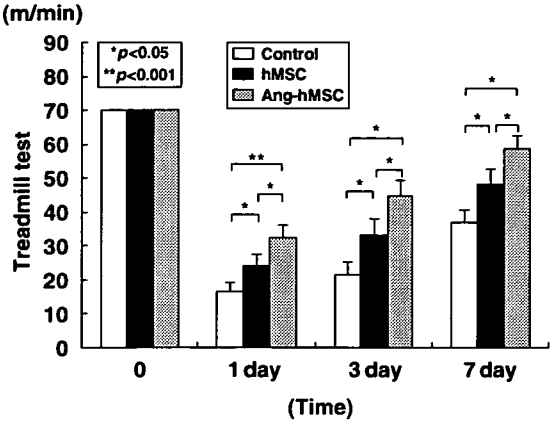

Figure 7 The treadmill stress test demonstrates that the maximum speed at which the rats could run on a motor-driven treadmill was faster in the hMSC- and the Ang-hMSC-treated rats than control. One day after MCAO, rats that received Ang-hMSC showed higher running speed compared with the medium alone and the hMSC group. From 3 days on after MCAO, the hMSC and Ang-hMSC groups attained higher velocities but the Ang-hMSC group was greater. The highest level of functional improvement was attained at 7 days.

it resistant to the damage and leak by VEGF or inflammatory challenges (Yancopoulos *et al*, 2000).

Vascular endothelial growth factor displays other effects such as an increase in the endothelium permeability (Bates *et al*, 2002). This effect could lead to vasogenic edema, particularly deleterious in cerebral ischemia. Indeed, early administration of VEGF after MCAO exacerbates blood–brain barrier leakage, causing massive brain edema (Zhang *et al*, 2000). Systemic delivery of Ang-1 by adenoviral gene delivery causes resistance to vascular leakage induced by VEGF in the brain (Zhang *et al*, 2002) and in the skin (Thurston *et al*, 1999).

Upregulation of endogenous Ang-1 in ischemic brain tissue was reported (Beck *et al*, 2000; Zhang *et al*, 2002), suggesting that Ang-1 might play an important role in endogenous repair in the ischemic lesion. However, therapeutic effects of exogenously applied Ang-1 on ischemic brain had not been studied. In this study, we applied Ang-1 by delivering hMSCs that hypersecrete Ang-1 to determine if Ang-1 would augment neovascularization in the post-ischemic brain and possibly improve functional outcome. Although in three-dimensional image analysis for angiogenesis, the Ang-hMSC-transplanted group showed small border areas of increased angiogenesis as compared with the hMSC transplanted and control groups, the effects were small and regional. Similarly, both hMSCs and Ang-hMSCs transplantation groups showed improvement of rCBF in the lesion, and only small areas of increased rCBF were observed in the Ang-hMSC transplantation group as compared with the hMSC group. No increase in cerebral edema was observed for either cell type as assayed by both *in vivo* MRI and histologic analysis. Thus, cellular delivery of Ang-1 to the ischemic brain had only modest effects on angiogenesis. One possibility to account for the modest effects is that the endogenous upregulation of Ang-1 in ischemic brain (Beck *et al*, 2000; Zhang *et al*, 2002) may be sufficient to support the observed angiogenesis after hMSC infusion, and additional Ang-1 is unnecessary. Our immunohistochemical data suggest that Ang-hMSCs do not result in extensive or dynamic angiogenesis. However, in studies using genetically modified hMSC to express trophic factors including brain-derived neurotrophic factor (Nomura *et al*, 2005), placental growth factor (PlGF) (Liu *et al*, 2006), or glial cell line-derived neurotrophic factor (Horita *et al*, 2006) lesion volume was reduced over that observed after non-modified hMSCs. Thus, a neuroprotective effect from hMSC delivery may be an important if not the dominant mechanism to account for reduction in lesion size and improved functional outcome.

Cell-based therapeutic approaches are being considered for a number of neurologic diseases (Chopp *et al*, 2000; Pluchino *et al*, 2003; Iihoshi *et al*, 2004; Nomura *et al*, 2005; Pluchino *et al*, 2005; Zhang *et al*, 2005; Honma *et al*, 2006; Horita *et al*, 2006, Liu *et al*, 2006). Suggested mechanisms include reduction of inflammatory infiltration, elevation of trophic factors that may be neuroprotective, and structural repair such as remyelination (Radtke *et al*, 2007). A cell-based therapy may have the advantage of exerting multiple therapeutic effects at various sites and times within the lesion as the cells respond to a particular pathologic microenvironment.

Our results indicate that infusion of non-genetically modified hMSCs results in significant reduction in ischemic lesion volume and improved functional outcome, and Ang-hMSC had a modest additional influence on angiogenesis and functional outcome. Thus, hMSCs without genetic modification to induce additional neovascularization may be a useful source of cells for a cell-based therapeutic approach in clinical studies.

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Childs Nerv Syst (2007) 23:1195–1198
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CASE REPORT

Symptomatic hyperperfusion after superficial temporal artery–middle cerebral artery anastomosis in a child with moyamoya disease

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Abstract
Object Surgical revascularization for moyamoya disease prevents cerebral ischemic attacks by improving cerebral blood flow (CBF). It is undetermined, however, how rapid increase in CBF affects ischemic brain at acute stage, especially in children.
Case report A 4-year-old girl with moyamoya disease underwent right superficial temporal artery–middle cerebral artery (STA–MCA) anastomosis. She suffered temporary left facial palsy 5 days after surgery. Postoperative *N*-isopropyl-*p*-[^{123}I]iodoamphetamine single-photon emission computed tomography (^{123}I-IMP-SPECT) revealed focal intense increase in CBF at the sites of anastomosis. Magnetic resonance imaging/angiography showed the apparently patent STA–MCA anastomosis as a thick high signal without ischemic changes. Her symptom improved 9 days after surgery, and single-photon emission computed tomography (SPECT) 2 months later showed normalization of CBF. Surgical revascularization completely relieved the transient ischemic attack on her left hand that was seen before surgery.
Conclusion We demonstrated, for the first time, that delayed focal neurological deficit after STA–MCA anastomosis can be caused by focal hyperperfusion in childhood moyamoya disease.

Keywords Moyamoya disease · Superficial temporal artery–middle cerebral artery anastomosis · Hyperperfusion · Single-photon emission computed tomography

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Introduction

Moyamoya disease is a chronic, occlusive cerebrovascular disease with unknown etiology characterized by bilateral steno-occlusive changes at the terminal portion of the internal carotid artery and an abnormal vascular network at the base of the brain [19]. Surgical revascularization for moyamoya disease is believed to prevent cerebral ischemic attacks by improving cerebral blood flow (CBF), and superficial temporal artery–middle cerebral artery (STA–MCA) anastomosis with or without indirect bypass is generally employed as the standard surgical treatment for moyamoya disease [6, 8, 16].

Cerebrovascular reconstruction surgery including carotid endarterectomy or STA–MCA anastomosis in patients with atherosclerotic cerebral steno-occlusive diseases can cause a rapid increase in CBF in the chronic ischemic brain, resulting in complications such as 'cerebral hyperperfusion syndrome.' 'Cerebral hyperperfusion syndrome' is characterized by unilateral headache, face and eye pain, seizures, and focal symptoms that occur secondary to cerebral edema or intracerebral hemorrhage [14, 17, 18]. Due to the relatively low flow revascularization obtained by surgery for moyamoya disease, postoperative hyperperfusion syndrome has been considered to be less common in this entity, except for limited reports [4, 12], compared to perioperative transient ischemic attack [5, 7, 15]. However, the exact

effect of STA–MCA anastomosis on ischemic brain of moyamoya disease at the acute stage is undetermined due to the lack of comprehensive CBF study immediately after revascularization for this entity.

To clarify this critical issue, we prospectively performed both *N*-isopropyl-*p*-[^{123}I] iodoamphetamine single-photon emission computed tomography (^{123}I-IMP-SPECT) and magnetic resonance (MR) imaging within 1 week after surgery during the past 2 years [3]. Among the 78 consecutive surgeries, we had an experience of a 4-year-old girl manifesting delayed transient facial palsy due to hyperperfusion 5 to 9 days after single STA–MCA anastomosis. Anatomical location and temporal profile of hyperperfusion were completely in accordance with her symptom. The reversibility of both her symptom and the SPECT finding further convinced the central role of hyperperfusion in her postoperative neurological deterioration.

