ョン法は，ES 細胞を用いた移植・再生医療の安全性の向上のために普遍的な応用が期待される．

E. 結論

(1) 未分化のサル ES 細胞をサル胎仔肝臓に移植すると，各組織に ES 由来細胞の“生着”が見られ，テラトーマは実質臓器外に限られた．漏れた細胞がテラトーマを作る可能性が高い．

(2) サル ES 細胞を前造血細胞に分化させてから移植すると，造血系を一部再構築できたが（2-5%），全例テラトーマをつくった．テラトーマ形成リスクは思いのほか高かった．サル同種移植の系は安全性の厳密な評価に適している．

(3) しかし，免疫不全マウスやヒツジ胎仔へ同じ細胞を移植しても腫瘍形成は稀である．

(4) SSEA4 陽性細胞を除去すると，生着能を妨げずに，テラトーマ形成は完全に予防できた．SSEA4 は臨床的なステムネス・マーカーといってよい．

F. 健康危険情報

なし．

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H. 知的財産権の出願・登録状況

なし.

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分担研究報告書

霊長類ES細胞の移植で生じる病理変化

分担研究者
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研究要旨

霊長類ES細胞移植による奇形腫の生育の状態の違いを、異種の各種免疫不全マウス、ヒツジおよび同種のカニクイザルで比較検討した。SCIDマウス、ヒツジ胎仔およびカニクイザルに形成された奇形腫は、NOGマウスとGM1投与のSCIDマウスの奇形腫より小型であった。組織学的にマウスでは充実性の多様な腫瘍組織が見られ、ヒツジでは充実性の多様な腫瘍組織に強い炎症反応を認めた。カニクイザルは多形な嚢胞に占められていた。SCIDマウスの移植で3ヶ月の奇形腫は、2ヶ月より高分化だった。ES細胞移植による宿主間の奇形腫形成の差異はそれぞれの免疫応答の違いによると考えられた。

A．研究目的

ES細胞の再生医療の成功のためには、適切に分化した細胞が適切な場所に定着する必要がある。本研究で用いられているカニクイザル由来ES細胞は、移植された宿主の環境内で、いかに増殖し、一方でいかに排除されるのか。この点を明らかにするため、異種ならびに同種動物への細胞移植によって形成された奇形腫を比較、移植後の大きさの変化、病理組織学的変化と分化程度の進行の度合いについて検索した。

B．研究方法

分化誘導されていないカニクイザル由来ES細胞CMK6を1）免疫不全であるSCIDマウスの皮下（n=17)、2）重度免疫不全であるNOGマウス皮下（n=5)、3）NK細胞を抑制するためにGM1が投与されたSCIDマウス皮下(n=3)、4）ヒツジ胎仔肝臓（n=1）に、接種した。一方、中胚葉へ分化誘導されたCMK6-D6細胞を5）SCIDマウスの皮下（n=2)、6）カニクイザル胎仔の肝臓（n=3）に接種した。1）は接種後2と3ヶ月後の腫瘤を、2)、3）および5）は接種後3ヶ月の腫瘤を、4）は出産後約半年と一年で形成された皮下腫瘤を、6）は流産胎仔の腹膜に形成された腫瘤を摘出した。摘出組織をホルマリン固定、パラフィン包埋し、組織学的ならびに免疫組織化学的に検索した。組織学的検索ではHE染色を施し、免疫組織化学的検索では外胚葉をAnti-Neuron Specific Enolase

(NSE)、中胚葉を Anti-Smooth Muscle Actin (SMA)、内胚葉を Anti-α1-fetoprotein (AFP)、移植細胞のタグを Anti-GFP (GFP)で確認した。

倫理面への配慮：当研究では十分に倫理面に配慮して実験された、専らホルマリン固定材料を用いるため、倫理面に関して何ら問題はない。

C．研究結果

1）CMK6×SCID マウス

検索例すべてに径約 2～3cm の充実した腫瘤が形成され、組織学的に接種後 2ヶ月（17 例中 2 例）では、NSE 陽性の神経管、軟骨、SMA 陽性の平滑筋、AFP 陽性の腺、その他に脈管などが見られた。接種後 3ヶ月（17 例中 15 例）では、さらにNSE陽性のメラニン含有細胞、SMA 弱陽性の横紋筋、AFP 陽性の分泌物含有の腺、毛包などが見られた。3ヶ月の方が明らかに分化した組織が確認された。

2）CMK6×NOG マウス

検索例すべてに径約 1cm 腫瘤が形成され、腫瘤の大きさは 1）の約半分であったが、組織学的および免疫組織化学的性状は 1）の接種後 3ヶ月とほぼ同様であった。

3）CMK6×GM1+ SCID

検索例すべてに径約 1cm 腫瘤が形成され、腫瘤の大きさ、組織学的および免疫組織化学的性状も 2）とほぼ同様であった。

4）CMK6×ヒツジ

出生時には腫瘤は観察されなかったが、生後半年で充実性の径約 5cm 大型腫瘤が形成され、組織学的には、NSE 陽性の神経管、軟骨、SMA 陽性の平滑筋、AFP 陽性の腺、その他に脈管などが見られたが、腫瘍細胞の壊死と炎症細胞浸潤が広範に認められた。さらに約半年後（出生 1 年後）、同個体に発生した腫瘍組織はわずかで、ほとんどが肉芽組織に置換されていた。

5）CMK6-D6×SCID マウス

検索例 10 例中 1 例のみに径約 5mm 非常に小型の充実性腫瘤が形成された。組織像は 1）の 2ヶ月のものと同等であった。

6）CMK6-D6×カニクイザル

3 例に嚢胞状の径約 1～3cm 腫瘤が形成され、嚢胞を裏打ちする細胞は NSE や AFP に陽性で、嚢胞間に神経網様構造は NSE に陽性、平滑筋様構造は SMA に陽性だった。

上記のいずれの奇形腫も GFP 陽性で移植細胞に由来することが明らかであった。

D．考察

NK 細胞を含むリンパ球欠損による免疫不全マウスの NOG マウスに対し、B および T リンパ球だけが欠損する SCID マウスに GM1 の NK 細胞機能抑制が加わることで、両者は同等の腫瘤退縮が引き起こされた。NK 細胞は一般的に腫瘍の排除に最も効果的な免疫細胞と考えられるが、ES 細胞移植では NK 細胞が働かない群の腫瘤は小型化し、一般論とは逆の現象が生じた。一方で、完全な免疫寛容の状態の移植であれば移植細胞は環境に馴化し、腫瘍形成は起こりに

くくなるはずで、今回の現象はこの考えには合致した。移植細胞の増殖と定着の関係には NK 細胞だけでなく別の要因にも支配されている可能性があるが、詳細については今後の検討課題である。

ヒツジは強烈な炎症反応が見られ、かつ時間経過に伴って慢性炎症の組織像に推移していった。明らかに出生後にできた腫瘤であるため、先行して起こった移植細胞の増殖に対し、新生仔の免疫能の発達とともに強烈な免疫応答が惹起されたものと考えられた。

分化誘導した細胞を移植したマウスの結果から、より分化した ES 細胞を移植することによって奇形腫形成は圧倒的に少なくなることが明らかとなった。一方で同種であるカニクイザルに奇形腫が形成されたことは、現実に同種移植が臨床応用されることを考えると、非常に問題である。カニクイザルと SCID マウスの間で異なる形状の腫瘤が形成されたことは、同じ免疫寛容の状態であったとしても、同種と異種の間の免疫応答に何らかの大きな違いがあることを示唆している。

未分化な ES 細胞移植では奇形腫形成の時間が長いほど、分化程度の高い腫瘍組織が観察されたが、この点については分化誘導した ES 細胞移植でも確認する必要がある。

