-cell-derived neural cells passed the corpus callosum and reached the injured cortex.

(D): Two color immunostaining of mouse brain with the neural cell graft 21 days after transplantation, and detection of the grafted cells in the cortical region of the damaged brain suggesting their migration to the lesion from the injection site. The grafted cells dispersed over the motor cortex.

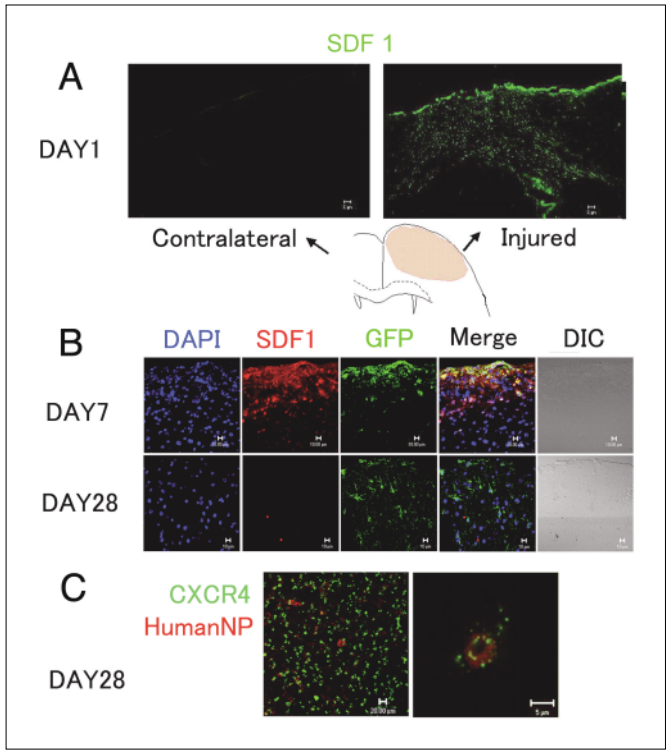

Fig.4 Expression of SDF1 in glial cells and its receptor CXCR4 expression in grafted neural cells derived from monkey ES cells in the damaged host brain tissue

(A): The expression pattern of SDF1 in the motor cortex of the damaged hemisphere and contra-lateral normal hemisphere was analyzed. The damaged hemisphere expressed SDF1. DIC indicates a differential interference contrast image.

(B): SDF1 was produced mainly by GFAP positive cells at day 7. At day 28, the production of SDF1 had subsided.

(C): After transplantation, the brains were recovered at day 28. The grafted neural cells derived from monkey ES cells were positive for anti-human nuclear protein antibody (red), which did not react with mouse cells, 28 days after transplantation. The cells simultaneously expressed CXCR4 (green) in the grafted brain.

were 7% on day3, 45% on day 7, 69% on day 14, and 94 on day 21 (a representative of three independent experiments). Eventually, the cells expressed NFM positive axons extensively over the injured cortex, suggesting that regeneration of the neural network had started (Fig.3D). These results suggested the transplanted cells had migrated from the site of injection to the injured cortex accompanying gradual elongation of NFM positive axons.

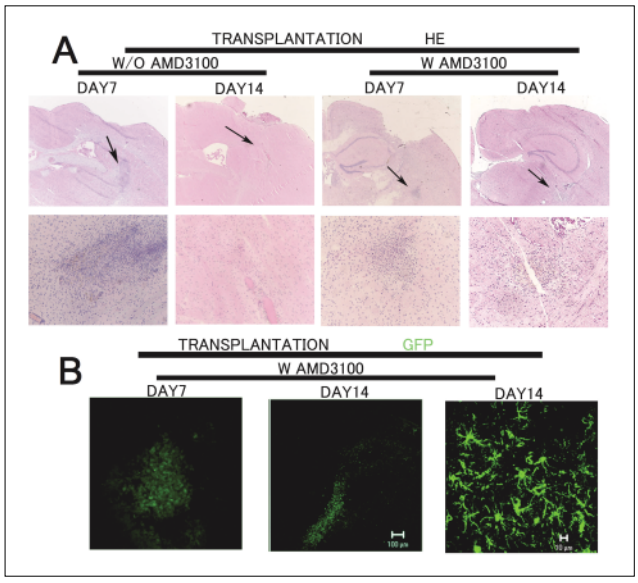

Fig.5 Effects of administration of AMD3100, a CXCR4 antagonist, on the migration of the grafted neural cells in the damaged host brain tissue