Case material

A 4-year-old girl with moyamoya disease presented with transient ischemic attach on her left hand. MR imaging and MR angiography demonstrated steno-occlusive changes at the terminal portions of the bilateral internal carotid arteries and the presence of abnormal network-like vessels at the bilateral basal ganglia, which satisfied the diagnostic criteria for moyamoya disease [19] according to the criteria of the Research Committee on Spontaneous Occlusion of the Circle of Willis of the Ministry of Health, Labor, and Welfare, Japan. Single STA–MCA anastomosis with encephalo-duro-myo-synangiosis (EDMS) and dural pedicle insertion [16, 21] were performed on the right. After exploring the parietal branch of the left STA, fronto-temporo-parietal craniotomy was performed. The recipient artery at the M4 segment of the MCA was then explored and anastomosis was performed between the stump of the STA (0.8 mm in diameter) and the proximal portion of the M4 segment (0.8 mm in diameter) that supplied the fronto-parietal lobe. Then EDMS and dural pedicle insertion were performed. She showed no neurological deficit immediately after surgery. The ^{123}I-IMP-SPECT 1 day after surgery showed no apparent change in CBF at the site of anastomosis (Fig. 1b) compared to the preoperative findings (Fig. 1a). Postoperative diffusion-weighted MR imaging 2 days after surgery showed no evidence of ischemic change (Fig. 2a). MR angiography showed the apparently patent STA–MCA bypass (Fig. 2c, arrow). She suffered left facial palsy and numbness on her left hand 5 days after surgery. The SPECT 7 days after surgery showed focal intense increase in CBF at the sites of anastomosis (Fig. 1c). MR imaging showed no ischemic changes, and MR angiography showed the thick high signal of STA (Fig. 2d, arrow) compared to that seen 2 days after surgery (Fig. 2c). We suspected the involvement of hyperperfusion in her neurological deterioration, and we kept her under intensive blood pressure control with the intravenous administration of

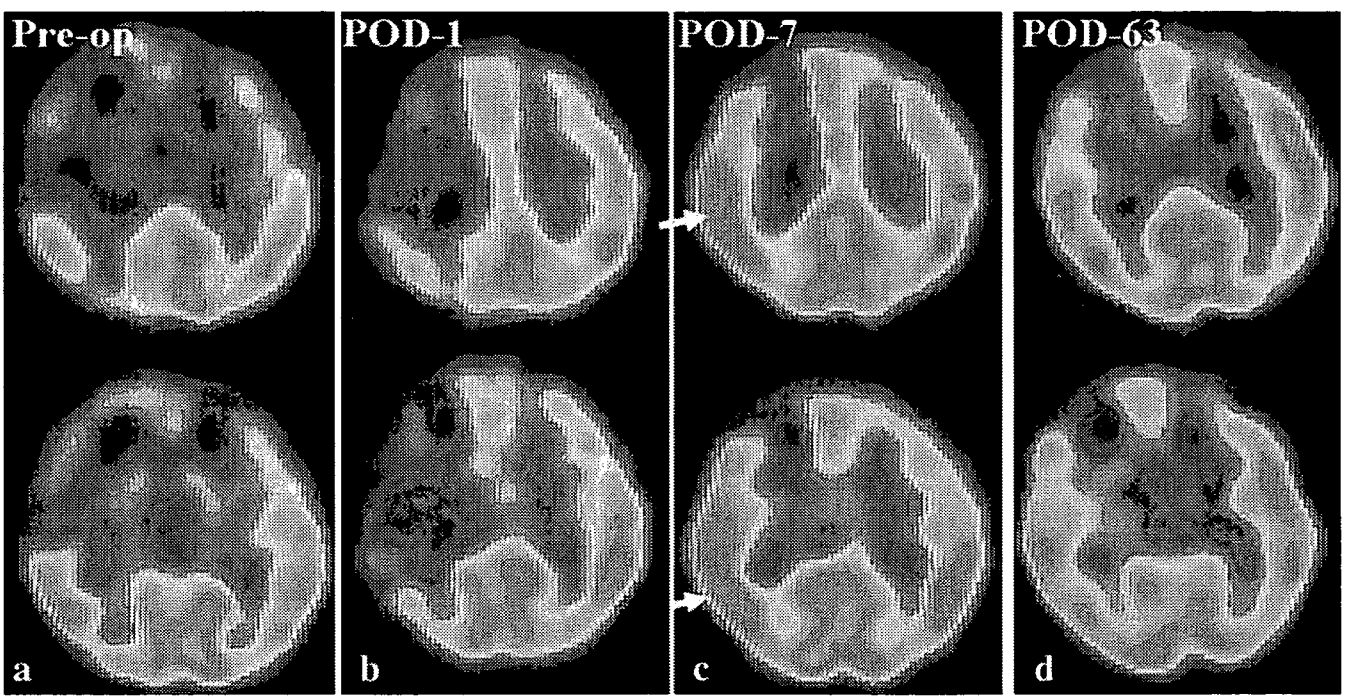

Fig. 1 ^{123}I-IMP-SPECT scans before surgery (**a**) and 1 (**b**), 7 (**c**), and 63 days (**d**) after right STA–MCA anastomosis. The focal intense increase in CBF at the site of anastomosis (*arrows* in **c**) was evident 7 days after surgery, when the patient suffered left facial palsy. Two months later, normalization of CBF at the territory of the right MCA was evident (**d**) in accordance with the recovery of her neurological deficit

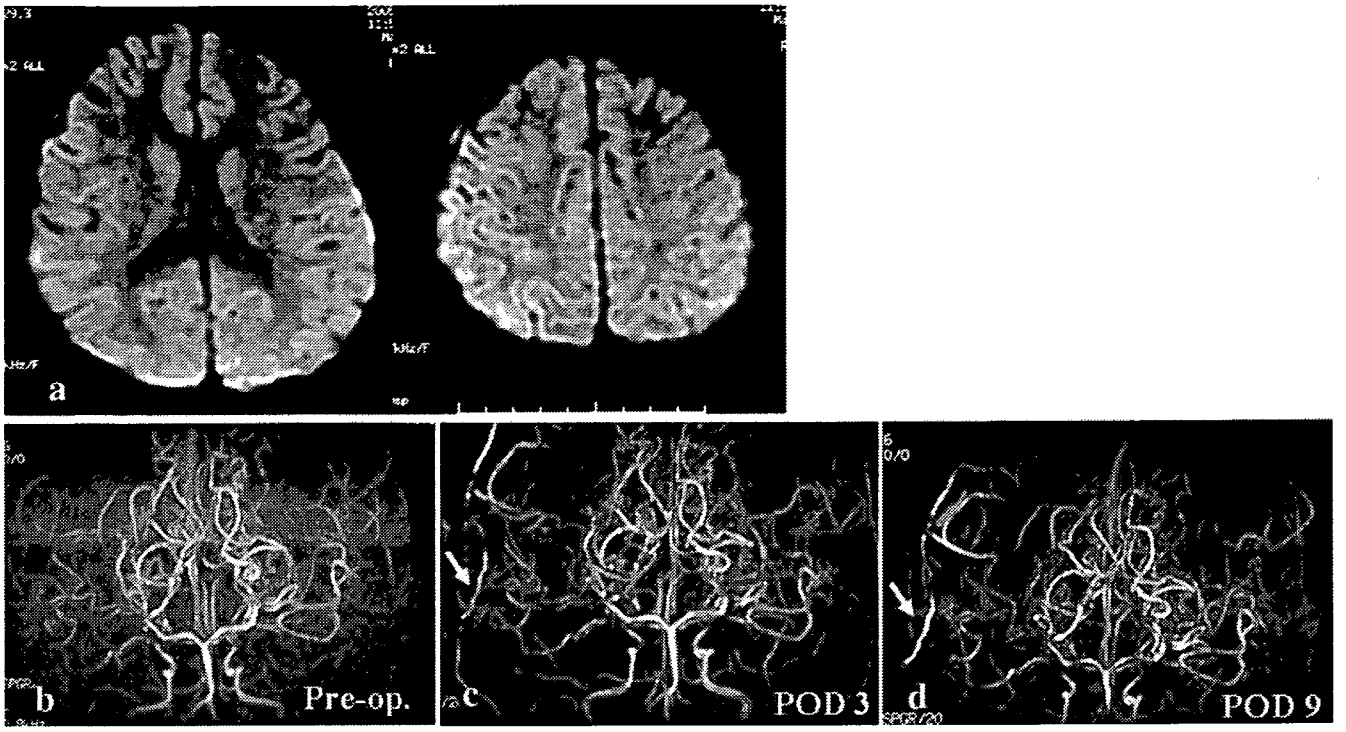

Fig. 2 Postoperative diffusion-weighted magnetic resonance images 2 days after surgery showing no evidence of ischemic change (**a**). Magnetic resonance angiogram before (**b**) and after surgery (**c**, **d**). Magnetic resonance angiogram 2 days after surgery showing the apparently patent STA–MCA bypass (*arrow* in **c**). Signal intensity of the STA–MCA bypass became more prominent 9 days after surgery (*arrow* in **d**)

free radical scavenger. Her facial palsy and numbness recovered completely 9 days after surgery. She was discharged without neurological deficit 11 days after surgery. One month later, she underwent surgical revascularization on the left side and was discharged without neurological deficit after an uneventful course. The SPECT performed 63 days after initial surgery showed normalization of CBF at the territory of the right middle cerebral artery (Fig. 1d). Surgical revascularization completely relieved her transient ischemic attack that was seen before surgery.

Discussion

Postoperative hyperperfusion syndrome has been considered to be less common in patients with moyamoya disease [5, 7, 15] due to the relatively low flow revascularization obtained by surgery for moyamoya disease. Furthermore, it was totally unclear how the rapid increase in CBF affects ischemic brain in childhood moyamoya disease, although substantial number of children with moyamoya disease, as much as 59.3% of patients with STA–MCA anastomosis, were reported to suffer transient neurological deterioration due to unknown mechanism [20]. We demonstrate, for the first time, a 4-year-old girl manifesting delayed transient facial palsy due to hyperperfusion 5 to 9 days after single STA–MCA anastomosis. Anatomical location and temporal profile of hyperperfusion, as shown by time sequential SPECT after surgery, were completely in accordance with her postoperative course. The reversibility of both her symptom and the SPECT finding further convinced the central role of hyperperfusion in her postoperative neurological deterioration.

