[2, 12, 16]. A similar phenomenon has been observed in familial migraine and cluster headache [17, 21]. These findings seem to suggest that genetic factors affect age at onset of familial moyamoya disease. The patients in the sporadic group more often present with cerebral infarct and intracranial hemorrhage than do those in the familial group (Table 1). The difference most likely results from the finding that mean age at onset is significantly higher in the sporadic than in the familial group.

Third, the current study reveals that mean age at onset is significantly lower in the second than in the first generation among the eight parent–offspring pairs. These results are the same with that of a recent literature review on familial moyamoya disease. Thus, the parent–offspring pairs of 16 pedigrees have previously been reported. Mean age at onset of the 16 parents is significantly higher than that of their 20 children, 39.5±12.8 and 12.7±8.0 years, respectively ($P<0.0001$; [15]). These results strongly suggest that anticipation may be closely associated with familial moyamoya disease.

Anticipation and expansion of repeat sequence

The clinical phenomenon of decreasing age at onset and/or increasing severity of symptoms of a disease in successive generations within a pedigree has been termed anticipation [1]. In total, 73 familial disorders have been reported to be linked to anticipation. Of these, responsible genes have previously been clarified in 20 familial disorders, most of which are neurological or neuropsychiatric disorders, such as myotonic dystrophy and Huntington's disease. Recent studies have strongly suggested that anticipation is caused by pathogenic unstable triplet repeat. In many of these disorders, repeat size correlates with severity and inversely with age at onset rather than penetrance. As the repeats tend to expand during transmission between generations, the age at onset tends to decrease and the severity tends to increase. This instability has led to the description of pathogenic repeat sequences as dynamic mutations [7, 18].

Of the eight parent–offspring pairs in the present study, all were maternal inheritance. There is increasing evidence that imprinting phenomenon may be associated with anticipation in some familial neurological disorders, including Huntington's disease. Genomic imprinting has been defined as "the differential expression of genetic material, at either a chromosome or allelic level, depending on whether the genetic material has come from the male or female parent" [6]. Previous studies have suggested that the methylation of CpG island that often functions as a strong promoter plays a central role in genomic imprinting [6]. Therefore, genomic imprinting may also affect the predominance of maternal inheritance in familial moyamoya disease.

Limitation of the current study

As described above, the responsible genes for familial moyamoya disease have not been determined, although microsatellite linkage analyses have shown the genetic loci on chromosomes 3, 6, and 17 [8, 9, 25]. Indeed, positional cloning analysis has failed to identify the possible genes [15]. Therefore, the present results can be a guiding principle in research efforts for elucidating the genes.

The present study is the first attempt to statistically analyze the clinical features of familial moyamoya disease and strongly suggests the association of anticipation. Of course, however, it should be reminded that the signs of anticipation may be attributed to several sampling and observation biases, including the tendency to select the parents with late onset and the offspring with early onset [3]. Another possible bias that may mimic anticipation can result from shared environmental factors because the affected individuals within families are not widely distributed geographically and across time. Therefore, a larger sample size of familial moyamoya disease would be necessary to minimize all possible biases, verifying the present results.

Another difficulty should also be taken into consideration in analyzing the clinical manifestations of familial moyamoya disease. Thus, only 40 years has passed since moyamoya disease was identified as a clinical entity [19], and it is very difficult to obtain accurate medical records of three- or four-generation families with moyamoya disease. A prospective follow-up study over several generations within families may clarify the clinical feature of familial moyamoya disease.

Conclusion

In this study, the authors statistically analyzed the clinical features of familial and sporadic cases of moyamoya disease. The results strongly suggest that anticipation may be closely related to familial moyamoya disease, although further studies are necessary. The present results may shed light on future research for identifying the genes responsible for familial moyamoya disease.

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Survival and differentiation of neural progenitor cells derived from embryonic stem cells and transplanted into ischemic brain

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Object. Cell replacement therapy including the use of embryonic stem cells (ESCs) may represent a novel treatment for damage from stroke. In this study, the authors transplanted neural progenitor cells (NPCs) derived from ESCs into ischemic brain and analyzed their survival and differentiation.