The GFP-labeled neural cells were transplanted into the injured mice. AMD3100 or vehicle was subcutaneously injected for 14 days after transplantation. Without (W/O) AMD3100 treatment, the neural cells migrated as shown in Fig. 3 and this figure. At day 14, the majority of cells had passed the corpus callosum and reached the injured cortex. In contrast, with (W) AMD3100 administration the neural cells stayed near the injection site for 14 days, periventricular area, far below the corpus callosum. The above finding was further confirmed with the observation of GFP-positive neural cells accumulating near the injection site at day 7 and day 14. Scale bar, 100 μm.

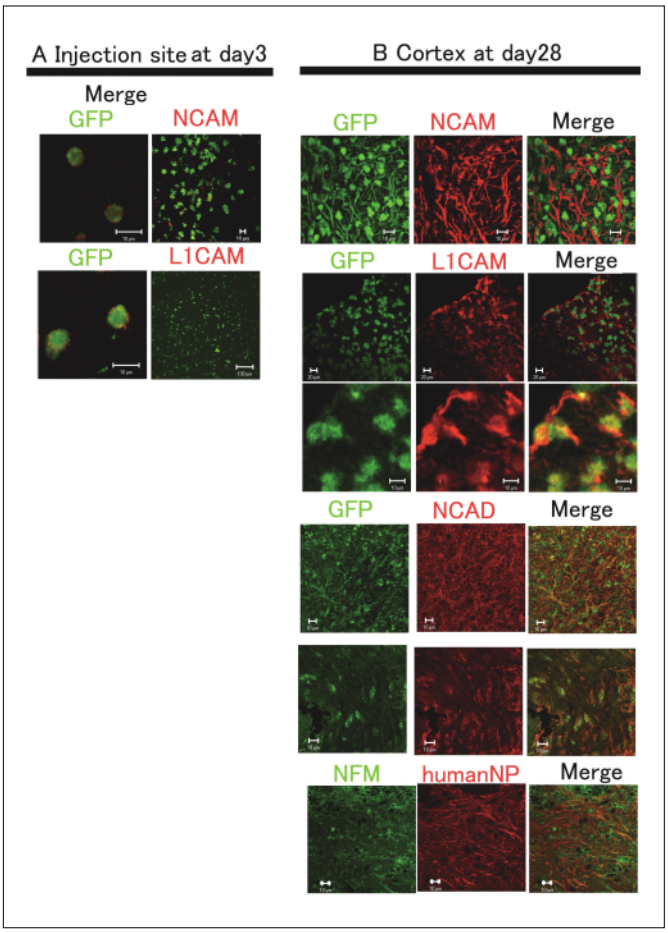

Fig.6 Expression of neural cell associated adhesion molecules of the monkey ES-cell-derived neural cells migrating to the damaged cortex of recipient mice

Confocal image of neural cells expressing GFP (green) 3 days after transplantation, the grafted cells at the injection site marginally expressed NCAM and L1CAM (red). N-Cadherin (NCAD) expression was not detected (data not shown). After reaching the damaged cortex the cells simultaneously expressed NCAM, L1CAM, N-Cadherin, and NFM (red) at 28 days after transplantation,. In some experiments, non-labeled neural cells were grafted, where anti-human nuclear protein (NP) antibody was used to detect monkey cells.

2) Role of chemokines in the migration of neural stem/progenitor cells toward the site of brain damage

In the CNS, the innate immune response involves the activation of astrocytes and microglia, resulting in the synthesis of a cascade of proinflammatory molecules. Because the neural cells expressed CXCR4 predominantly, it is important to study whether the cells in the injured area produce SDF1 *in vivo*.