The clinical manifestation due to hyperperfusion in our case was distinct from the 'cerebral hyperperfusion syndrome' seen after carotid endarterectomy or STA–MCA anastomosis for atherosclerotic cerebral occlusive disease, which is characterized by unilateral headache, face and eye pain, seizures, and focal symptoms secondary to cerebral edema or intracerebral hemorrhage [14, 17, 18]. The clinical presentation of our case was characterized by simple focal neurological deficit without headache and seizure. Also in adult patients with moyamoya disease, we observed similar transient neurological deficit without headache due to postoperative focal hyperperfusion in our same series [3].

The underlying mechanism of such specific manifestations of hyperperfusion in patients with moyamoya disease remains undetermined. Certain specific biological conditions, such as differences in reactive oxygen species (ROS) production after vascular reconstruction, vulnerability to ROS, and the expression of antioxidant enzymes in the chronic ischemic cortex may be involved in these intrinsic responses to vascular reconstruction against chronic ischemic brain in moyamoya disease because ROS has been implicated in cerebral ischemia/

reperfusion injury [1, 2]. Antioxidant agent is reported to prevent hyperperfusion syndrome after carotid endarterectomy in patients with atherosclerotic occlusive disease and markedly affected cerebrovascular reserve capacity [11]. Therefore, we treated her with edaravone, a novel free radical scavenger, to ameliorate the unfavorable effects of hyperperfusion on the affected brain.

Long-term follow-up of the neurological function including cognitive functions and the histological changes in the affected brain is needed because exposure to sublethal levels of ROS can affect organelles, and thus lead to delayed neuronal damage by apoptosis [2]. Postoperative cerebral hyperperfusion may be associated with impairment of cognitive function in patients undergoing carotid endarterectomy [13]. These characteristics of moyamoya disease remain to be elucidated. Identification of the predictors for cerebral hyperperfusion in patients with moyamoya disease is clinically important. Preoperative cerebrovascular reserve capacity [10], intraoperative ischemic period [9], difference in vascular anatomy, and patient age may affect postoperative cerebral hyperperfusion. Investigation of a larger number of patients is required.

Conclusion

We demonstrated, for the first time, that transient focal neurological deficit after STA–MCA anastomosis can be caused by focal hyperperfusion in a child with moyamoya disease. Routine CBF measurement by SPECT is recommended for the differential diagnosis between hyperperfusion and transient ischemic attack because the treatments for these conditions are contradictory.

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PAPER

Inheritance pattern of familial moyamoya disease: autosomal dominant mode and genomic imprinting

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Background: Although the aetiology of moyamoya disease (MMD) has not been fully clarified, genetic analysis of familial MMD (F-MMD) has considerable potential to disclose it.
Objective: To determine the inheritance pattern and clinical characteristics of F-MMD to enable precise genetic analyses of the disease.
Methods: 15 highly aggregated Japanese families (52 patients; 38 women and 14 men) with three or more affected members were examined. The difference in categories of age at onset (child onset, adult onset and asymptomatic) between paternal and maternal transmission was compared by χ^2 statistics.
Results: In all families there had been three or more generations without consanguinity, and all types of transmission, including father-to-son, were observed. Among a total of 135 offspring of affected people, 59 (43.7%) were patients with MMD or obligatory carriers. Affected mothers were more likely to produce late-onset (adult-onset or asymptomatic) female offspring ($p=0.007$).
Conclusions: The mode of inheritance of F-MMD is autosomal dominant with incomplete penetrance. Thus, in future genetic studies on F-MMD, parametric linkage analyses using large families with an autosomal dominant mode of inheritance are recommended. Genomic imprinting may be associated with the disease.

Moyamoya disease (MMD) is an idiopathic progressive angiopathy characterised by progressive stenosis or occlusion and affecting the terminal portions of the bilateral internal carotid artery and the circle of Willis. Collateral vessels develop at the base of the brain to compensate for the progressive stenosis. These enlarged collaterals appear as a puff of smoke on angiography, which gives the disease its name.

MMD is predominantly found in East Asian populations, with most reported cases originating from Japan, Korea and China. The estimated annual incidence in Japan in 1994 was 0.35 per 100 000 population,[1] whereas that in Europe was one tenth of this.[2] It is well known that MMD has been observed predominantly in women, with a female-to-male ratio of 1.8.[1 3] Recently, MMD has been diagnosed by magnetic resonance imaging (MRI) and magnetic resonance angiography (MRA),[4 5] which makes it possible to detect asymptomatic patients. Accordingly, familial occurrence has been increasing; it was observed in 12.1% of the patients in 2004.[3] The female predominance, East Asian distribution and familial occurrence of the disease imply the existence of genetic risk factors.

To clarify the genetic background of MMD, several non-parametric linkage analyses using mainly affected sibling pairs have been carried out, showing linkages to 3p24.2–p26, 6q25, 8q23, 12p12 and 17q25.[6–9] The association analysis of tissue inhibitor of metalloproteinase 2 in 17q25 showed that a polymorphism in the promoter region was markedly associated with familial MMD (F-MMD).[10]

To make further progress in genetic analysis of the disease, it is important to enrol highly aggregated families with MMD to characterise the clinical features and inheritance patterns in F-MMD. Some unresolved issues are as follows:

(1) The mode of inheritance has not yet been determined.
(2) Some reports mention that paternal transmission led to earlier onset than maternal transmission.[11] However, the difference between maternal and paternal transmission has not been proved by a statistical analysis.

The aim of this study is to resolve these issues, to enable precise genetic analyses of F-MMD.

METHODS

Study population

This study was approved by the ethics committee of the Kyoto University Institutional Review Board (Kyoto, Japan), and written informed consent was obtained from all the participants. We collected cases of F-MMD by collaborating with hospitals. These patients were diagnosed according to the official diagnostic criteria of the Research Committee on the Spontaneous Occlusion of the Circle of Willis of the Ministry of Health and Welfare, Japan.[4] A clinical interview and 1.5-T MRI and MRA examination were carried out for all available relatives. We ascertained the medical history, age, age at onset, age at diagnosis, first sign at onset, course of the disease, treatment and associated diseases. For a few twin cases of MMD, we reviewed the previous case reports. We searched PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed) from 1968 to 2005 and the Japan Medical Abstract Society (Japana Centra Revuo Medicina, http://login.jamas.or.jp/enter.html) from 1983 to 2005 (full data) using the keyword "moyamoya twin" ("moyamoya souseiji" in Japanese). From both searches, only Japanese families were included in this study.

Data analysis

We assumed the nearest common ancestor of the affected people to be the founder of the family. We put the founder in the first generation in the pedigree chart (fig 1). So, family 1

Abbreviations: F-MMD, familial moyamoya disease; MMD, moyamoya disease; MRA, magnetic resonance angiography; MRI, magnetic resonance imaging