Methods. Multipotential NPCs were generated from ESCs by using the stromal cell–derived inducing activity method. These cells could differentiate in vitro into neurons, glia, and oligodendrocytes, thus revealing them to be neural stem cells. The NPCs were then transplanted into ischemic brain. At 2 weeks postischemia, the transplanted cells occupied 18.8 ± 2.5% of the hemispheric area; by 4 weeks postischemia, 26.5 ± 4% of the hemisphere. At 4 weeks after transplantation, green fluorescent protein (GFP)–positive transplanted cells showed mature neuronal morphological features. The authors also investigated the expression of differentiation markers and various neurotransmitters. Transplanted cells were immunopositive for neuronal nuclei, β-tubulin-III, and glial fibrillary acidic protein. Of the GFP-positive cells, 33.3 ± 11.5% were positive for glutamate decarboxylase, 13.3 ± 5.8% for glutamate, 2.1 ± 2.5% for tyrosine hydroxylase, 1.8 ± 2% for serotonin, and 0.4 ± 0.2% for choline acetyltransferase.

Conclusions. The authors confirmed the survival and differentiation of ESC-derived NPCs transplanted into the ischemic brain. Surviving transplanted cells expressed several neural markers and neurotransmitters. These findings indicate that these cells can function in the brain.

KEY WORDS • **neural progenitor cell** • **embryonic stem cell** • **brain ischemia** • **stroke** • **mouse**

STROKE affects millions of people worldwide, with more than 500,000 new patients per year in the US alone. Although fewer than one third of strokes are fatal, approximately 60% of patients show significant residual impairments and the prevalence of stroke-related morbidity is expected to increase as the population ages because there is no therapy to reverse these effects. Cell replacement therapy, including the use of ESCs,[8] neural stem cells,[19,20,23,26,29] and bone marrow stromal cells,[3,6,7,15,16,32] may represent a novel treatment for stroke damage.[4,13]

Embryonic stem cells can be expanded to large numbers while maintaining their potential to differentiate into various somatic cell types of the three germ layers, and the in vitro differentiation of ESCs thus can provide donor cells for transplantation therapies. Indeed, ESCs have been found to differentiate in vitro into many clinically relevant cell types, including hematopoietic cells, cardiomyocytes, insulin-secreting cells, neurons, and glia.[4,13] Following transplantation into the central nervous system, ESC-derived neural precursors have been shown to integrate into host tissue and, in some cases, to promote functional improvement.[8] After brain ischemia, many types of cells are lost, including neurons, glia, and oligodendrocytes. It is hoped that NPCs can produce replacements.[26,31] Recently, Kawasaki and colleagues[12] reported that the SDIA method could easily produce NPCs from ESCs. In the present study, we generated NPCs from ESCs, expanded them as neurospheres, and transplanted them into the ischemic brain. To our knowledge, this is the first report on the use of ESC-derived neurons for transplantation into the ischemic brain.

Abbreviations used in this paper: ChAT = choline acetyltransferase; CNPase = cyclic nucleotide phosphodiesterase; ESC = embryonic stem cell; GAD = glutamate decarboxylase; GalC = galactocerebroside; GFAP = glial fibrillary acidic protein; GFP = green fluorescent protein; LIF = leukemia inhibitory factor; MCA = middle cerebral artery; NeuN = neuronal nuclei; NPC = neural progenitor cell; PBS = phosphate-buffered saline; rCBF = regional cerebral blood flow; SDIA = stromal cell–derived inducing activity; TH = tyrosine hydroxylase; TuJ1 = β-tubulin-III.

Materials and Methods

Induction of Neural Differentiation of ESCs

Undifferentiated murine ESCs (G4-2) were maintained on gelatin-coated dishes in Glasgow minimum essential medium (Sigma, St.