E. 結論

良好な免疫寛容の状態であるほど奇形腫が小さく、移植細胞の異常な生育は抑えられることが明らかになった。奇形腫内では時間の経過とともに、腫瘍細胞の分化が進行していくことが示唆された。

F. 健康危険情報

なし。

G. 研究発表

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H. 知的財産権の出願・登録状況

なし。

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Transplantation of neural cells derived from retinoic acid-treated cynomolgus monkey embryonic stem cells successfully improved motor function of hemiplegic mice with experimental brain injury

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We induced neural cells by treating cynomolgus monkey embryonic stem (ES) cells with retinoic acid. The treated cells mainly expressed βIIItubulin. They further differentiated into neurons expressing neurofilament middle chain (NFM) in elongated axons. Half of the cells differentiated into Islet1+ motoneurons in vitro. The monkey ES-derived neural cells were transplanted to hemiplegic mice with experimental brain injury mimicking stroke. The neural cells that had grafted into periventricular area of the mice distributed extensively over the injured cortex. Some of the transplanted cells expressed the neural stem/progenitor marker nestin 2 days after transplantation. The cells expressed markers characteristic of mature motoneurons 28 days after transplantation. Mice with the neural cell graft gradually recovered motor function, whereas control animals remained hemiplegic. This is the first demonstration that neural cells derived from nonhuman primate ES cells have the ability to restore motor function in an animal model of brain injury.

Keywords: Motor neuron; Nonhuman primate; Embryonic stem cells; Hemiplegia; Differentiation

Introduction

The limited capacity for structural and functional repair in human central nervous system (CNS) damaged by stroke is partly explained by the inability of mature CNS to regenerate new cellular components in response to damage (Cao et al., 2002). ES cells are derived from the inner cell mass of blastocysts (Alison et al., 2002; Czyz et al., 2003), and differentiate by forming complex 3 dimensional cell aggregates termed embryoid bodies (EBs), which may contain pluripotent cells and cells in various stages of differentiation. ES cells and their progeny proliferate robustly with a normal karyotype (Shamblott et al., 2001). Mouse embryonic stem (ES) cells have been shown to differentiate preferentially into neural cells. To initiate neural differentiation of mouse ES cells, all-*trans* retinoic acid (RA) and basic fibroblast growth factor (bFGF) have often been used (Appel and Eisen, 2003; Gottlieb, 2002; Guan et al., 2001; Wernig and Brustle, 2002). We found that the RA treatment induced predominantly neurons, especially motoneurons and small numbers of glial cells from mouse ES cells (Chiba et al., 2003, 2004). Recently, human ES cells are predicted to be a valuable source for producing ES cell-derived therapeutic spare tissues to treat diseases by controlling their growth and differentiation (Odorico et al., 2001; Thomson et al., 1998).

To understand the full potential of human ES cells, it will be necessary to characterize the mechanism that controls self-renewal and differentiation into neural lineage. For such analysis, characterization of nonhuman primate ES cells is also useful as their similarity with human ES cells. As for neural cells, dopaminergic neurons, oligodendrocytes and peripheral neurons were induced

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from mouse, nonhuman primate and human ES cells (Amit and Itskovitz-Eldor, 2002; Kawasaki et al., 2002; McDonald and Howard, 2002; Mizuseki et al., 2003; Ostenfeld and Svendsen, 2003). Some of the induced neural cells from mouse ES cells were transplanted to injured site of CNS and functional recovery has been obtained in such studies (Kim et al., 2002; McDonald and Howard, 2002). We have also reported functional recovery of hemiplegic mice by transplantation of neural cells derived from mouse ES cells by treatment with RA (Chiba et al., 2003, 2004). Transplantation of dopaminergic neurons derived from primate ES cells to damaged brain were reported (Kawasaki et al., 2002). However, reports of functional consequences of animals transplanted with primate ES were scant.

In this study, we induced neural cells including motoneurons by treating cynomolgus monkey ES cells with RA (Renoncourt et al., 1998). The transplanted neural cells adapted to the host environment and differentiated into mature neural cells. Motor function of the hemiplegic mice with experimental brain injury was significantly improved by transplantation of the neural cells compared to the PBS-injected control mice.

Materials and methods

Cynomolgus monkey ES cells

The cynomolgus monkey ES cell line, CMK 6.4-6 (Passage number 53-122, normal karyotype), was used in this study (Suemori et al., 2001). Undifferentiated ES cells were maintained in growth medium consisting of DMEM/F12 supplemented with 1× non-essential amino acids, 1× pyruvate, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine (all purchased from Gibco BRL, Tokyo, Japan), and 20% Knockout serum replacement (KSR). The cells were cultured on feeder layer consisting of mitomycin C-treated mouse fetal fibroblasts.

Induction of neural differentiation of cynomolgus monkey ES cells by RA

We induced neural differentiation of the ES cells in the presence of all-*trans* RA (Sigma, Tokyo, Japan). In brief, the ES cells in the maintenance culture were recovered (at day 0) and cultured in DMEM/F12 supplemented with 20% KSR on bacterial grade Petri dishes. 4 days later, floating cell aggregates, EBs, were transferred onto fresh Petri dishes to which 1 μM RA was added twice at day 4 and day 6. The RA treatment successfully induced neural differentiation of the ES cells (please see below). For morphological and immunochemical analyses, the EB recovered at day 8 were cultured in DMEM/F12 supplemented with N2 supplement on fibronectin-coated 8 chamber dishes without mitogens nor growth factors until day 22. For transplantation, the EB was recovered at day 8 (totally cultured for 8 days in vitro) and a single cell suspension of the EB was immediately used as a graft. It contained feeder cells less than 0.01%.

RT-PCR

Total RNA extraction and cDNA synthesis have been reported (Kashiwakura et al., 1999). Cycling parameters were hot starting 94°C for 120 s, denaturing at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 60 s The reaction was repeated 30 times and followed by elongation at 72°C for 10 min. Because sequence data of cynomolgus monkey mRNAs of the following markers were not available, we designed the primers according to human sequences. β-actin (expected size, 661 bp), sense tgacggggtcacccacactgtgcccatcta, antisense ctagaagcattgcggtggacgatggaggg; nestin (177 bp), sense agacttccctcagctttcagg, antisense gcctggaggaattcttggtt; NFM (209 bp), sense: tagcacatttgcaggaagca, antisense: cggccaattcctctgtaatg; MAP2 (197 bp), sense agaccaccattgacgactcc, antisense agtggaaattctgcctctcc; Islet1 (203 bp), sense gttaccagccaccttggaaa, antisense: tgaatgttcctcatgcctca; GFAP (323 bp), sense tgcagacctgacagacgctgctg, antisense ctgctagagggcgaggagaaccg; galactocerebrosidase (GalCase) (256 bp), sense tgactataaagtgtgatgtt, antisense agcatgccagaggcgaaatg. We have confirmed the internal DNA sequences of the amplified products by TA cloning method and subsequent DNA sequencing.

Flow cytometry

The RA-treated cells were collected at day 8 of culture. The cells were suspended in PBS containing 0.01% azide and 1 mM EDTA. The single cell suspension was incubated with anti-NCAM antibody (Becton Dickinson, NJ) or mouse IgG1, followed by incubation with phycoerythrin conjugated secondary antibody. After washing, stained cells were analyzed by a flow cytometer (Cytoron, Ortho-Clinical Diagnostics, Tokyo, Japan).

Experimental brain injury by cryogenic injury and transplantation

Female C57BL/6 mice (6–8 weeks old) were used as transplant recipients (Chiba et al., 2003, 2004). All subsequent procedures were conducted along with the institutional guideline. Mice were anesthetized and 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 s four times.