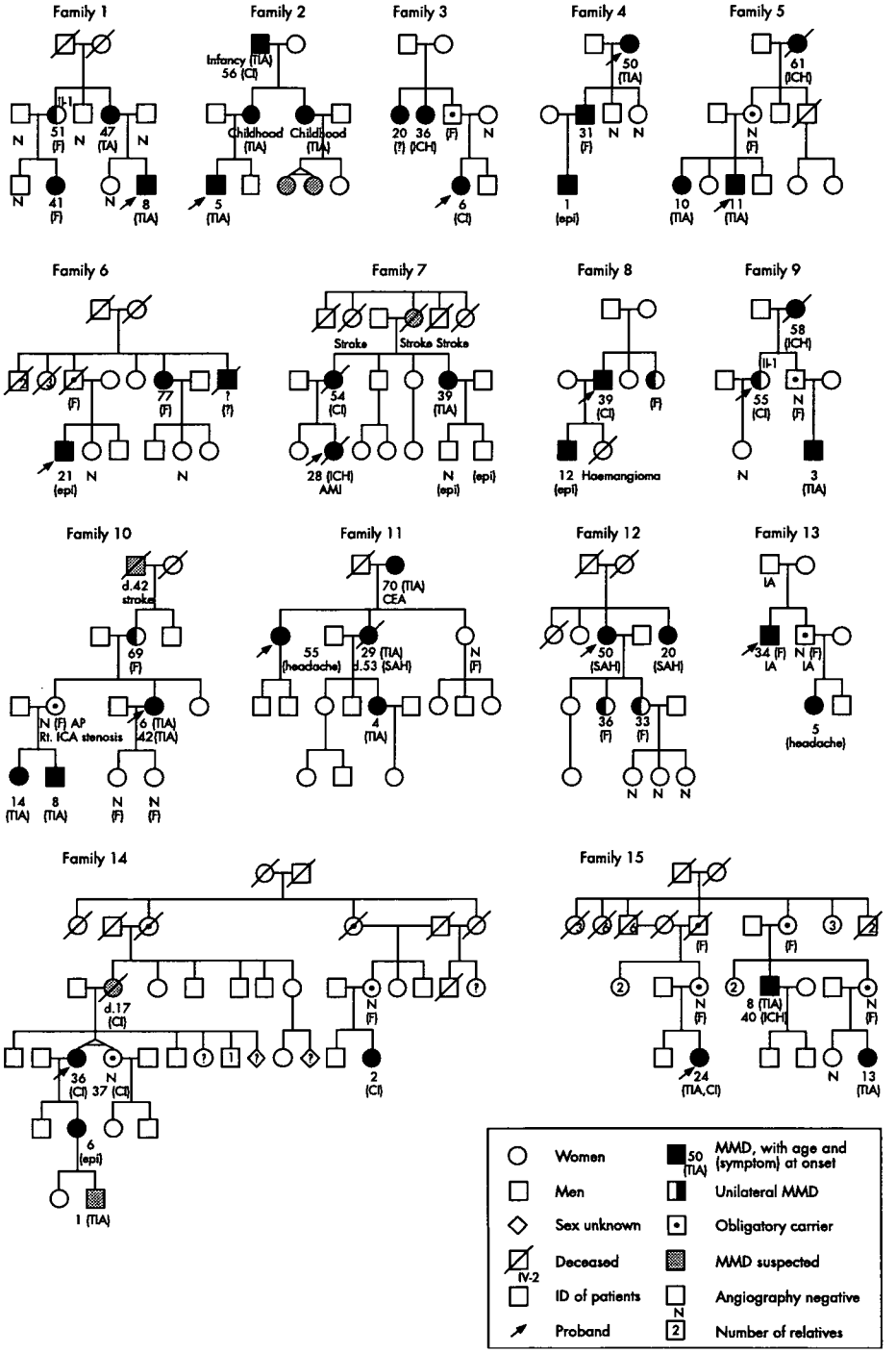

Figure 1 Families with familial moyamoya disease (MMD). AMI, acute myocardial infarction; CEA, carotid endoarterectomy; CI, cerebral infarction; epi, epileptic attack; (F), symptom free; IA, intracranial aneurysm; ICH, intracerebral haemorrhage; Rt. ICA, right internal carotid artery; SAH, subarachnoid haemorrhage; TIA, transient ischaemic attack. Filled symbols indicate patients with MMD; half-filled symbols, patients with unilateral MMD; dotted symbols, obligatory carriers; gray-shaded symbols, people suspected to be affected with MMD; circles, women; squares, men; diamonds, people of unknown sexuality; crossed symbols, deceased people; arrows, probands; numbers in the right upper angle of symbols, ID of patients; numbers in symbols, number of relatives; N, people who were confirmed to be unaffected by angiography. Numbers below the circle show age at onset, and symptom at the onset was shown in a parenthesis.

and family 2 were treated as three-generation families, and family 14 was treated as a five-generation family. People who were suspected to be affected according to their symptoms but whose diagnosis had not been confirmed by MRI and MRA or conventional angiography were treated as unaffected. Patients with MMD and obligatory carriers were treated as affected. Age at onset was divided into three groups: childhood onset (<15 years old), adult onset (≥15 years old) and adult asymptomatic patients (including obligatory carriers). The difference in age at onset between the four types of transmission (mother-to-daughter, mother-to-son, father-to-daughter and father-to-son) was analysed statistically using the χ^2 test or Fisher's exact test. All statistical analyses were carried out using SAS software V.8.2.

RESULTS

We studied 15 families (fig 1), including five families previously reported elsewhere.[12–16] Twelve families were three-generation families, two were four-generation and one was a five-generation family. No families showed parental consanguinity. We studied a total of 52 patients (38 women and 14 men; sex ratio 2.71) and 14 obligatory carriers (9 women and 5 men). Among 14 obligatory carriers, 8 were diagnosed as not having MMD by MRI and MRA or angiography, and 6 did not receive diagnostic examinations (some may have been asymptomatic patients). The number of children of affected people was 135 (84 women and 51 men; sex ratio 1.65), among whom 59 (43.7%) were affected. All types of transmission, including father-to-son, were observed (table 1). The ratio of maternal transmission to paternal transmission was 3.44, showing maternal predominance, and mother-to-daughter transmission was most commonly seen (60.0%). No difference in the category of age at onset was observed between paternal and maternal transmission, although a significant difference was observed between mother-to-daughter transmission and the other patterns of transmission (table 1; $p = 0.028$). Adult onset and asymptomatic patients were more commonly seen with mother-to-daughter transmission than with the other types of transmission ($p = 0.007$) (see fig 1).

Familial occurrence of both unilateral and bilateral MMD was observed in five families. There were six patients with unilateral MMD, and all of them showed adult onset. In family 1, patient II-1 was diagnosed with probable MMD at 51 years of age and remained unilateral for >18 years. In family 9, patient II-1 remained unilateral for >4 years. No follow-up data were available for the other patients (see fig 1).

In the literature, we found 17 twin pairs, including ours.[17–30] Of 17 pairs, 14 were monozygotic, two were dizygotic and one was of unknown zygosity. The ratio of monozygotic to dizygotic twinning was about 2:1 in the general population,[31] whereas that in patients with MMD was 4.7–7.5:1, showing monozygotic predominance. All 12 monozygotic twins were female except for two pairs of unknown sexuality. Among the 12 monozygotic twins, 5 pairs showed discordant phenotypes (symptomatic *v* asymptomatic, bilateral *v* unilateral, or patients *v* non-patients). In family 14, for example, one of the twins was a patient whereas the other was not.

DISCUSSION

Mode of inheritance

Owing to the lack of highly aggregated families with MMD, its mode of inheritance has remained undetermined. We studied as many as 15 large families with three or more generations. Parental consanguinity was absent from these families, which excluded the possibility of autosomal recessive inheritance. The families exhibited all types of transmission, including father-to-son, which made X-linked inheritance unlikely. As for the proportion of affected people among the total offspring, 50% indicates an autosomal dominant mode and 25% indicates an autosomal recessive mode of inheritance. In our patients, nearly one half of the children of affected people have become affected, which supports an autosomal dominant model with incomplete penetrance. Although the pedigree analysis carried out by Seol *et al*[32] yielded no specific pattern of inheritance in F-MMD, they recruited only small families with two affected members in each family, which made it difficult to tell the pattern of inheritance.

Genomic imprinting and epigenetic modification

We have shown that transmission is predominantly maternal and that affected mothers were more likely to produce late-onset (adult-onset or asymptomatic) female offspring. This non-mendelian sex-related pattern of inheritance is referred to as "parent-of-origin effect". One explanation for the effect is genomic imprinting, the molecular basis of which is epigenetic mechanisms such as DNA methylation and histone acetylation.[33] It is noteworthy that the Prader–Willi syndrome and Angelman's syndrome, which are well known for their genomic imprinting, are reported to be associated with moyamoya angiopathy.[34]

It is well known that patients with MMD are predominantly female. We have shown that the children of affected people are also predominantly female, whether affected or not, and that most monozygotic twins were female. This suggests that a gene responsible for MMD may be associated with sex determination.

We have shown that the proportion of monozygotic twinning is higher in MMD than in the general population. Most monozygotic twin pairs were female, and there were several pairs of monozygotic twins who were discordant for MMD. A good example of female-dominant monozygotic twins with discordant phenotypes is the Beckwith–Wiedemann syndrome, which is also characterised by genomic imprinting. Weksberg *et al*[35] reported that a loss of imprinting in the pre-implantation period would be a credible aetiology for the twinning as well as the discordances between monozygotic twins. Another example is Noonan's syndrome, which is caused by a mis-sense mutation in *PTPN11*.[36] The *PTPN11* gene has been implicated in oogenesis in *Caenorhabditis elegans*, and is thought to be associated with oogenesis and twinning in humans.[36] Noonan's syndrome is known to cause moyamoya angiopathy,[37] indicating that MMD might be associated with the genes that control twinning.

Table 1 Differences in age at onset with four types of transmission

	F–F	F–M	M–F	M–M	Maternal	Paternal	p Value	F–F	Non-F–F	p Value
Total number	25	7	6	4	32	10		25	17	
Class of age at onset										
Child onset (0–14 years)	7	5	4	3	12	7	0.193*	7	12	0.028*
Adult onset (≥15 years)	10	1	0	1	11	1		10	2	
Asymptomatic†	8	1	2	0	9	2		8	3	
Child onset					12	7	0.108	7	12	0.011
Adult onset					11	1		10	2	
Child onset					12	7	0.144	7	12	0.007
Non-child onset					20	3		18	5	

F–F, female-to-female transmission; F–M, female-to-male transmission; M–F, male-to-female transmission; M–M, male-to-male transmission. Non-F–F consists of F–M, M–F and M–M.
*Calculated comparing three classes of age at onset; †includes asymptomatic patients with MMD and obligatory carriers. Non-child onset indicates adult onset, asymptomatic and obligatory carriers. p Values were calculated by χ^2 statistics or Fisher's exact test.

Thus, in addition to genetic risk factors, epigenetic factors such as genomic imprinting, sex determination and twinning may be associated with the disease.