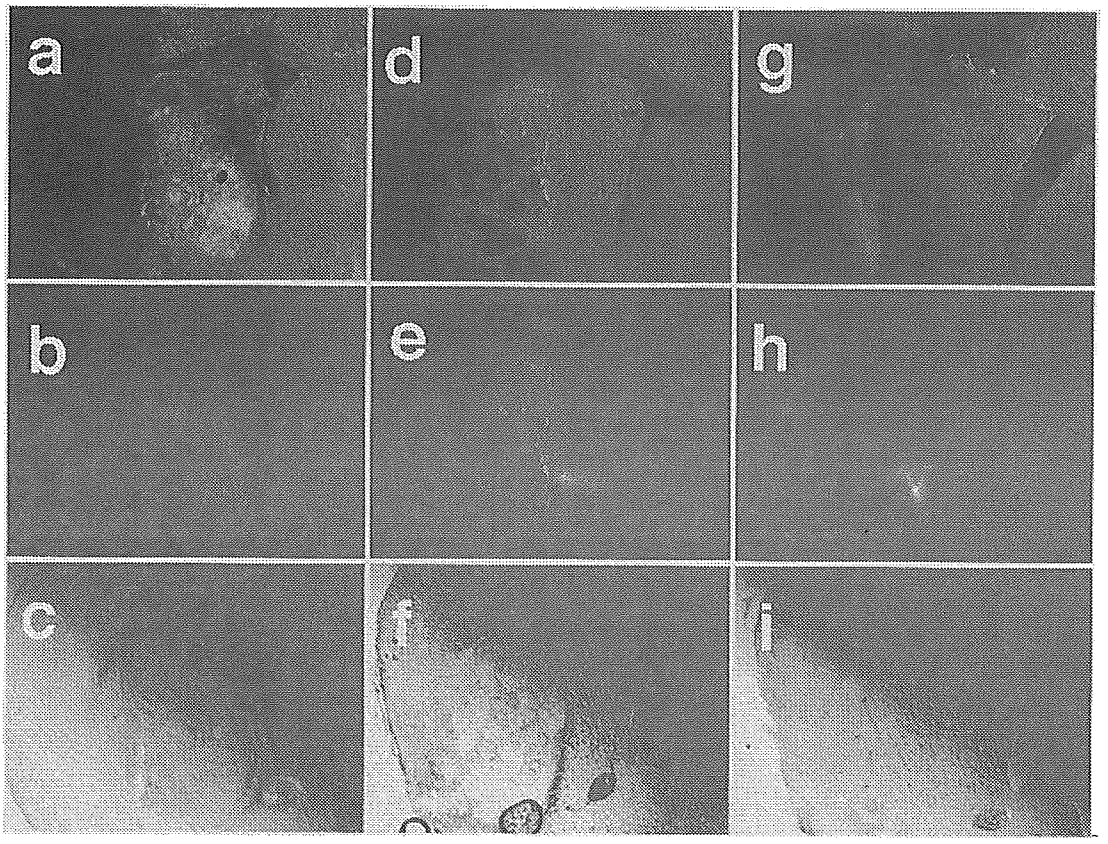

FIG. 2. Images demonstrating survival in the ischemic brain of transplanted neurospheres made from mouse ESCs 2 weeks after transplantation (a–c), 4 weeks after transplantation (d–f), and in sham controls (g–i). Red indicates NeuN (a, d, and g); green, GFP (b, e, and h). The GFP-positive cells occupied the ischemic areas (a–f), especially 4 weeks after ischemia (d–f). Interference differential microscopic images (c, f, and i) demonstrate the area of infarction, which was located in the lateral striatum after ischemia (c and f). Original magnification × 40.

shown in Fig. 2, surviving cells occupied a small area (Fig. 2g–i). Infarct volumes were not significantly different between the specimens prepared 2 weeks after ischemia and those 4 weeks after ischemia (data not shown).