When mice were exposed to cold injury three times or less (compression of the metal probe treated with liquid nitrogen for 30 s 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 could reliably reproduce hemiplegia by administering cold injury four times in each mouse (Cook et al., 1998; Katano et al., 1998; Morita-Fujimura et al., 1999).

2 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 26-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 (Chiba et al., 2004). In some experiments, dead graft cells were used as control. The RA-treated EB at day 8 was fixed with 10% formaldehyde for 30 min, washed extensively with PBS, and 1.0×10^5 dead graft cells were transplanted. The mice

did not show functional recovery (Chiba et al., 2004). When we used undifferentiated moues ES cells as a graft, the mice did not show their functional recovery (Chiba et al., 2004). Thus, we did not try to use undifferentiated cynomolgus monkey ES cells as a graft. 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) 1 h 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.

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 (Allen et al., 2000; Fox et al., 1998; Hamm et al., 1994; Zausinger et al., 2000). 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.

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 (Fox et al., 1998; Hamm et al., 1994; Rozas et al., 1997; Zausinger et al., 2000; Zhang et al., 2000). 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 s 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 s. 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 a 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.

Immunochemical and immunofluorescence staining

For all immunohistochemical procedures, adjacent sections served as negative controls and were processed using identical procedures, except for incubation without the primary antibody in each case. The reactivity was visualized with diaminobenzidine tetrahydro-chloride and counterstained with hematoxylin. Cells cultured on chamber slides and cryostat sections (5 μm thick) of whole brains were fixed in 4% paraformaldehyde for 15 min. All subsequent procedures were performed using DAKO LSAB®2 Kit (Dako, Carpinteria, CA) or M.O.M.™ Kit (Vector laboratory, Burlingame, CA). Endogenous peroxidase activity was blocked by incubating sections with 0.3% hydrogen peroxide. The samples were incubated with primary antibodies and followed by peroxidase conjugated second antibodies. The primary antibodies included anti-human nestin (catalogue #MAB5326, Chemicon, Temecula, CA), anti-βIIItubulin (catalogue #G7121, Promega, Madison, WI), anti-Islet1 (clone #40.2D6, Hybridoma bank, Iowa City, IA), anti-NFM (catalogue #AB1987, Chemicon), anti-GalC (catalogue #AB142, Chemicon), anti-GFAP (clone #6F2, Dako), anti-synaptophysin (catalogue #U0037, Dako), and anti-human nuclei (catalogue #MAB1281, Chemicon) antibodies. Especially, anti-human nestin and anti-human nuclei antibodies specifically react to cynomolgus monkey cells but not to mouse cells, therefore, the cells stained by these antibodies in the transplanted brain tissue are proved to be grafted cynomolgus monkey cells. Expression of some neural antigens was examined by immunofluorescence staining. In this method, the sections were blocked for 2 h in PBSTG (0.2% Tween 20, 0.2% gelatin in PBS). The sections were incubated overnight with appropriate primary antibodies, then with biotinylated second antibody (catalogue #E0413/E0431, DakoCytomation, Kyoto, Japan) and finally with Alexa488-conjugated streptavidin (Molecular Probes, Eugene, OR) and Cy3-conjugated streptavidin (Jackson Immuno Research, West Grove, PA). If the primary antibody was mouse monoclonal antibody, M.O.M.™ Kit was used for the staining. Fluorescence was recorded with a confocal laser microscope (Carl Zeiss, Jena, Germany). Appropriate control antibodies were included in all experiments.

To calculate the cells expressing neural cell specific molecules in vitro, we performed immunocytochemistry as described above and counted at least 500 cells in each experiment. In the case of in vivo study, we prepared serial sections of the brain transplanted with neural cells and stained every three sections with the antibody specific for NFM, Islet1 or synaptophysin. We carefully counted the cells positive for human nuclei and that positive for NFM, Islet1 or synaptophysin under the confocal laser microscope, and calculated the ratio of double positive cells. The minimum number of the counted cells in vivo study was also 500 in each experiment.

Magnetic resonance imaging (MRI) study

Lesion area was determined from proton-density and T2-weighted images obtained with a standard spin-echo pulse sequence on a 4.7-T imaging spectrometer (Unity Inova 200/4350, Varian Inc., Palo Alto, CA). The imaging parameters were TR = 2000 ms, TE = 18 ms (proton-density) and 60 ms (T2-weighted), matrix size = 128 × 128, 20 mm × 20 mm and slice thickness = 0.8 mm. The images consisted of twelve slices with the slice positions chosen so that the majority of the lesion appeared in the second to forth slices. Perfusion-weighted MR images were acquired with a arterial spin-labeling sequence based on the well-known flow-sensitive alternating inversion recovery (FAIR) protocol (Kim, 1995; Kwong et al., 1995). A hyperbolic-secant inversion pulse was followed by a centric-ordered FLASH image acquisition (Pell et al., 1999). The images covered a 2-mm-thin slice through the middle of the lesion as determined from the proton-density and T2-weighted images. The imaging parameters were TR = 10 ms, TE = 5 ms, TI = 1.5 s, matrix size = 64 × 64, FOV = 20 mm × 20 mm. The width of the selective inversion slice for the selective image was 10 mm and the slice-selection gradient was turned off for the non-selective images. Fifty selective-nonselective image pairs were acquired over a period of

8 min. A predelay of 3 s was inserted between each image acquisition. The average selective and nonselective images were subtracted to obtain the flow-weighted image. The number of mice used for each experiment was 8 for neural transplantation after the injury, 6 for PBS injection control after the injury and 3 for normal without injury.

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.

Results

Differentiation of cynomolgus monkey ES cells into neural cells by RA

Colonies of undifferentiated cynomolgus monkey ES cells (Fig. 1A) looked flat as compared with those of undifferentiated mouse ES cells. The undifferentiated cynomolgus monkey ES cells were recovered from the maintenance culture, and were cultured on a non-coated dish in the presence of 10% FCS for 4 days (first culture). They became to form floating cell aggregates, EBs, during the first culture (Fig. 1B). The EB was cultured for another 4 days, during which RA was given at day 4 and day 6 (second culture) to potentiate neural differentiation. When the culture was further continued on a gelatin coated dish after the addition of RA, axon-like long processes were induced at the periphery of EB at day 17 and their morphology was similar with neural cells (Fig. 1C). Interestingly, the axon-like processes came to connect to those of other colonies at day 17 (Fig. 1D), resembling neural network formation in vitro.

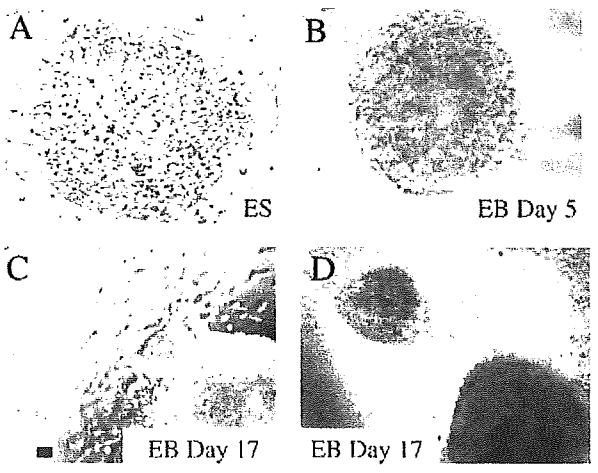

Fig. 1. Differentiation of cynomolgus monkey ES cells treated with RA in vitro. (A) A colony of undifferentiated cynomolgus monkey ES cells showed flat configuration as compared with that of undifferentiated mouse ES cells. (B) Cynomolgus monkey ES cells became to form floating cell aggregates, EBs, at day 5. (C) RA-treated cells cultured on a dish coated with gelatin at day 17 showed axon like processes at the periphery of EB. (D) The axon like processes looked interacting each other and came to overlay to those of another EB at day 17. The scale bar represents 20 μm (A–C) and 40 μm (D).