Unilateral MMD

Although MMD is defined as a bilateral lesion, unilateral involvement also occurs. Most paediatric patients with unilateral MMD develop a bilateral lesion within 1–2 years, whereas lesions in adults tend to remain unilateral.[38] The official diagnostic criteria of the Research Committee on the Spontaneous Occlusion of the Circle of Willis of the Ministry of Health and Welfare classify adult cases with bilateral occlusive lesions as "definite" MMD and those with unilateral involvement as "probable" MMD. We investigated six cases of familial occurrence of probable MMD. The coincidence of probable and definite MMD in a single family indicates that they reflect different phenotypes caused by the same genetic defects.

Future linkage analysis

Although previous studies shared several pedigrees in their non-parametric linkage analyses, they showed different linkage regions. There seem to be three main explanations for this phenomenon. Firstly, MMD may be caused by several different mechanisms (disease heterogeneity). Secondly, MMD exhibits different modes of inheritance—namely, autosomal dominant and autosomal recessive (genetic heterogeneity). Thirdly, several genetic factors in different loci can cause the same disease (locus heterogeneity).

In addition to the issue of heterogeneity, we have shown that the disease may be susceptible to epigenetic modifications. Therefore, the only feasible approach to identify the gene responsible would be positional cloning. To solve the issue of genetic heterogeneity, we need to collect large families of three or more generations without consanguinity, in which the autosomal dominant mode can be safely assumed. Linkage analysis of large families gives us a relatively high logarithm of odds score in each family, which makes clear the existence of disease heterogeneity or locus heterogeneity. Given the reduced penetrance, affected member-only analysis may be the most rational approach, in which obligatory carriers should be treated as "affected". Patients with unilateral MMD should also be included in the analysis.

In the genetic study on intracranial aneurysm, we applied a similar approach by collecting 29 highly aggregated Japanese families. We were able to successfully show the linkage to chromosome 17 and identified *TNFRSF13B* as a candidate gene.[39–41]

CONCLUSIONS

Pedigree analysis of highly aggregated Japanese families with MMD indicates that the mode of inheritance is autosomal dominant with reduced penetrance. Thus in future genetic studies on F-MMD, parametric linkage analyses using large families in which the autosomal dominant mode is safely assumed are recommended. Epigenetic modification such as genomic imprinting may be associated with the disease.

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Case report

Pediatric moyamoya disease presenting with intracerebral hemorrhage—Report of three cases and review of the literature

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Abstract

Intracerebral hemorrhage in patients with moyamoya disease is rare in children. We report three unique cases of pediatric moyamoya disease with hemorrhagic onset. Two 7-year-old girls and a 9-year-old girl were admitted to our hospital because of intracerebral hemorrhage associated with angiographically verified moyamoya disease. Two of them did not demonstrate either an ischemic episode or cerebral infarct on the magnetic resonance images. A decreased regional cerebral blood flow was revealed on single photon emission computed tomography in two patients, who developed cerebral infarction in the acute stage following hemorrhage. They underwent superficial temporal artery-middle cerebral artery anastomoses combined with encephalo-myo-synangiosis, and have not experienced any further ischemic episodes thereafter. Hemodynamic insufficiency associated with moyamoya disease could cause intracerebral hemorrhage even in children. Adequate management in the acute stage of hemorrhage and revascularization surgery are recommended to prevent cerebral infarction, which may easily occur in pediatric patients with moyamoya disease.

Keywords: Moyamoya disease; Pediatric patients; Intracerebral hemorrhage; Cerebral infarction; Edaravone

1. Introduction

Moyamoya disease is characterized by the progressive occlusion of the internal carotid arteries with spontaneous development of a collateral vascular network [1]. Pediatric patients mainly present with cerebral ischemia and they very rarely bleed [2,4,14,15]. Although the benefits of surgical revascularization for ischemic moyamoya disease have been well recognized, the indications for such treatment in hemorrhagic type to prevent rebleeding have not yet been established [2,6,13,20,22,27]. We herein report three children aged <10 years presenting with intracerebral hemorrhage associated with moyamoya disease, two of which were accompanied with early ischemic complications followed by revascularization surgery.

2. Case report

2.1. Case 1

A previously healthy 7-year-old girl suddenly suffered severe headache and vomiting on November 17, 2004. She developed moderate weakness in her right extremities, and was admitted to our hospital by ambulance. Computed tomography (CT) demonstrated an intracerebral hemorrhage in the left globus pallidus and no cerebral infarction was evident on magnetic resonance image (MRI) (Fig. 1). Cerebral angiography revealed angiographic moyamoya disease (right side, Stage 2; left side, Stage 3 according to the angiographic criteria described by Suzuki and Kodama [24]) without any cerebral aneurysms. Both vascular blush in the

Abbreviations: CT, computed tomography; MRI, magnetic resonance imaging; SPECT, single photon emission computed tomography; IMP, *N*-isopropyl-4 iodoamphetamine; CBF, cerebral blood flow.

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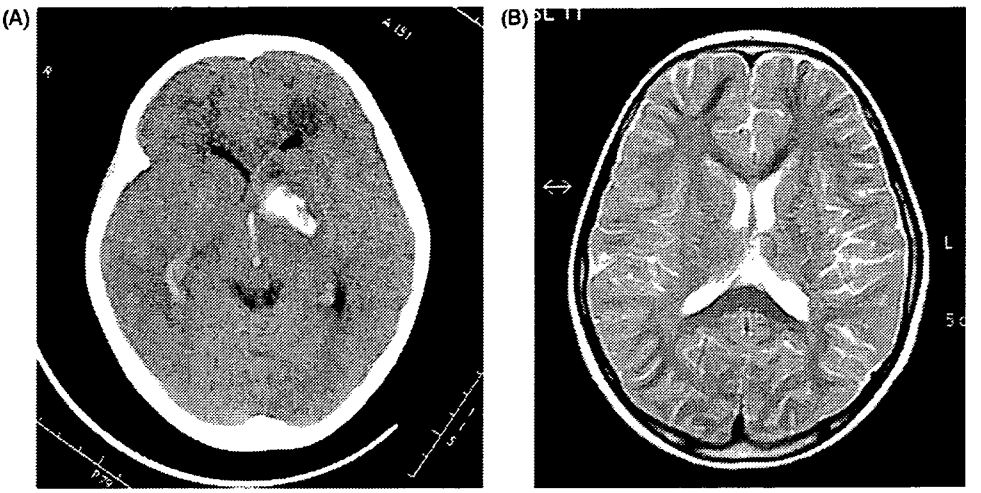

Fig. 1. (A) A computed tomographic scan demonstrated an intracerebral hemorrhage in the left globus pallidus and intraventricular hemorrhage. (B) No cerebral infarction was evident on the T2-weighted magnetic resonance image.

lenticulostriate arteries and dilatation of the anterior choroidal artery were prominent in the left side. Single photon emission computed tomography (SPECT) using ^{99m}Tc-ethyl cysteinate dimer (ECD) obtained on the 9th hospital day showed a modest, and not significant, reduction in the regional cerebral blood flow (CBF) in the left frontoparietal cortex. The cerebral vascular reactivity to acetazolamide remained normal. She was managed conservatively with hydration and her hemiparesis gradually improved. She has not experienced any further neurological episodes and she is not on medication during the 28-month follow-up after the first ictus.

2.2. Case 2

A previously healthy 7-year-old girl experienced severe headache and vomiting on June 9, 2005. She was taken to a local hospital, and then transferred to ours because of her decreased level of consciousness. CT on admission demonstrated an intracerebral hemorrhage in the right paraventricular white matter and intraventricular clots, but no evidence of cerebral infarction was observed on MRI (Fig. 2(A and B)). Cerebral angiography revealed angiographic moyamoya disease (right side, Stage 3; left side, Stage 1) without aneurysmal formation. Both vascular blush in the lenticulostriate arteries and dilatation of the anterior choroidal artery were prominent on the right side. SPECT using *N*-isopropyl-4 iodoamphetamine (^{123}I-IMP) obtained on the 4th hospital day showed a marked reduction of regional CBF in the right parietal cortex (Fig. 2(C)). She was treated with the intravenous administration of a hyperosmotic agent (400 ml/day glycerol, 10%, w/v, concentrated glycerin solution). Thereafter, she became alert and her headache subsided.

On the 16th hospital day, she complained of a severe headache and began to cry. Soon thereafter, she demonstrated mild left hemiparesis. MRI revealed multiple infarcts in the right frontal and bilateral parietal subcortexes (Fig. 2(D and E)). A free radical scavenger, edaravone [25], was intravenously administered (60 mg/day) for 5 days, and her left hemiparesis gradually improved. She underwent a superficial temporal artery-middle cerebral artery (STA-MCA) anastomosis combined with encephalo-myo-synangiosis (EMS) on the right side on the 47th day after the last ictus. She has not experienced further ischemic episodes without any medication during a 20-month follow-up after surgery. Postoperative cerebral angiography showed a good patency of the direct bypass, and an increased regional CBF in the right cerebral hemisphere was evident on SPECT using ^{123}I-IMP.