In this experiment, we used non-labeled monkey ES cells for the induction of neural cell differentiation and subsequent neural cell transplantation. We examined the expression of SDF1 in injured brains in mice. From day 1 (Fig.4A, Day 1), the expression of SDF1 was wide-spread in the damaged motor cortex. The contralateral uninjured cortex of the same mouse did not produce SDF1. Next, we found that endogenous GFAP positive cells were present at the site of the injury and displayed small round or spindle shapes (Fig.4B, Day 7). The cells seemed to produce SDF1 in the injured cortex, which subsided by day 28 (Fig.4B, Day 28).

We stained anti-human CXCR4 as green and anti-human nuclear protein as red (Fig.4C, Day 28). The cells similarly expressed CXCR4 on day 7 and day 14 (data not shown). Thus, the

monkey ES-cell-derived neural cells that were actively migrating away from the injection site toward the damaged cortex strongly expressed the receptor. Virtually all of the migrating cells stained positively for CXCR4.

3) A CXCR4 antagonist AMD3100 inhibits neural cell migration *in vivo*

It was suggested that neural stem/progenitor cells that expressed CXCR4 migrated toward the site of brain injury where SDF1 was secreted. This hypothesis was tested by injecting AMD3100, a specific antagonist of CXCR4[21,34,35] twice a day into mice after receiving neural cell transplantation. Injured mice transplanted with neural cells that received PBS injections in a similar manner served as a control treatment group. The neural cells transplanted into the periventricular region migrated toward the injured cortex in the PBS injected mice at day 14 (Fig.3 and Fig.5, W/O AMD3100).

In mice receiving AMD3100 the neural cell graft remained near the injection site for up to 14 days. No effect on cell viability was observed with AMD3100, and the grafted cells expressed GFP strongly (Fig.5, W AMD3100). Therefore, CXCR4/SDF1 activation seems to play a crucial role in neural cell migration necessary for neural regeneration. Collectively, these results indicate that SDF1/CXCR4 is primarily responsible for the migration of neural stem/progenitor cells where neural regeneration has occurred.

4) Expressions of neural cell associated adhesion molecules in monkey ES-cell-derived neural cells upon migration to the damaged cortex of the recipient mice

When we conducted histological analysis of brains with GFP-negative grafts, we found NFM (green) expressing grafted cells that were positive for anti-human nuclear protein (NP) antibody (red) in the damaged cortex, suggesting that the grafted cells accumulated in this area and maturated to express NFM (Fig.6). Similar findings were obtained when we used GFP-positive neural cells as a graft (Fig.6). Almost all DAPI positive cells locating in the damaged cortex were GFP positive and had NFM positive axons.

In other experiments, we focused on neural cell associated adhesion molecule expression in the graft cells because these molecules are important for axon elongation[36-38]. Grafted cells expressing GFP at the injection site scarcely expressed L1CAM and NCAM 3 days after transplantation (Fig.6), and N-Cadherin was not expressed at all (data not shown). Neural cells migrated to the damaged cortex and simultaneously expressed NCAM, L1CAM, and N-Cadherin at 28 days after transplantation. Along with migration, the graft cells seemed to start expressing neural cell associated adhesion molecules, L1CAM, NCAM, and N-Cadherin, which are important for axon elongation.

Discussion

We have reported previously on the successful transplantation of monkey ES-cell-derived neural cells. The grafted neural cells brought about functional improvement of hemiplegic mice. The grafted cells expressed synaptophysin protein suggesting the formation of synaptic connections between the grafted cells and host cells and regeneration of neural network[9]. However, the precise mechanisms governing the regeneration process after cell transplantation remains largely unknown. We found that GFAP positive cells within the damaged cortex produced SDF1, and the grafted neural stem/progenitor cells expressing CXCR4 migrated from the injection site toward the damaged motor cortex. Therefore, the SDF1/CXCR4 pathway seems to be important for the migration and regeneration processes. Furthermore, two important neural cell adhesion molecules, L1CAM and NCAM, were expressed extensively on the grafted neural tissue after reaching the damaged cortex. It is possible that these molecules controlled neurite outgrowth and contributed to the regeneration process.