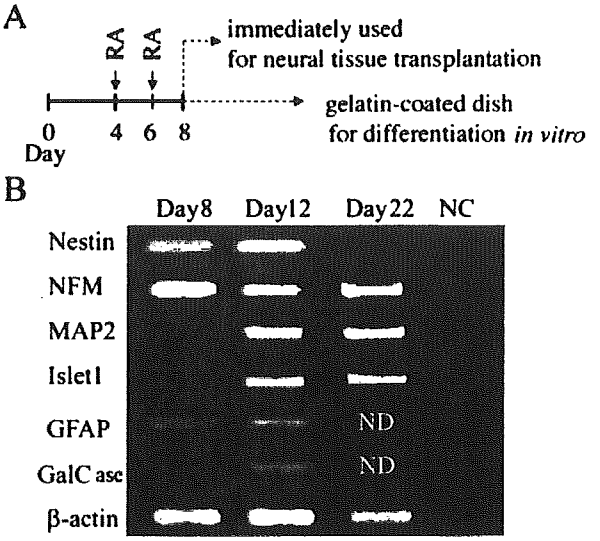

Fig. 2. Expression of neural markers in RA-treated cynomolgus monkey cells by RT-PCR. (A) Schedule for induction of neural cells from cynomolgus monkey ES cells and their transplantation to mice with cryogenic brain injury. Undifferentiated ES cells were cultured in a floating fashion for 4 days. 1 μM RA was added twice to the cell culture at day 4 and day 6. 1 × 10^5 RA-treated cells at day 8 were transplanted to underneath the motor cortex having cryogenic injury. Immunosuppressants were given to the recipients from day of the transplantation to that of sacrifice. RT-PCR was conducted using mRNAs from the RA-treated cells at days 8, 12 and 22. (B) mRNA expression of several neural cell markers in the RA-treated cells was determined by RT-PCR. The RA-treated cells derived from cynomolgus monkey ES cells at day 8, day 12 and day 22 were used. Diethylpyrocarbonate (DEPC)-treated water served as negative controls (NC). The RA-treated cells at day 8 (cultured for 4 days without RA, followed by 4 days culture with RA) that expressed nestin, was used for transplantation. ND, not done.

The schedule for neural cell induction and transplantation was shown in Fig. 2A. We examined mRNA expression of neural cell markers by RT-PCR (Fig. 2B). RA-treated cells at day 8 strongly expressed mRNA of nestin, a neural stem and progenitor cell marker. The RA-treated cells at day 22 did not express mRNA of nestin, but strongly expressed those of mature neural cell markers, NFM and MAP2. They also expressed mRNA of a motoneuron marker, Islet1, suggesting that a considerable portion of the induced cells has differentiated to motoneuron lineage. To estimate neural cell induction by RA treatment of cynomolgus monkey ES cells, flow cytometric analysis was conducted. We found that more than a half of the cells expressed panNCAM (50–65%).

We next examined protein expression of neural specific markers in the RA-treated cells. The RA-treated cells at day 8 had axon like processes (Fig. 3A). They expressed βIII tubulin (Fig. 3A). At day 14, the RA-treated cells became to express NFM (Fig. 3B). The percentage of βIII tubulin-positive neurons was 33% at day 8 and NFM-positive neurons was 64% at day 14 (data showed representatives of each staining). We have already tested a variety of culture conditions employing different culture media and growth factors, and found that medium containing 1 μM RA was most suitable for inducing differentiation into neurons, especially motoneurons, with high cell viability (data not

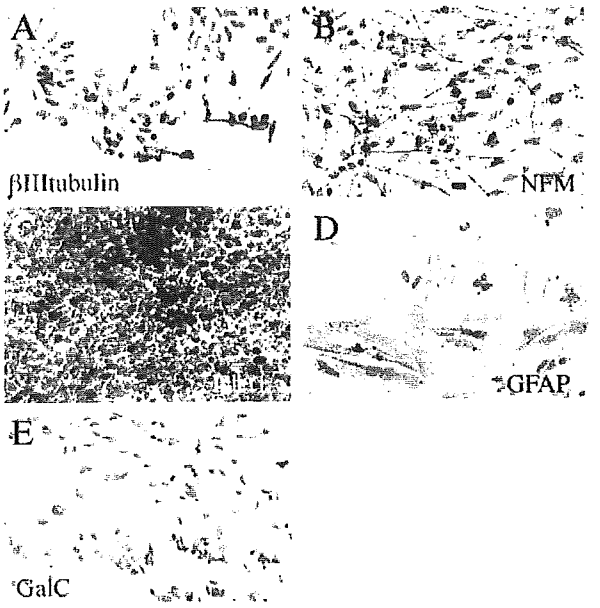

Fig. 3. Expression of neural cell antigens in RA-treated cynomolgus monkey ES derived neural cells in vitro by immunocytochemical staining. The cells were cultured on the 8-well chamber slides. After fixation, the cells were stained with the indicated antibody. Brown color indicates immunopositive cells and blue color represents counter staining for nuclei with hematoxylin. (A) Some of the RA-treated cells derived from cynomolgus monkey ES cells at day 8 were βIII tubulin-positive with axon like processes. (B) The RA-treated cells at day 14 had axons expressing NFM. (C) Approximately 50% of the cells expressed Islet1 at day 14, suggesting their differentiation into motoneurons. Islet1-positive cells were typically observed here. (D) A few of the cells locating peripheral site of EB expressed GFAP, suggesting that some of them differentiated to glial cells. (E) GalC-positive cells were hardly found in the culture period throughout, even though we have detected weak expression of GalCase mRNA in the cells (Fig. 2B). The scale bar represents 10 μm (A, B, C, D, E).

shown). We found that the RA-treated cells became Islet1-positive motoneuron lineage (Fig. 3C). When we calculated the number of the Islet1-positive cells at the periphery of EB cultured in chamber slide, Islet1-positive cell was never observed at day 8. However, the percentage of the Islet1-positive cells was approximately 50% at day 14. Noggin treatment induced differentiation of the monkey ES cells into Islet1-positive motoneurons with low efficiency (data not shown). Only a few of them became Islet1 positive with the noggin treatment, thus we decided to use RA for induction of neural cells transplantable for hemiplegic mice with experimental brain injury. Small numbers of GFAP expressing glial cells appeared outer side of the EB treated with RA (Fig. 3D). We did not find GalC expressing oligodendrocytes in the cultures throughout (Fig. 3E), even though weak expression of GalCase was detected by RT-PCR (Fig. 2B). Cells expressing those neural markers were rarely found in nontreated EB (not shown). The results suggested that the RA-treated cells at day 8 was predominantly shared by neural stem/progenitor cells, whereas at days 12 and 22, they have differentiated into more mature neural cells containing motoneurons and a small number of glial cells. From these results, we concluded that the RA-treated EB at day 8 mainly contained neural cells suitable for transplantation.