Differentiation of Transplanted ESC-Derived NPCs

At 4 weeks after transplantation, GFP-positive cells demonstrated mature neuronal morphological features (Fig. 3a). In addition, we investigated the expression of both the differentiation markers and the various neurotransmitters (Fig. 3 and Table 2). Transplanted cells were immunopositive for NeuN (Fig. 3b), TuJ1, and GFAP (Fig. 3c). Of the GFP-positive cells, 60 ± 10% were NeuN-positive, 40 ± 10% TUJ1-positive, and 22 ± 7.2% GFAP-positive. Only 0.4 ± 0.5% of the GFP-positive cells were GalC-positive. Next we examined neurotransmitter expression of the transplanted cells. Of the GFP-positive cells, 33.3 ± 11.5% were GAD-positive (Fig. 3d), 13.3 ± 5.8% glutamate-positive (Fig. 3e), 2.1 ± 2.5% TH-positive (Fig. 3g), 1.8 ± 2% serotonin-positive (Fig. 3h), and 0.4 ± 0.2% ChAT-positive (Fig. 3e) cells.

TABLE 1

*Occupied area of transplanted cells in the brain**

Group	% of Hemisphere Occupied
controls	4.0 ± 1.2
2 weeks postischemia	18.8 ± 2.5
4 weeks postischemia	26.5 ± 4.0

* Data are expressed as the means ± standard deviation.

Discussion

In this study, we generated multipotential NPCs from ESCs and transplanted them into ischemic mouse brain. These cells could survive and expand widely in the ischemic area. Moreover, the transplanted NPCs differentiated into various types of neural cells.

TABLE 2

*Differentiation of transplanted cells in the ischemic brain**

Marker	% GFP
NeuN	60.0 ± 10.0
TuJ1	40.0 ± 10.0
GFAP	22.0 ± 7.2
GalC	0.38 ± 5.3
TH	0.6 ± 0.36
GAD	33.3 ± 11.5
Glutamate	13.3 ± 5.8
Serotonin	0.5 ± 0.44
ChAT	0.4 ± 0.2

* Data are expressed as the means ± standard deviation.