2.3. Case 3

A 9-year-old girl suddenly experienced a severe headache and became lethargic on October 29, 2005. She was taken to a local hospital, and then was referred to our institution. She had a previous history of occasional headaches since 3 years of age and transient weakness of the right upper extremity at 8 years old. CT revealed a tiny intracerebral hemorrhage in the right paraventricular white matter and clots in the right lateral ventricle (Fig. 3(A)). MRI on admission did not show any other ischemic lesions (Fig. 3(B)). Cerebral angiography revealed angiographic moyamoya disease (right side, Stage 2; left side, Stage 3) without aneurysmal formation. The dilatation of the left anterior choroidal artery was prominent, while neither the anterior choroidal artery nor the posterior communicating artery showed abnormal dilatation and branching in the right side. SPECT using ^{123}I-IMP obtained on the 6th hospital day showed a modest, and significant, reduction of regional CBF in the left frontoparietal cortexes (Fig. 3(C)), which was on the opposite side to the bleeding. She was treated with the intravenous administration of a hyperosmotic agent (400 ml/day glycerol, 10%, w/v, concentrated glycerin solution). She became alert and her headache subsided.

On the 7th hospital day, she had a recurrence of the headache and manifested transient right hemiparesis several

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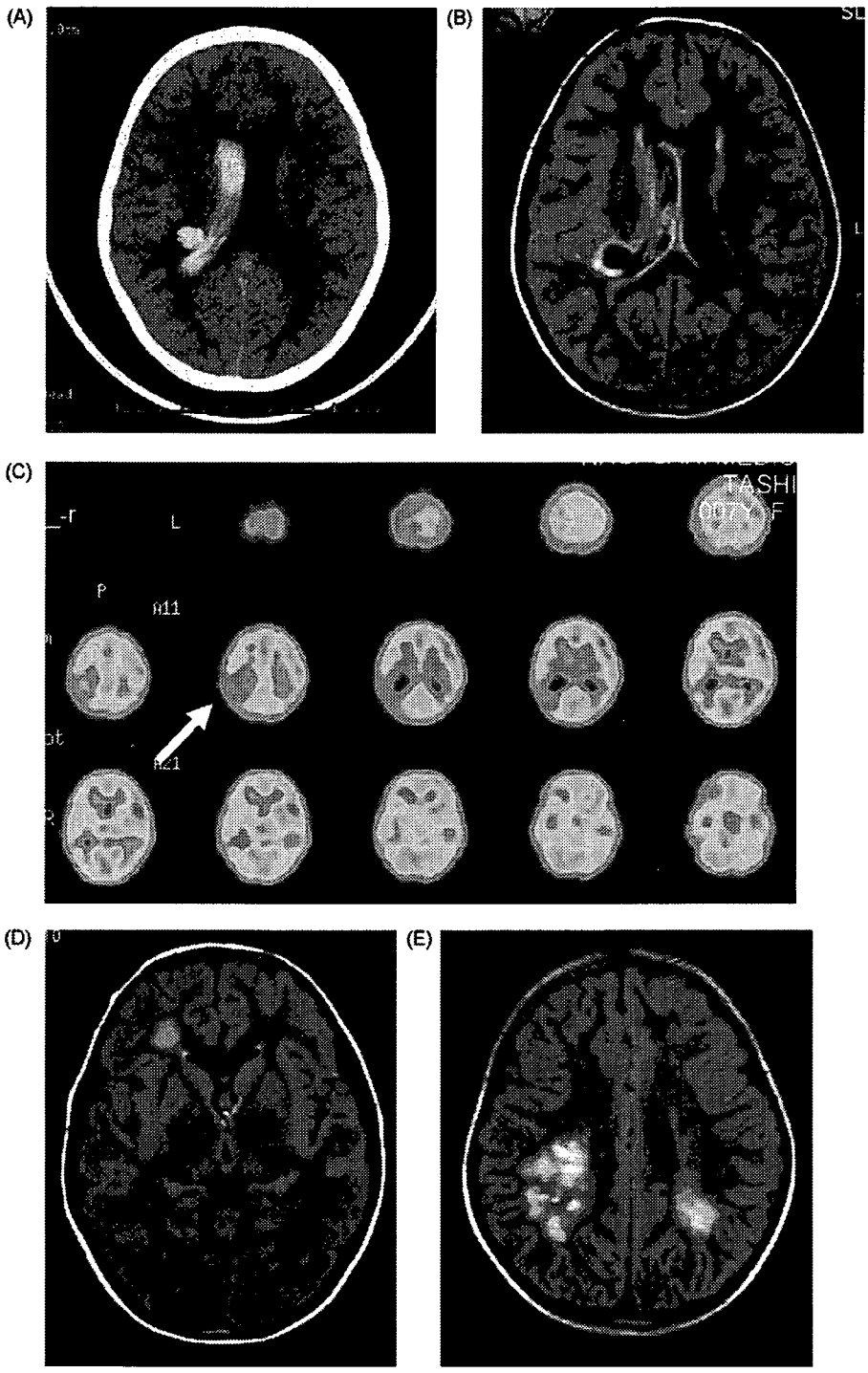

Fig. 2. (A) A computed tomographic scan demonstrated an intracerebral hemorrhage in the right paraventricular white matter and intraventricular clots. (B) No cerebral infarction was evident on fluid attenuated inversion recovery (FLAIR) magnetic resonance image. (C) Single photon emission computed tomography (SPECT) using *N*-isopropyl-4 iodoamphetamine (^{123}I-IMP) showed a marked reduction in the regional cerebral blood flow in the right parietal cortex (arrow). (D and E) FLAIR magnetic resonance images revealed multiple infarcts in the right frontal and bilateral parietal subcortexes.

times. Diffusion-weighted MRI revealed an infarct in the left frontal subcortex (Fig. 3(D)). Edaravone [26] was intravenously administered (30 mg/day) for 5 days, and her ischemic attacks did not progress to a complete stroke. She underwent STA-MCA anastomosis with EMS on the left side on the 40th day after the last ictus. She has not experienced any further ischemic episodes without medication during a 15-month follow-up after surgery. Postoperative cerebral angiography showed a good patency of the direct bypass, and an increased regional CBF in the left cerebral hemisphere was evident on SPECT using ^{123}I-IMP.

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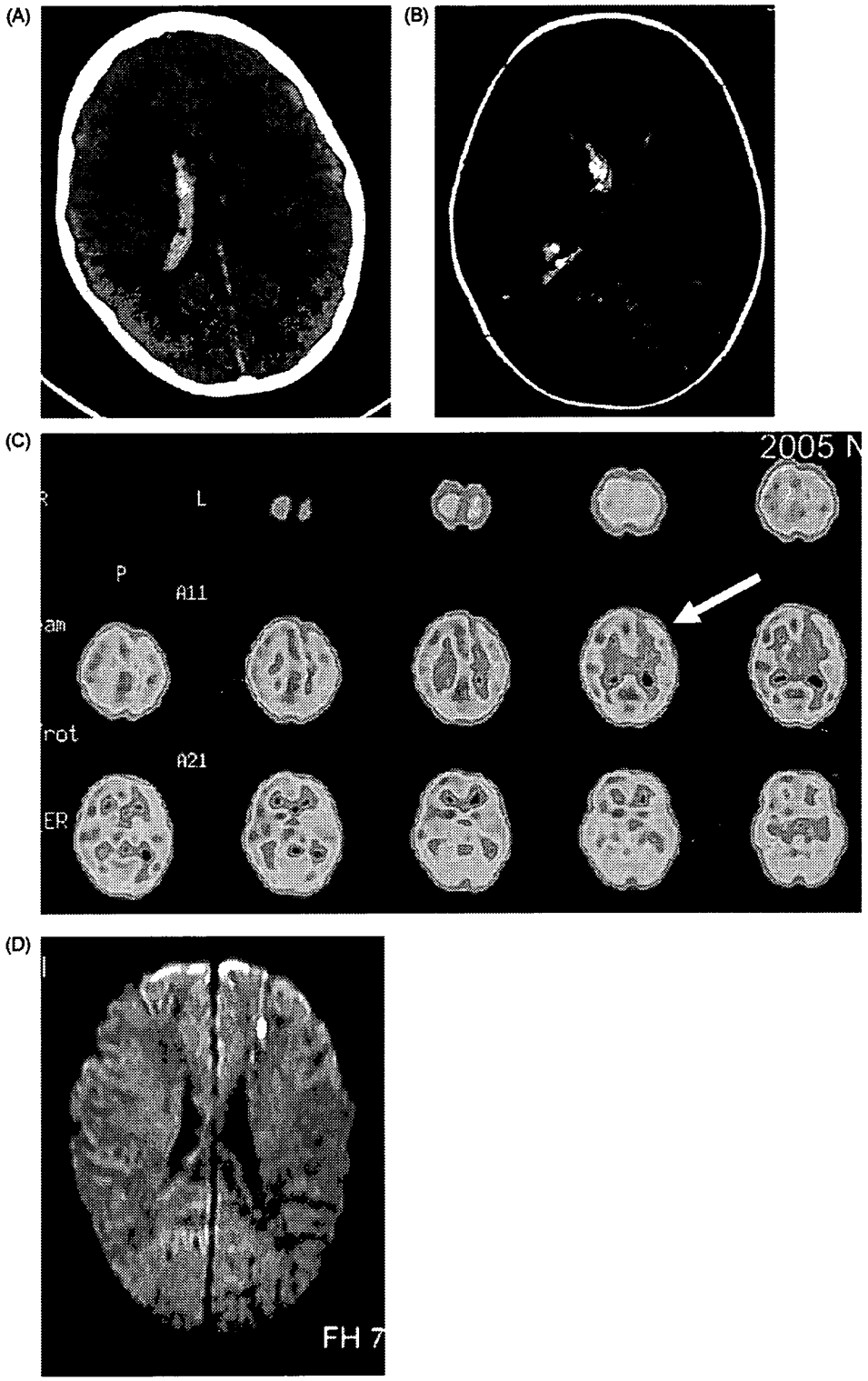

Fig. 3. (A) A computed tomographic scan revealed a tiny intracerebral hemorrhage in the right paraventricular white matter and clots in the right lateral ventricle. (B) FLAIR magnetic resonance images did not show any other hemorrhagic or ischemic lesions. (C) SPECT using ^{123}I-IMP showed a modest, and significant, reduction of the regional CBF in the left frontoparietal cortexes (arrow). (D) Magnetic resonance imaging showed restricted diffusion in the left frontal subcortex on the diffusion-weighted image.