Improved motor function of hemiplegic mice having experimental brain injury with transplantation of the neural cells derived from cynomolgus monkey ES cells

We studied whether the neural cells induced from cynomolgus monkey ES cells were functionally relevant for use as a graft. The hemiplegic mice were generated by cryogenic injury and were used as a model of brain injury. Left motor cortex area was injured at day 1. We used single cell suspension of the RA-treated EB at day 8 as graft cells because the cells mainly contained neural (stem/progenitor) cells, including motoneuron lineage (Fig. 3). 1×10^5 of the neural cells or PBS was injected underneath the injured motor cortex area at day 8. We evaluated motor function of the transplanted mice by beam walking test and rotarod test (Fig. 4). Transplantation of the neural cells significantly recovered motor function of the hemiplegic mice with brain injury. Rotarod test measures the duration time during which a mouse stays on the rotating rod and reflects refined motor function and coordination. The duration time was significantly longer in the mice transplanted with the neural cells compared to that in the mice treated with PBS after day 22 (ANOVA $P < 0.01$, Fig. 4). We have also

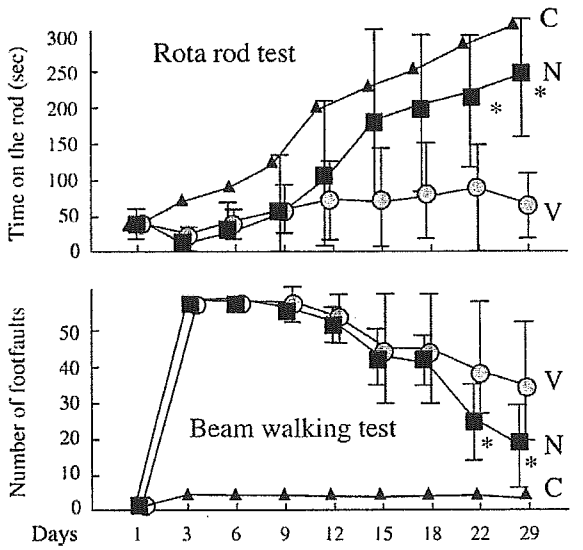

Fig. 4. Recovery of motor function in mice with experimental brain injury transplanted with the cynomolgus monkey ES cell derived neural cells. The motor cortex of normal mice was damaged by cryogenic injury at day 1. Mice were transplanted with either the graft cells or vehicle at day 8. Motor function was evaluated by the beam walking test and the rotarod test. In the experiments, RA-treated cells or vehicle (PBS) were injected. Data shown were mean ± SD at each point after injection. The numbers of footfaults in 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 22 (* in beam walking test; day 22–day 29, $P < 0.01$). 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 22–day 29, $P < 0.05$). However, it is evident that neural cell transplantation did not restore their motor function completely when compared with control mice without brain injury ($n = 9$). The SD of the control mice without brain injury were less than 5% of the mean, thus were omitted. We have conducted the experiments three times and the representative result was presented. V, vehicle; N, neural cell transplantation; C, uninjured control.

performed the beam walking test that counts the foot faults during walking 50 steps. The scores were significantly improved in the transplanted mice after day 22, compared to those in the control PBS injected mice (ANOVA $P < 0.01$, Fig. 4). There was spontaneous recovery in beam walking test over time in the PBS-treated group and it was probably due to the training and learning by repeated tests. However, the statistical difference was significant between two groups of mice, confirming the improvement of motor function by neural cell transplantation. Taking together, transplantation with the neural cells induced from cynomolgus monkey ES cells successfully recovered motor function of the hemiplegic mouse model.

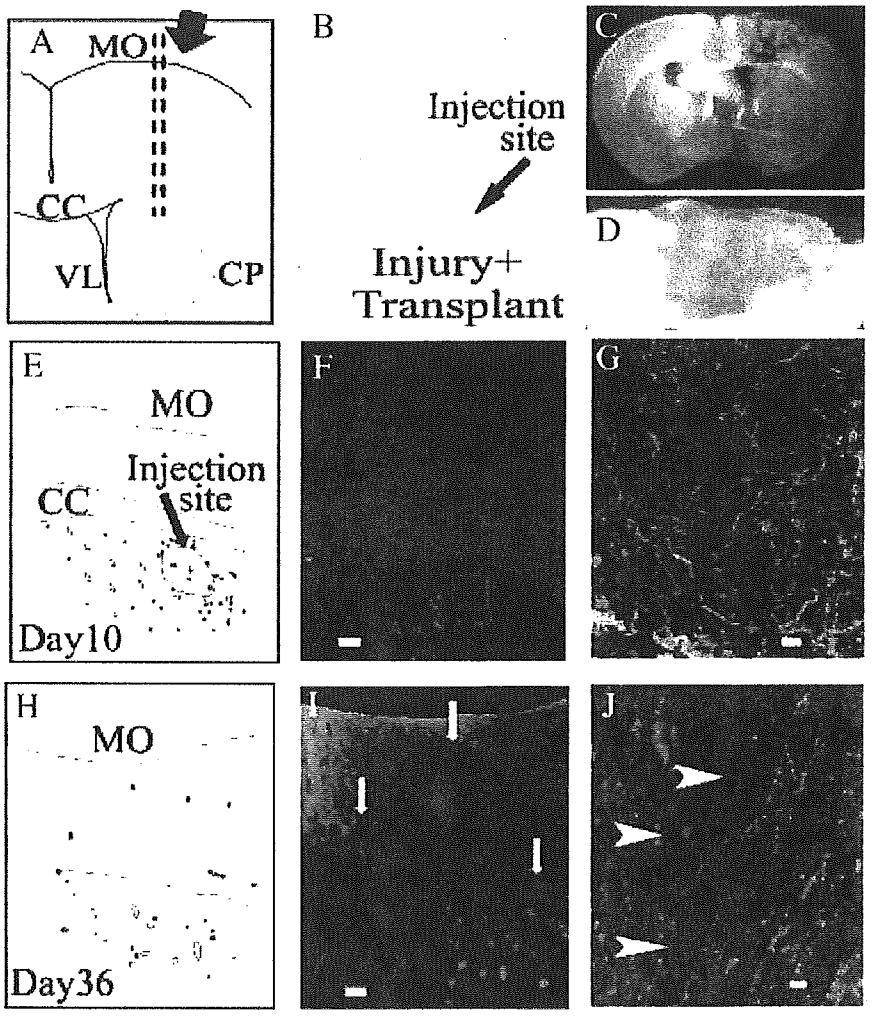

Fig. 5. Adaptation of the neural cells derived from cynomolgus monkey ES cells to mouse brain tissue. (A) Schematic representation of panel B. The brain was cryoinjured (a closed arrow) for use as a model of brain damage. The graft cells were injected to the periventricular area of left hemisphere (underneath the lesion). The dotted parallel lines indicate the position of needle insertion. The abbreviations used in the panels were as follows; VL, lateral ventricle; CP, caudate putamen; CC, corpus callosum; MO, motor cortex. (B) The RA-treated neural cells were injected underneath the motor cortex with cryogenic injury. A representative result (day 14) with HE staining was shown. (C) A microscopic image of the mouse brain with cryogenic injury. The motor cortex of left hemisphere was widely damaged, and the depth of the cryogenic injury was restricted to cortex and exactly reached to the upper surface of corpus callosum. A representative of five independent experiments was shown. (D) Higher magnification of the damaged motor cortex in panel C. (E) Schematic representation of panel F. Red dots indicate the location of the transplanted cells. (F) Two color immunostaining of mouse brain with the neural cell graft at day 10, 2 days after transplantation. Single cell suspension of the monkey ES cell derived neural cells at day 8 was used as a tissue graft. Green color was anti-NFM antibody and red color was anti-human nuclei antibody. A cluster of the grafted cells located near the injection site (a white box). (G) Higher magnification of the boxed area in panel F. (H) Schematic representation of panel I. Red dots indicate the location of the transplanted cells. (I) Two-color immunostaining of mouse brain with the neural cell graft at day 36, 28 days after transplantation. Green color was anti-NFM antibody. Red color was anti-human nuclei antibody, detecting the grafted cells in the cortical region of the damaged brain (white arrows), suggesting their migration to the lesion from the injection site. The grafted cells dispersed over the motor cortex. (J) Higher magnification of panel I. White arrowheads indicate the anti-human nuclei antibody-positive transplanted cells in the injured cortex. Three individual mice both at day 10 and at day 36 were analyzed and representative results obtained from each group of mice were shown. The scale bars represent 100 μm (BCD), 50 μm (FI) and 10 μm (G, J).