Numerous papers have demonstrated the homing of neural progenitors to sites of brain injury. These cells clearly express diverse chemokine receptors[5,19]. Involvement of SDF1/CXCR4 and MCP1/CCR2 in the migration of neural progenitors after cellular transplantation in the CNS has been reported. SDF1/CXCR4 signaling is important in regulating the migration and proliferation of neural cells[16,18,39,40]. SDF1 is involved in regulating path finding by TrkA-positive axons of developing sensory neurons[39]. SDF1/CXCR4 has been shown to regulate interneuron migration in the developing cortex[15]. Mice that lack either SDF1 or CXCR4 show similar developmental abnormalities[18,41].

The neutralization of SDF1/CXCR4 signaling by AMD3100 disrupted migration of the neural cells *in vitro* and in the damaged brain. Our findings are consistent with several previous observations[3-5,19,42-44].

We found that the CCR2 and CX3CR1 chemokine receptor mRNAs were not expressed in the neural cells derived from monkey ES cells. Similarly mRNAs of other chemokine receptors examined so far were not detected. Because receptors for the chemokines are reported to be expressed by neural progenitors and microglial cells[20,45,46], it was not completely clear why our neural stem/progenitor cells derived from mouse and mon-

key ES cells did not express them. It is possible that neural cells in the embryonic stage of the differentiation pathway preferentially use SDF1/CXCR4 signaling for their migration. Further effort is needed to clarify this issue.

We found that two important neural cell adhesion molecules, L1CAM and NCAM, were expressed extensively in the monkey ES-cell-derived neural cells 28 days after transplantation, even though they were expressed only at a low level at the time of transplantation (Fig.2C and Fig.6). L1CAM and NCAM are members of the immunoglobulin superfamily[47], and they are widely expressed in neural tissues during development[48]. L1CAM and NCAM mediate homophilic adhesion and heterophilic adhesion[47,48]. Cell adhesion molecules were assigned an important role in neurite outgrowth[36]. L1CAM plays an important role for neurite extension, and NCAM for growth cone protrusion[49]. L1CAM knockout mice have enlarged ventricles and dramatic hypoplasia of the corticospinal tract[50,51]. NCAM knockout mice showed defects primarily in embryonic neuronal migration and in axon growth and fasciculation[52]. These reports support the importance of L1CAM and NCAM for neural development. We found that L1CAM, NCAM, and N-Cadherin were expressed extensively in the regenerating neural tissue by the grafted monkey ES-cell-derived neural stem/progenitor cells. RT-PCR (Fig.2C) and immunostaining (Fig.6) suggested that the neural cells did not express L1CAM and NCAM at the time of and 3 days after transplantation. It is possible that after neural cell migration reaching the damaged cortex, the grafted cells then express adhesion molecules sufficiently. It is possible that these adhesion molecules play important roles in the regeneration of the damaged cortex by the grafted neural cells[53,54].

There has been several publications concerning growth and differentiation of human ES cells *in vitro*[55-57]. The diverse differentiation of human ES cells makes them excellent candidates for transplantation therapies. To establish transplantation therapies using human ES cells, allogeneic and xenogeneic transplantation models of nonhuman primate ES cells are useful. Primate and human ES cells share unique characteristics such as the formation of flat colonies, a tendency to produce the trophectoderm lineage, and a different pattern of cell surface antigen expression[23]. Transplantation of neural cells originating from primate and human ES cells into rodents has been attempted. The donor-derived neurons demonstrated widespread distribution but not restricted to sites exhibiting neurogenesis[57]. In a rodent model of traumatic brain injury, transplanted human neural progenitor cells proliferated, migrated even into the contralateral cortex, and showed neuronal and astrocytic differentiation, and might also improve survival of host cells[58].

Collectively, our findings suggested an important role for SDF1/CXCR4 signaling in directing the migration of motor neuron progenitors to the injured cortex and subsequent neural regeneration.

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