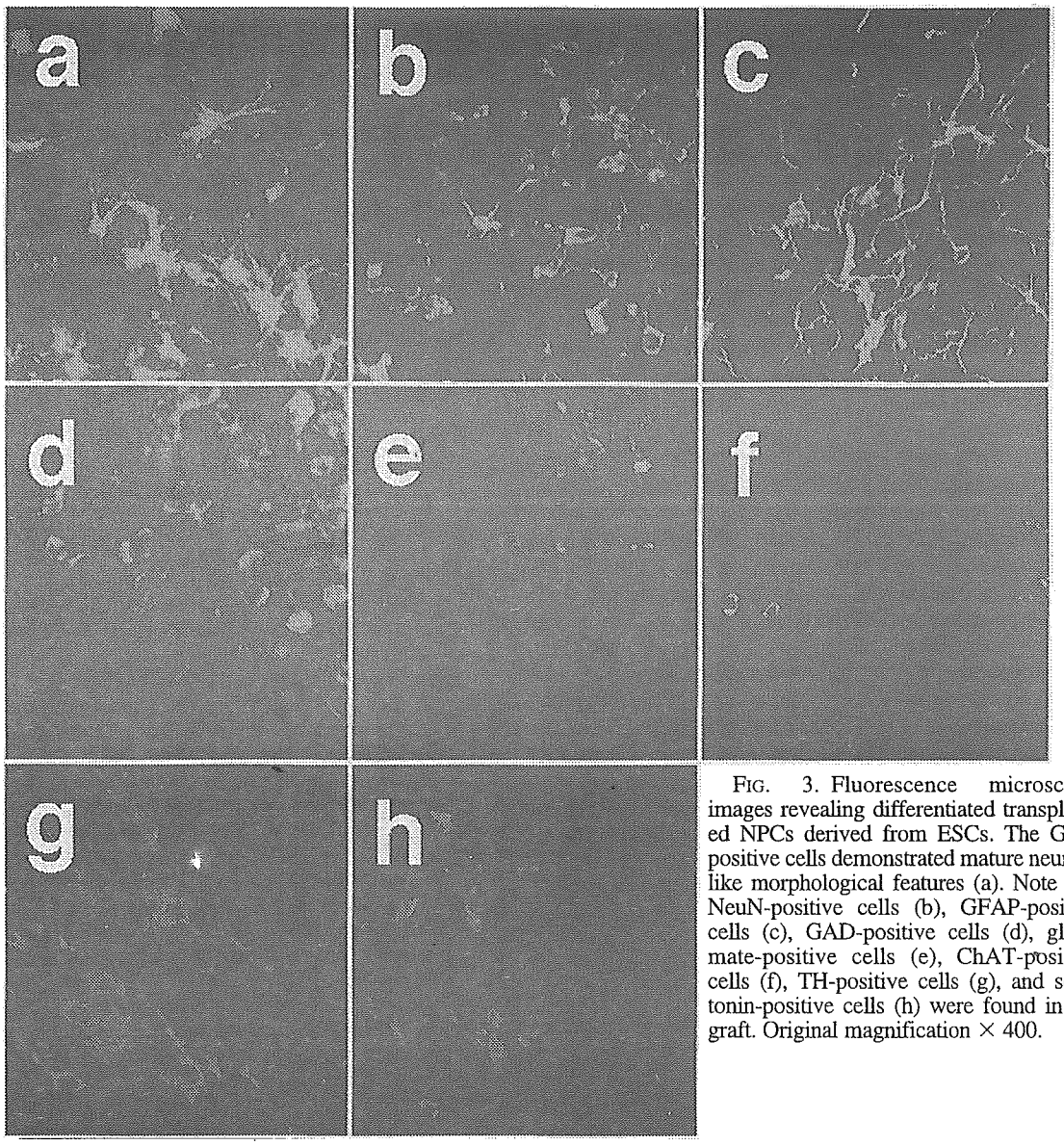

FIG. 3. Fluorescence microscopy images revealing differentiated transplanted NPCs derived from ESCs. The GFP-positive cells demonstrated mature neuron-like morphological features (a). Note that NeuN-positive cells (b), GFAP-positive cells (c), GAD-positive cells (d), glutamate-positive cells (e), ChAT-positive cells (f), TH-positive cells (g), and serotonin-positive cells (h) were found in the graft. Original magnification × 400.

Embryonic stem cells have many characteristics required for an optimal source for cell replacement therapy.[4] The ESCs are self-renewing, multipotent cells derived from the inner cell mass of the preimplantation blastocyst.[4] Kawasaki and colleagues[12] previously reported a strong neuralization-inducing activity present on the cell surface of stromal cells and named it "SDIA." In the absence of exogenous BMP4, mouse ESCs were shown to differentiate efficiently into NPCs and neurons when cultured on SDIA-possessing mouse stromal cells (PA6 cells) for 1 week.[12] Recently, the SDIA method has also become applicable for primate ESCs. After having been cultured on PA6 cells for 2 weeks, the majority of primate ESC colonies contained a large number of NPCs and postmitotic neurons.[12] Neural progenitor cells have multipotent, self-renewing capacities and can be cultured as neurospheres.

In this study, we analyzed several neurotransmitters' expressions. Note that GAD is a γ-aminobutyric acid synthetic enzyme. The γ-aminobutyric acidergic neurons in the cerebellum are rich in ventral mesencephalon or Purkinje cells. Choline acetyltransferase is a synthetic enzyme of acetylcholine. Cholinergic neurons are rich in the basal forebrain and associated with Alzheimer disease. Glutamate is used by descending pathways originating from neocortical pyramidal cells. The dorsal raphe nucleus is known to be rich in serotonin, which is implicated in emotion, fear, and cognition. Dopaminergic neurons of the substantia nigra are lost because of Parkinson disease. Tyrosine hydroxylase expression is linked to the secretion of levadopa, which is a dopamine precursor.[25,27,30] To determine cell types, we used several markers. Beta-Tubulin-III is expressed in postmitotic neunrons at an early stage of development. The NeuN is a nuclear protein that is a marker of a mature neuron. Both GalC and CNPase are thought to be mature oligodendrocyte markers. As an astrocyte marker, GFAP is detectable during fetal glial development.[2]