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3. Discussion

Hemorrhagic type moyamoya disease is frequently seen in adults [1], and quite a low incidence of hemorrhage in patients aged <16 years has been reported both in Japan [2,15] and Korea [4,14]. In adults, rebleeding is not uncommon and it could worsen the prognosis following hemorrhage [7,27]. In contrast, the natural course following the first hemorrhage in children remains unclear because of the limited number of reports regarding this kind of episode. Morioka et al. [17] reported a long-term observation following hemorrhage in two children who had not undergone revascularization surgery, and one of whom experienced recurrent hemorrhage in patient's 30s. In contrast, others have reported neither recurrent hemorrhage nor any ischemic events following hemorrhage among children who had undergone surgery, despite the absence of either long-term observations or precise descriptions [8,10,12,27].

The increased bleeding risk in adults is considered to result from the fragility of the basal moyamoya vessels and the medullary arteries derived from the choroidal arteries, both of which have developed as long-term compensation for decreased regional CBF [8]. However, in our cases, only one had a previous ischemic episode and none had ischemic lesions demonstrated on the initial MRI. In terms of the bleeding hemisphere, the predominant angiographic findings in adults have been reported as disease progression to Stage 3 according to Suzuki's angiographic criteria [15,24]. In our case 3, bleeding appeared in the hemisphere where the angiographic staging was less advanced and the regional CBF remained normal (Table 1). It was also noted that both the anterior choroidal artery and the posterior communicating artery did not show the type of abnormal dilatation and branching that have been reported to be predominant in young patients with hemorrhage as well as adults [18]. These findings suggest that hypoperfusion is not necessarily responsible for bleeding, and that non-chronically hemodynamic stress may cause intracerebral bleeding from the especially vulnerable collateral vessels, even in children with moyamoya disease.

Subsequent ischemic complications can be a major concern in the acute phase of bleeding in patients with moyamoya disease. Suspected hemodynamic insufficiency and an increased intracranial pressure following hemorrhage can induce cerebral ischemia. In addition, both hyperventilation following crying and dehydration following the use of hyperosmotic agents may thus play a critical role in the cerebral hemodynamic conditions right after bleeding in pediatric patients. In our cases 2 and 3, the ischemic events appeared within 16 days of the first ictus in the region where regional CBF had decreased (Table 1). We successfully used edaravone [26] to prevent further ischemic events instead of using antiplatelet agents that may have adversely affected the intracerebral hemorrhage. Iwama et al. [10] and Rafay et al. [21] reported similar cases to ours with ischemic complications. These findings raise the possibility that subsequent ischemic complications may be easily induced in pediatric patients following intracerebral hemorrhage. Adequate sedation [19], hydration [10], and management against increased intracranial pressure [21] are crucial factors in the acute phase of bleeding in children with moyamoya disease.

The efficacy of revascularization surgery for ischemic moyamoya disease is generally recognized, while that for the hemorrhagic type in adults to prevent rebleeding has not yet been established [25]. Although some reports have demonstrated the effects of direct revascularization surgery to prevent recurrent bleeding [13], or at least subsequent ischemia [20], the long-term efficacy of revascularization surgery following hemorrhage in adult patients remains controversial [2,6,22,27]. On the other hand, moyamoya disease in childhood manifests cerebral infarction more frequently than in adults. Because of the possible limitations regarding the oral intake of antiplatelet agents following intracranial bleeding, surgical revascularization for the region where regional CBF had decreased should be justified to prevent cerebral infarction in pediatric patients with hemorrhage. In our cases 2 and 3, in whom a subsequent infarction had developed, we performed direct revascularization combined with indirect procedures in order to obtain both a rapid improvement in CBF to prevent further ischaemic attacks [3,9,11,16] and the long-term patency of the bypass [5]. They have not experienced any further ischemic episodes thereafter. Another concern is whether revascularization surgery performed in childhood for hemorrhagic moyamoya disease can reduce the rebleeding risk when patients reach the age range of 46–50 years, which is the age range with the highest likelihood of rebleeding [17]. Although the long-term effect of revascularization surgery on the prevention of hemorrhagic episodes in pediatric patients with ischemia has been reported [7,8,23], the follow-up findings of those with hemorrhage should thus be clarified by further long-term follow-up studies.

In conclusion, hemodynamic insufficiency could cause not only cerebral infarction but also intracerebral hemor-

Table 1
Summary of the cases; ICH, intracerebral hemorrhage; IVH, intraventricular hemorrhage; Bil, bilateral; Angiographic Staging, staging according to Suzuki's criteria [24]

Pt. no.	Age (years)/ sex	Past history	Onset	Infarct on MRI	Angiographic staging	Regional CBF on SPECT	Subsequent infarction	Revascularization surgery	Follow-up (month)
1	7/F	–	ICH(L)	–	(R)2, (L)3	Normal	–	–	28
2	7/F	–	ICH(R), IVH	–	(R)3, (L)1	(R)decreased	+(Bil)	+(R)	20
3	9/F	TIA	ICH(R), IVH	–	(R)2, (L)3	(L)decreased	+(L)	+(L)	15

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rhage even in children with moyamoya disease. Adequate management in the acute phase of bleeding and revascularization surgery are therefore recommended to prevent cerebral infarction which may easily occur in pediatric patients with hemorrhagic onset. Because the efficacy of revascularization surgery in preventing rebleeding remains unclear, further long-term follow-up studies are thus called for.

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The Phosphodiesterase Inhibitor Rolipram Promotes Survival of Newborn Hippocampal Neurons After Ischemia

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Background and Purpose—Brain ischemia stimulates neurogenesis. However, newborn neurons show a progressive decrease in number over time. Under normal conditions, the cAMP–cAMP responsive element binding protein (CREB) pathway regulates the survival of newborn neurons. Constitutive activation of CREB after brain ischemia also stimulates hippocampal neurogenesis. Thus, activation of cAMP-CREB signaling may provide a promising strategy for enhancing the survival of newborn neurons. We examined whether treatment of mice with the phosphodiesterase-4 inhibitor rolipram enhances hippocampal neurogenesis after ischemia.

Methods—Both common carotid arteries in mice were occluded for 12 minutes. Bromodeoxyuridine (BrdU) was used to label proliferating cells. Mice were perfused transcardially with 4% paraformaldehyde, and immunohistochemistry was performed. To evaluate the role of CREB in the survival of newborn neurons after ischemia, intrahippocampal injection of a CRE-decoy oligonucleotide was delivered for 1 week. We examined whether the activation of cAMP-CREB signaling by rolipram enhanced the proliferation and survival of newborn neurons.

Results—Phospho-CREB immunostaining was markedly upregulated in immature neurons, decreasing to low levels in mature neurons. The number of BrdU-positive cells 30 days after ischemia was significantly less in the CRE-decoy treatment group than in the vehicle group. Rolipram enhanced the proliferation of newborn cells under physiologic conditions but not under ischemic conditions. Rolipram significantly increased the survival of nascent BrdU-positive neurons, accompanied by an enhancement of phospho-CREB staining and decreased newborn cell death after ischemia.