Histological analyses of the brains with experimental brain injury transplanted with the neural cells

We have conducted histological analysis of brains with the transplantation. The mouse brains were recovered 2 and 28 days after transplantation. We found cell aggregate at periventricular region that was corresponding to the transplantation site (Figs. 5B, E, F and G), which located just beneath the injured cortex (Figs. 5C, D). We next analyzed localization of the grafted cells using confocal laser microscope. We stained anti-NFM antibody with green and anti-human nuclei specific antibody with red. The latter antibody recognizes cynomolgus monkey ES-derived cells, but not mouse cells. We detected an area with a dense expression of NFM at the transplantation site with positive anti-human nuclei-specific antibody staining at day 10, 2 days after transplantation. The control PBS-injected group lacked nuclear staining of cynomolgus monkey ES derived cells (data not shown). The higher magnification of panel F in Fig. 5 revealed that the grafted cells accumulated to this area and surely expressed NFM (Fig. 5G). At day 36, 28 days after the transplantation, the cell aggregates disappeared from the grafted site (Fig. 5I). When we focused on the damaged cortex, we found the NFM expressing grafted cells migrating to the damaged area that were positive for anti-human nuclei specific antibody (arrows in Fig. 5I and arrowheads in Fig. 5J). The calculated numbers of the anti-human nuclei antibody-positive cells at the transplantation site and the cortex were 500 and 400 cells/mm2, respectively (Figs. 5G and J). The percentages of anti-human nuclei antibody-positive cells at the transplantation site at day 2 and at day 28 after transplantation were 91% and 3%, respectively. In contrast, those in the cortex at day 2 and at day 28 were 2% and 67%, respectively. These results suggested the migration of the transplanted cells from the injected site to the injured cortex. In addition, we observed migration of ES-derived mouse neural cells from the transplanted site to the injured cortex over corpus callosum (Ikeda et al., 2004). Moreover, we never observed any transplanted cells surviving in the brain without immunosuppression. These findings suggested that the disappearance of the grafted cells from the periventricular region was not because of their rejection but because of their dispersion to the damaged motor cortex. The cells both positive for anti-human nuclei antibody and glial markers (GFAP and GalC) were seldom or never observed at day 10 and day 36 (data not shown). In a preliminary experiment, we were unable to detect the grafted cells in the brain without immunosuppression. These histological findings were consistent throughout the transplanted mice.

When we focused on the brain recovered 2 days after the transplantation, small clusters of anti-human nestin antibody-positive cells were present around the injection site (Fig. 6A) (confer Fig. 5E). The antibody recognized monkey and human nestin but not mouse nestin, thus, the grafted cells were found to keep nestin positive for 2 days after the transplantation. Some of the grafted cells were also positive for β III tubulin 28 days after the transplantation (Figs. 6B, C and D). Synaptophysin-positive and Islet1-positive grafted cells (arrows in Figs. 6G and J) appeared in the damaged cortex (Figs. 6E, F, G, H, I and J), neighboring synaptophysin-positive and Islet1-positive host cells (arrowheads in Figs. 6G and J). At day 14 after the transplantation, the percentages of the transplanted cells positive for Islet1 and synaptophysin were 31% and 7%, while 43–50% and 14–15% at day 28, respectively (data showed representatives of each staining). Thus, reconstitution of functional neural network between the graft and the host neural cells may have occurred. These cells may contribute to the recovery of the motor function in the grafted mice.

Evaluation of blood flow on the grafted region by perfusion MRI

To monitor the regional blood flow that reflects activities of the grafted neural cells in the damaged cortex, functional MRI study was conducted. Perfusion of the damaged areas of the grafted mice was analyzed. Fig. 7B showed a representative blood flow of normal mouse brain. As shown in Fig. 7F, the injured area of the grafted mice after 7 days of the transplantation (day 15) showed the perfusion pattern resembling to the normal brain. However, the perfusion pattern of the control PBS injected site seemed to be disturbed as compared to that of normal brain (Fig. 7D). The recovery of regional blood flow pattern may reflect adaptation of the grafted cells to the recipient motor cortex and may suggest improved regional neural cell activity.

Discussion

Here, we describe a successful induction of transplantable neural cells enriched by motoneurons from cynomolgus monkey ES cells using RA (Chiba et al., 2004). Transplantation of the monkey ES-derived neural cells brought about functional improvement of hemiplegic mice with experimental brain injury.

There has been many publications presenting growth and differentiation of human ES cells in vitro (Amit and Itskovitz-Eldor, 2002; Carpenter et al., 2001; Odorico et al., 2001; Ostenfeld and Svendsen, 2003; Reubinoff et al., 2001; Schuldiner et al., 2001). The diverse differentiation of human ES cells makes them excellent candidates for transplantation therapies (Draper and Fox, 2003). 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 showed unique characteristics such as a formation of flat colonies, a tendency to produce the trophectoderm lineage and a different pattern of cell surface antigen expression (Nakatsuji and Suemori, 2002). They also showed no response to the LIF and gp130 signals that suppress spontaneous differentiation of mouse ES cells. Based on the fact, the culture conditions optimized for mouse ES cells to induce differentiation into specific lineage were not easily applicable for primate ES cells.

Primate and human ES cells can differentiate into neural lineage including neurons, dopaminergic neuron and astrocytes (Kawasaki et al., 2002; Mizuseki et al., 2003; Reubinoff et al., 2001; Schuldiner et al., 2001; Zhang et al., 2001). Culture conditions required for induction of neural cells from primate and human ES cells included MEDII medium, 3 dimensional collagen matrixes, astrocyte conditioned medium and withdrawal of fibroblast growth factor (FGF)2 (Calhoun et al., 2003; Chen et al., 2003; Kuo et al., 2003; Nakayama et al., 2003; Zhang et al., 2001; Zwaka and Thomson, 2003).

The RA treatment mainly generated neurons from the cynomolgus monkey ES cells, while small number of glial cells was found but oligodendrocytes were hardly detected in the cultures. In contrast, the same RA treatment of mouse ES cells induced neurons, astrocytes and oligodendrocytes in cultures (Chiba et al., 2004). Thus, neural cells induced by RA treatment of ES cells may have different characteristics between mouse and cynomolgus monkey.