The use of neural transplantation for the treatment of neurological diseases first became a potential therapeutic modality in 1979 when Bjorklund and colleagues[5,13,24] demonstrated that implanting dopaminergic-containing neurons

into the rat striatum improved functional deficits induced by damage to the nigrostriatal pathway. Since then, advances in neural transplantation have moved from the animal model to the human model, with varying degrees of success.[11,19,20,23,29] In the animal models, authors examined a wide variety of disease states—from degenerative diseases to trauma and stroke—and the tissues used for transplantation—from fetal tissue to tumor lines to stem cells.[19,20,23,26,29] In some models, implants provide a source of neurotrophic factors.[6,7,15] Successes in animal models have led to transplant trials in the human population. Patient trials have been focused on transplantation for Parkinson disease, Huntington disease, spinal cord injury, and stroke.[4,13] As research in animal models progresses, transplant trials may be initiated for the treatment of multiple sclerosis, traumatic brain injury, cerebral palsy, amyotrophic lateral sclerosis, Alzheimer disease, and other disorders.[4,13,26]

In patients disabled by stroke, the concept of restoring function by transplanting human neuronal cells into the brain is a novel one. Data obtained from a rat model of transient focal cerebral ischemia demonstrated that transplantation of fetal tissue restored both behavioral and motor functions.[17,19,20,29] As for studies in humans, Kondziolka and colleagues[14,18] reported the results of a clinical trial using human neuronal cells. In examining 12 patients in this trial, their initial objective was to demonstrate the safety and feasibility of the neuronal cell implantation procedure. Among the treatment groups, mean National Institutes of Health Stroke Scale total scores decreased and mean European Stroke Scale total scores increased—both changes indicating improvement.[14,18] The transplanted cells were proposed to have improved neurological function through a number of different mechanisms, including provision of neurotrophic support, production of neurotransmitters, reestablishment of local interneuronal connections, cell differentiation and integration, and improvement of regional O_2 tension.

In the present study, we used ESC-derived NPCs for transplantation. The advantage in ESCs is that they can be expanded easily compared with neural stem cells. We also confirmed the differentiation of ESC-derived NPCs. During ischemia, various types of neurons as well as glial cells and oligodendrocytes are lost. The ESCs could supply these cells. Interestingly, in the sham-operated control brains, the transplanted cells occupied only a small area. On the contrary, in the ischemic brain, the transplanted cells spread throughout the ischemic lesion. This result indicates that the fate of the graft is dependent on the host environment. After ischemia, several cytokines and growth factors are known to be released. Today, the family of growth and trophic factors has been proposed to affect the survival and development of neuroprogenitor cells. Among them, LIF and ciliary neurotrophic factor in addition to more traditional growth factors, such as platelet-derived growth factor, are considered to be potent promoters of neuroprogenitor cell proliferation and their eventual differentiation.[1,22] Moreover, brain-derived neurotrophic factor, another member of the neurotrophin family (which includes nerve growth factor, neurotrophin-3, and neurotrophin-4/5), was shown to have great potency in modulating the growth and survival of dopaminergic cells and their precursors.[1,22] Glial-derived neurotrophic factor has similar or even enhanced trophic effects on dopaminergic neurons and their precursors.[1,22]

In using a 30-minute ischemia model, we did not examine behavioral improvement after transplantation, because such a model demonstrates only a slight behavioral deficit, thus making it difficult to assess behavior. Therefore we will use a longer period of ischemia in the next study and will examine network formation.

Conclusions

In summary, we confirmed the survival and differentiation of ESC-derived NPCs transplanted into the ischemic brain. We used the SDIA method on murine ESCs. Note that this method is also effective on primate and even human ESCs. Our findings indicated that ESC-derived NPCs can function in the brain.

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