Conclusions—CREB phosphorylation regulates the survival of newborn neurons after ischemia. Chronic pharmacological activation of cAMP-CREB signaling may be therapeutically useful for the enhancement of neurogenesis after ischemia. (***Stroke.*** **2007;38:1597-1605.)**

Key Words: CREB ■ hippocampus ■ neurogenesis ■ ischemia

Neurogenesis continues throughout adulthood in the subgranular zone (SGZ) of the hippocampus and in the subventricular zone.[1,2] Brain ischemia also enhances neurogenesis in the hippocampus and also induces the migration of neuroblasts into lesions of nonneurogenic areas such as the striatum.[3] However, only a small fraction of these newborn neurons derived from proliferating progenitors survive.[2,3]

The transcription factor, cAMP responsive element binding protein (CREB), mediates diverse responses in the nervous system, such as learning, memory, neuronal plasticity, and cell survival.[4] In addition to its function in mature neurons, CREB regulates cell proliferation, differentiation, and survival in the developing brain. Phosphorylation of CREB at Ser 133 is crucial for CREB-dependent transcription.[5]

Under normal conditions, the cAMP-CREB pathway regulates multiple aspects of adult hippocampal neurogenesis.[6,7] Activation of CREB by expression of constitutively active VP16-CREB after brain ischemia also stimulates hippocampal neurogenesis.[8] A therapeutic pharmacological approach to increase cAMP and activate cAMP-CREB signaling involves inhibition of the degradation enzyme phosphodiesterase (PDE). Among the PDE subfamily, PDE4 represents 70% to 80% of PDE activity in neuronal tissue.[9]

cAMP-CREB signaling in hippocampal neurogenesis is very important. However, the effect of pharmacological activation of the cAMP-CREB signaling pathway after ischemia has not been elucidated. In this study, we sought to determine whether pharmacological activation of this pathway by the PDE4 inhibitor rolipram enhances survival of newborn neurons in the hippocampal dentate gyrus under ischemic conditions.

Materials and Methods

Animals

Adult male C57BL/6 mice (11 to 12 weeks old) were used in this study. The experimental protocol was approved by the institutional

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animal care and use committee of Osaka University Graduate School of Medicine. They were fed standard laboratory chow and had access to water ad libitum before and after all procedures. Animal care was given according to the guidelines of the animal center of Osaka University Graduate School of Medicine.

Bromodeoxyuridine Labeling Protocols and Immunohistochemistry

Bromodeoxyuridine (BrdU; Roche Diagnostics, Indianapolis, Ind), a thymidine analog, was used to label proliferating cells. To quantify BrdU-positive cells and evaluate the phenotype of postmitotic cells, mice were injected intraperitoneally with BrdU (50 mg/kg) 4 times every 2 hours for 6 hours and killed at 1, 3, 7, 14, or 30 days after BrdU administration. Mice were killed under deep pentobarbital anesthesia and perfused transcardially with 4% paraformaldehyde, and the brains were removed and fixed in 4% paraformaldehyde at 4°C.

The protocol for BrdU immunohistochemistry was as described previously.[10] In brief, sections were treated with 50% formamide and 2× saline-sodium citrate buffer and then incubated in 2N HCl. After a wash, sections were incubated with rat monoclonal anti-BrdU antibody (1:100, Harlan Sera-Laboratory, Loughborough, UK) at 4°C overnight. After a wash, sections were incubated with biotinylated secondary antibody, washed, and then incubated with streptavidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, Calif). Sections were reacted with 0.05% 3'3-diaminobenzidine in the presence of 0.01% H_2O_2.

For double immunofluorescence, free-floating sections (40 μm) were incubated with primary antibody diluted in Tris-buffered saline/0.1% Triton X-100 containing 1.5% normal serum at 4°C overnight. The following primary antibodies were used for immunofluorescence: rat monoclonal anti-BrdU antibody (1:100, Harlan Sera-Labo), mouse monoclonal anti-BrdU antibody (1:200, Amersham, Piscataway, NJ), rabbit polyclonal anti–phospho-CREB (pCREB) antibody (1:200, Upstate Biotechnology, Lake Placid, NY), rabbit polyclonal anti-CREB antibody (1:200, Cell Signaling, Beverly, Mass), mouse monoclonal anti-NeuN antibody (1:200, Chemicon, Temecula, Calif), rabbit polyclonal anti-glial fibrillary acidic protein antibody (1:200, Sigma-Aldrich, St. Louis, Mo), goat polyclonal anti-doublecortin (DCX) antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, Calif), rat monoclonal anti–Musashi-1 (Msi-1) antibody clone 14H1[11] (1:200), and mouse monoclonal polysialylated neural cell adhesion molecule (PSA-NCAM) antibody (1:200, Pharmingen, San Jose, Calif).

For double labeling of BrdU and cell markers (NeuN for mature neurons; glial fibrillary acidic protein for astrocytes; DCX and PSA-NCAM for migrating neuroblasts and immature neurons; and Msi-1 for neuronal progenitors, neuronal stem cells, and astrocytes), sections were subjected to DNA denaturation and incubated with the appropriate anti-BrdU antibody plus antibody to 1 of the aforementioned cell markers at 4°C overnight. Sections were then incubated with an appropriate anti-IgG or anti-IgM secondary donkey antibody conjugated to fluorescein isothiocyanate (FITC) or rhodamine (1:200, Chemicon) for 90 minutes at room temperature. After a rinse in Tris-buffered saline, sections were mounted with Vectorshield (Vector Laboratories), and staining was visualized with a confocal microscopy system (LSM-510, Zeiss, Oberkochen, Germany).

Transient Forebrain Ischemia

General anesthesia was maintained with 1% halothane by means of an open facemask. A polyacrylamide column for measurement of cortical microperfusion by laser Doppler flowmetry (Advanced Laser Flowmetry) was attached to the skull, 3 mm lateral to the bregma on the right side, with dental cement. Body and skull temperatures were monitored and maintained at 36.5°C to 37.5°C with a heat lamp and mat. Both common carotid arteries were occluded for 12 minutes with microaneurysm clips and then reperfused. As described previously, only mice that showed <13% of baseline control microperfusion during the first minute of occlusion were used in subsequent experiments.[12] We injected BrdU (50 mg/kg, IP) 9 days after ischemia, as previously reported.[10] Thereafter, mice subjected to ischemia were processed under the same schedule as those under normal conditions.

CRE-Decoy Oligonucleotide Administration

To evaluate the importance of CREB for the survival of newborn neurons after ischemia, intrahippocampal injection of a CRE decoy was delivered by means of a miniosmotic pump starting 7 days after BrdU labeling and continuing for 1 week. Treatment with the CRE-decoy oligonucleotide was performed as described previously with some modification.[13–15] CRE-decoy and control oligonucleotides used in this experiment were phosphorothioate oligonucleotides (OligoExpress, Sigma, Tokyo, Japan). Their sequences were as follows: 24-mer CRE-decoy, 5'-TGACGTCATGACGTCATGACGTCA-3' and 24-mer nonsense-sequence control, 5'-CTAGCTAGCTAGCTAGCTAGCTAG-3'. Because the CRE *cis*-element, TGACGTCA, is palindromic, it was shown that the CRE-decoy oligonucleotide self-hybridized to form a duplex hairpin and competed with CRE enhancers for binding transcription factors and specifically interfered with CRE-directed transcription in vitro.[13]

Anesthetized mice were placed in a stereotaxic frame, and a mixture (100 μL) consisting of 10 μmol/L oligonucleotide dissolved in *N*-[1-(2,3-Dioleoyloxy)]-*N*,*N*,*N*-trimethylammonium propane methylsulfate (DOTAP) solution was infused unilaterally into the hippocampus with an osmotic pump (1007D, Alza Corp, Mountain View, Calif) attached to a cannula (brain infusion kit II, Alza Corp.) implanted at 1.5 mm lateral and 2.0 mm caudal to the bregma to a depth of 2.5 mm from the dura. The CRE decoy was infused 7 days after BrdU labeling for 7 days at a flow rate of 0.5 μL/h. Intrahippocampal injection was confirmed by noting the presence of injection scars on fixed brain slices. For immunohistochemical examination of hippocampal CRE-decoy oligonucleotide distribution, brains were removed 24 hours after FITC-labeled CRE-decoy injection. To quantify the survival of newborn neurons, mice were anesthetized and perfusion-fixed with 4% paraformaldehyde as described earlier. The number of surviving newborn neurons was compared with that at 30 days after BrdU labeling.

Rolipram Treatment

To evaluate the effect of rolipram on the proliferation of newborn cells, normal mice received injections of rolipram daily for 7 consecutive days. Ischemic mice received injections of rolipram (3 mg/kg or vehicle IP) starting 3 days after ischemia and daily for 7 consecutive days. BrdU was given 4 times every 2 hours for 6 hours at 2 hours after the last rolipram injection. Both group of mice were killed 1 or 28 days after the last BrdU injection. Next, to evaluate the effect of rolipram on the survival of newborn cells, BrdU was given 4 times every 2 hours for 6 hours at 9 days after ischemia. Rolipram (1 or 3 mg/kg or vehicle IP) was administered starting 7 days after BrdU labeling and daily for 21 consecutive days. At 30 days after BrdU labeling, the number of surviving newborn neurons was compared.

TUNEL Staining

To identify cell apoptosis, terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) labeling was performed. The brain was removed rapidly en bloc and quickly frozen in liquid N_2 vapor. Fourteen-micron-thick sections were cut on a cryostat and postfixed in 1% paraformaldehyde for 10 minutes. The Apoptag fluorescein in situ apoptosis detection kit (S7110, Chemicon, Temecula, Calif) was then applied. For immunofluorescein double labeling of TUNEL signal and BrdU, the TUNEL-fluorescein labeling was performed first, followed by incubation in 2N HCl for 30 minutes at 37°C, followed by application of a rat monoclonal anti-BrdU antibody.

Quantification

To count BrdU-positive cells, 5 sections from the hippocampus were cut every 120 μm beginning 1.4 mm caudal and 1.9 mm caudal to the bregma. In the hippocampus, the granular cell layer (GCL) and SGZ,