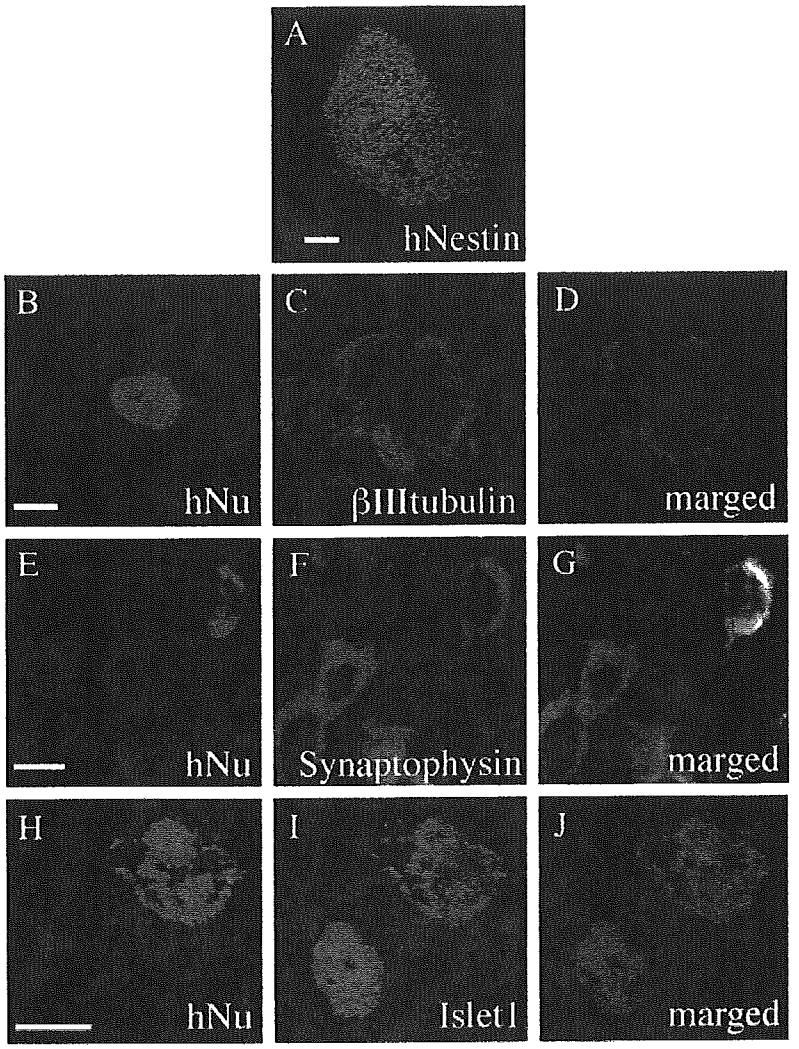

Fig. 6. Expression of neural markers in transplanted RA-treated cynomolgus monkey cells in host brain tissue. After transplantation of the RA-treated neural cells whose nuclei were anti-human nuclei antibody positive, the brains were recovered at days 10 and 28 for analyses. Panel A was higher magnification of the injection site (a boxed area in Fig. 5F). Panels B, C, D, E, F, G and H, I, J were higher magnifications of the injured cortical region (arrows in Fig. 5I). (A) Neural cells derived from cynomolgus monkey ES cells were positive for anti-human nestin antibody, which did not react with mouse nestin, on day 10, 2 days after transplantation. (B, C, D) Two color staining with anti-human nuclei (red) and anti βIIItubulin (green). The anti-human nuclei antibody-positive neural cells derived from cynomolgus monkey ES cells were positive for the neural marker on day 28. (E, F, G) Two-color staining with anti-human nuclei (red) and anti-synaptophysin (green). The anti-human nuclei antibody-positive nucleus was surrounded by synaptophysin immunostaining (an arrow). Circular synaptophysin immunostaining lacking anti-human nuclei antibody staining (an arrowhead) located near the anti-human nuclei antibody-positive nucleus (an arrow), suggesting possible synaptic connection between the host and the grafted neural cells. (H, I, J) Two-color staining with anti-human nuclei (red) and anti-Islet1 (green). The anti-human nuclei antibody-positive nucleus was simultaneously positive for Islet1 immunostaining (an arrow). Islet1 immunostaining lacking anti-human nuclei antibody staining (an arrowhead) located near the anti-human nuclei antibody-positive nucleus (an arrow). Three individual mice both at day 10 and at day 36 were analyzed and representative results were shown. The scale bars represent 5 μm (A, B, C, D) and 10 μm (E, F, G, H, I, J).

We have also succeeded in induction of neural lineage from cynomolgus monkey ES cells by using noggin (data not shown). However, the neural cells induced by noggin contained a few Islet1-positive motoneurons. Thus, we decided to use RA to induce the differentiation of primate ES cells into transplantable neural precursor cells enriched for motoneurons. They further differentiated into mature motoneurons after transplantation in injured cerebral cortex of mice, leading to motor functional recovery. In accordance with our observation, it was reported that earlier RA exposure was important for development of motoneuron precursors (Gallo et al., 2002). Collectively, we successfully established the induction method of sufficient amount of neurons including motoneurons from cynomolgus ES cells for neural transplantation to improve motor function of mice with experimental brain injury.

Transplantation of the neural cells originated from primate and human ES cells, and other precursor cells into rodents have been attempted and the details of the transplanted cells in the host were analyzed. However, the functional consequence of the transplanta-

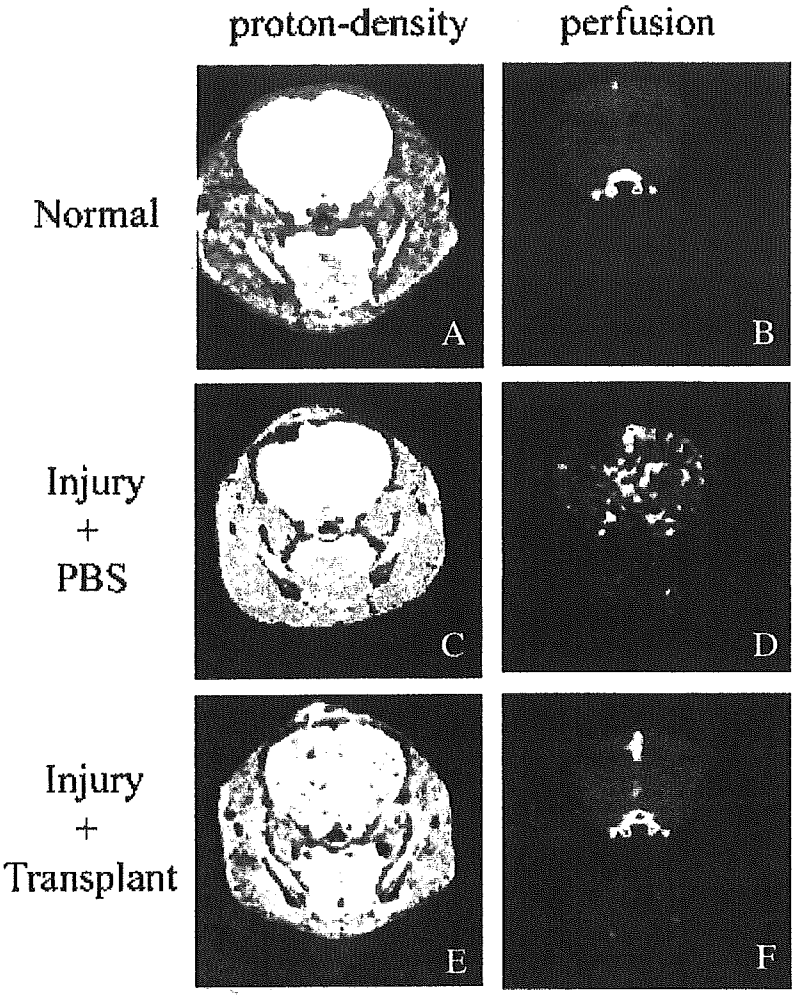

Fig. 7. Perfusion MRI studies revealed recovery of the local perfusion pattern by the transplantation of cynomolgus monkey ES cell-derived neural cells. The blood flow patterns in the brain of normal mice ($n = 3$) (A, B), the mice with cryogenic injury and PBS injection ($n = 6$) (C, D), and the mice transplanted with neural precursor cells ($n = 8$) (E, F) were analyzed at day 14 after the injection/transplantation by functional MRI study as described in Materials and methods. Representative results of proton-density images (A, C, E) and perfusion images (B, D, F) of each group of mice were shown.

tion has scarcely been reported. Previous reports demonstrated that human ES cell-derived neural progenitor cells transplanted into newborn mouse brain became incorporated into a variety of brain regions, where they differentiated into both neurons and astrocytes, however, mature oligodendrocytes were not detected. The donor-derived neurons demonstrated widespread distribution but not restricted to sites exhibiting neurogenesis (Reubinoff et al., 2001; Zhang et al., 2001). 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 but not oligodendrocytic differentiation, and might also improve survival of host cells (Wennersten et al., 2004).

Other cells, including bone marrow stromal cells and human adipose tissue-derived stromal cells, also successfully transplanted into rodent ischemic brain injury by middle cerebral artery occlusion and migrated into the injured cortex and differentiated into neurons and astrocytes (Kang et al., 2003; Lee et al., 2003). In rats transplanted with bone marrow stromal cells after traumatic brain injury, significant improvement of motor function was observed (Mahmood et al., 2004). We demonstrated here that primate ES derived neural cells transplanted into host brain survived and further differentiated into mature functional motoneurons but not glias, leading to improvement of motor functions. However, the transplantation did not change the size of the injured lesion. Synaptophysin immunostaining may support the possibility of reconstituting neural network with host neurons (Fig. 6). Furthermore, MRI study showed preferential recovery of the perfusion in damaged area in the grafted animal, demonstrating that the recovery of blood flow may have an advantage for functional activity of the neural graft in the damaged area. In this study, we first demonstrated that the neural cell transplantation derived from primate ES cells into damaged brain showed improvement of motor function of hemiplegic mice.

One important but unavoidable problem in allogeneic transplantation therapies using human ES cells is immunological rejection. Immunological reactions in brain were generally regarded as not so critical as other organs. In this study, however, we achieved successful xenogeneic transplantation and functional recoveries using immunosuppressants including cyclosporine. Our unpublished data showed that without the daily injection of

cyclosporine, the transplanted neural cells were unable to survive in the recipient brain, disappeared thereafter and the functional recoveries were not observed. So far, only limited studies have actually investigated the therapeutic effect of transplantation. To our knowledge, this is the first study to transplant neural cells derived from primate ES cells into damaged brain and showed functional recovery of hemiplegia.

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ヒト胚性幹細胞（ES細胞）

Human Embryonic Stem Cells

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Human Embryonic Stem Cells

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2003年，京都大学再生医科学研究所で国内初のヒトES細胞が3株樹立され，国産のヒトES細胞株の分配体制も整った。今後，国内のヒトES細胞研究は一層盛んになるものと思われる。本稿では，ヒトES細胞をとりまく研究の現況や今後の課題について，特に再生医療への応用の観点から概説してみたい。

1. はじめに

ES細胞（embryonic stem cell，胚性幹細胞）は，胚の初期段階である胚盤胞の内部細胞塊から分離される特殊な細胞であり，未分化状態を保持したまま分裂増殖して無限に自らのコピーを作り出せる能力（自己複製能）と体を構成するあらゆる細胞に分化する能力（多能性）とを併せもつ。

ES細胞は1981年にマウスで初めて試験管内で増殖できる細胞株として樹立されて以来[1,2]，分化・発生機構を解明するための基礎研究材料として，また遺伝子改変技術を応用した遺伝子の機能解析や疾患モデル作製のための研究ツールとして分子生物学の分野で広く利用されてきた。そして現在までに，マウス以外のさまざまな動物種でもES細胞の樹立が報告されてきている。霊長類に関しては，1995年にアカゲザルとコモンマーモセットからES細胞が樹立されたのが最初であり[3,4]，その後筆者らも，カニクイザルからのES細胞の樹立に成功している[5]。

ヒトES細胞としては，1998年11月，米国ウィスコンシン州立大学のトムソンらによってその樹立がなされ，科学雑誌『*Science*』に掲載されたのが最初となる[6]。この発表により，それまで一般の人にはあまり馴染みのなかったES細胞という言葉が一躍，“市民権”を得ることになり，以来，再生医療の切り札として関心を集め，各国の多くの施設でヒトES細胞を用いた研究が盛んに進められている。

2. ヒトES細胞

ヒトES細胞株はマウスES細胞株と比較していくつかの相違点がある。形態的には，マウスに比べてコロニー形態が扁平である（写真1）。マウスES細胞を未分化のまま培養・維持するためには，LIF（leukemia inhibitory factor）を培養液に添加しなければならないが，LIFにはヒトES細胞を維持する効果が認められていない。また，ES細胞の細胞表面マーカーであるSSEA（stage specific embryonic antigen）やTRA-1（tumor rejection antigen-1）等の発現パターンも，ヒトとマウスでは異なっている。これらの差異は，ヒトとマウスでは胚体組織や胚体外組織の構造など，胚発生の過程で異なる面があることに起因するのかもしれない。筆者らはカニクイザルES細胞の樹立に成功しているが[5]，サルのES細胞は，形態，培養条件，マーカー分子の発現パターンなど，ヒトES細胞のものとほぼ一致している。

ヒトES細胞に関する各国の考え方は，その作製を認めていないドイツのような国から，ヒトのクローン胚研究までもを認めるイギリスまでさまざまである。NIH（National Institutes of Health；アメリカ）が公表した資料によると，アメリカ，オーストラリア，スウェーデン，インド，イスラエル，韓国の6か国・14機関の企業や大学で分離された75株のヒトES細胞がリストされている（表1）。アメリカではヒト胚作製や，ヒト胚を破壊，廃棄し，故意に傷つけ，または死に至らしめる研究に対して連邦資金を支出することを禁じており，ヒトES細胞の樹立やクローン技術による胚の作製も禁止の対象に含まれている。実際にトムソンらは公的資金には頼らず，米ジェロン社が提供した民間資金を利用してES細胞の樹立に成功しており，これをきっかけにアメ

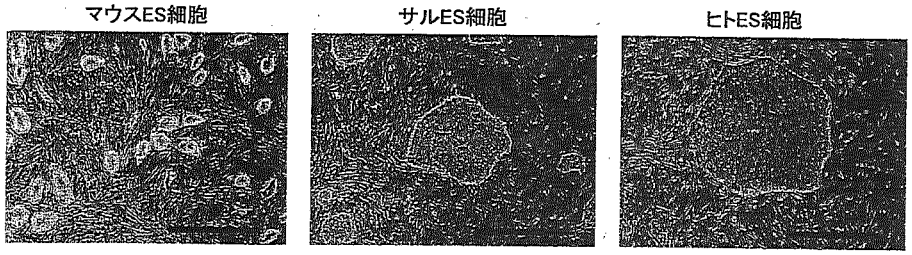

写真1　ES細胞コロニー形態の比較（カラー写真参照）
（Scale Barは1mm）

表1　ヒトES細胞株の樹立機関と所有細胞株数

樹立機関	細胞株数
Wisconsin Alumni Research Foundation（米国ウィスコンシン州 Madison）	5
California大学（米国カリフォルニア州 San Francisco）	2
CyThera社（米国カリフォルニア州 San Diego）	9
Geron Corporation（米国カリフォルニア州 Mcnlo Park）	7
BresaGen社（豪州・米国支店はジョージア州 Athenes）	4
ES Cell Internatinal社（豪州 Melbourne）	6
Technion-Israel Institute of Technology（イスラエル Haifa）	4
Reliance Life Sciences（インド Mumbai）	7
Pochon CHA University（韓国 Seoul）	2
Maria Biotech社（韓国 Seoul）	3
Seoul National University（韓国 Seoul）	1
Cellartis社（スウェーデン Goteborg）	3
Karolinska Institute（スウェーデン Stockholm）	6
Goteborg大学（スウェーデン Goteborg）	16

米国NIHの資料より

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