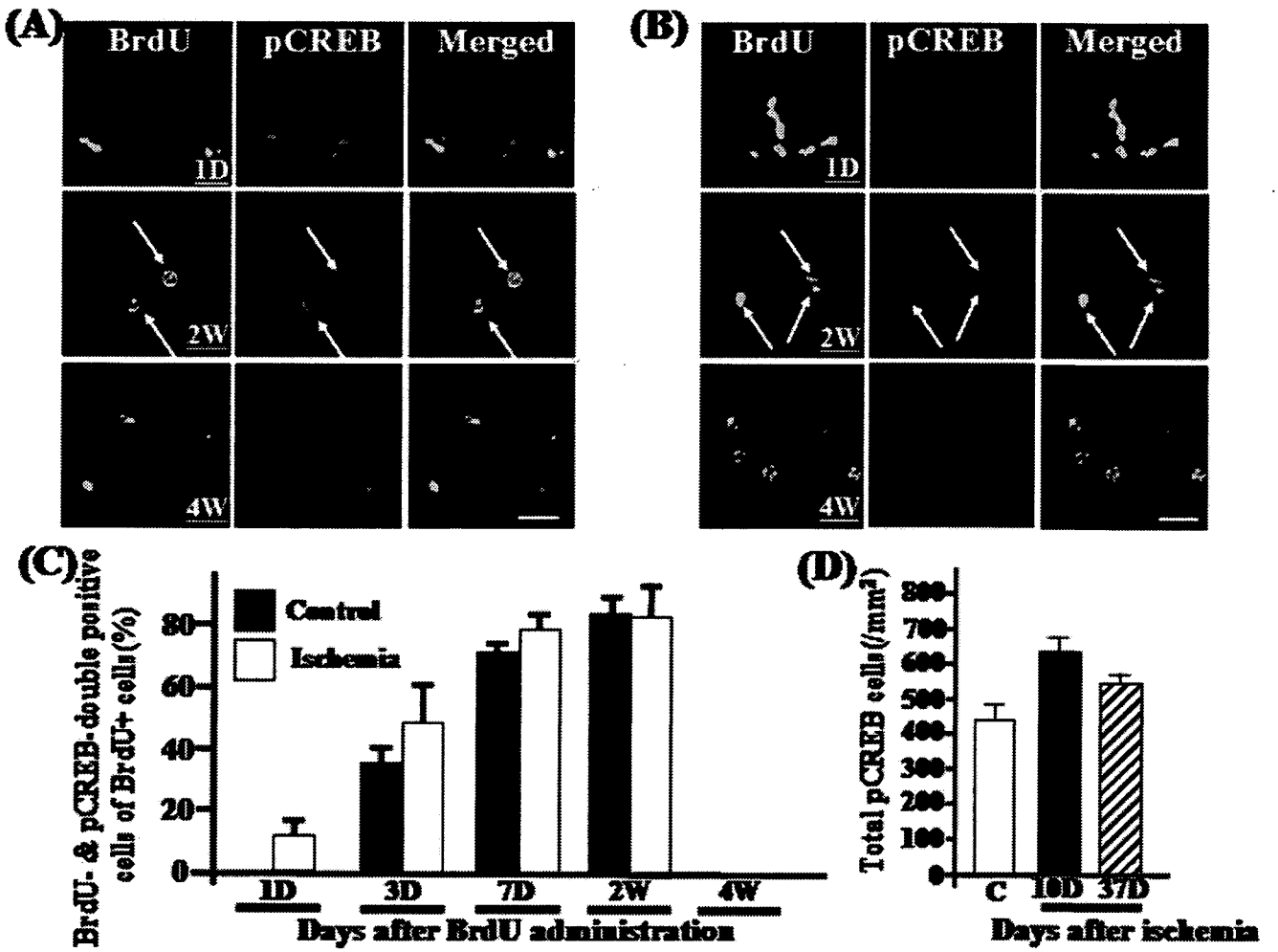

Figure 1. Double-immunofluorescence staining of BrdU and pCREB under normal conditions (A) and under ischemic conditions (B). Colocalization of BrdU and pCREB is shown (A, B, arrow). C, Temporal profiles of BrdU- and pCREB-double-positive cells under normal conditions and after ischemia (n=6). D, Total number of pCREB-positive cells. C indicates control. Bars=20 μm in A and B.

defined as a zone 2 cell bodies wide along the border of the GCL and hilus, were considered together for quantification. The mean density of BrdU-positive cells in each mouse was calculated as the number of labeled nuclei divided by the area.

To assess the phenotype of BrdU-positive cell in double immunofluorescence, a mean value for each marker was obtained from 10 sections from 5 to 8 mice. Data in the text and figure are described mean±SD. Multiple comparisons were evaluated statistically by ANOVA, followed by Scheffé's post hoc tests.

Results

To assess BrdU-positive newborn cells in the dentate gyrus over time, we determined the number of BrdU-positive cells and the phenotype of postmitotic cells at 1 and 30 days after BrdU administration. Under normal conditions, the number of BrdU-positive cells showed a progressive decrease (1 day, 39.4±8.6/mm^2; 30 days, 9.6±3.6/mm^2). Under ischemic conditions, the numbers of BrdU-positive cells was 115.5±23.7/mm^2 at 1 day and 38.7±13.1/mm^2 at 30 days.

To examine the expression of pCREB in newborn hippocampal neurons, analysis of pCREB staining at various times after BrdU administration was carried out (Figure 1). Only a few BrdU-positive cells in the SGZ and GCL showed pCREB staining 1 day after BrdU administration (Figure 1A). pCREB staining was detected as early as 3 days after BrdU administration and increased in number thereafter. At 14 days after BrdU administration, a majority of BrdU-positive cells showed pCREB staining (Figure 1A). pCREB staining of BrdU-positive cells decreased dramatically by 30 days after BrdU injection. The profile of BrdU-positive cells showing pCREB staining under ischemic conditions over time was similar to that under normal conditions (Figure 1B). Semiquantitative analysis showed the following with respect to BrdU-plus-pCREB–positive cells in the SGZ and GCL: 0% at 1 day, 35.3±6.6% at 3 days, 70.0±3.1% at 7 days, 81.0±6.6% at 14 days, and 0% at 30 days in the normal group and 12.1±5.2% at 1 day, 52.5±9.0% at 3 days, 77.1±4.7% at 7 days, 80.5±10.4% at 14 days, and 0% at 30 days in the ischemia group (Figure 1C). Also, there was an increase in the total number of pCREB-positive cells at 10 days (9+1 days) or 37 days (9+28 days) after ischemia (control, 433.3±54.9/mm^2; 10 days, 638.9±59.2/mm^2; 37 days, 553.5±32.0/mm^2; Figure 1D).

To identify the phenotype of pCREB-positive cells, we performed double immunolabeling with BrdU antibody and for neuronal progenitor/neuronal stem cell (Msi-1), immature neuron (DCX, PSA-NCAM), or mature neuron

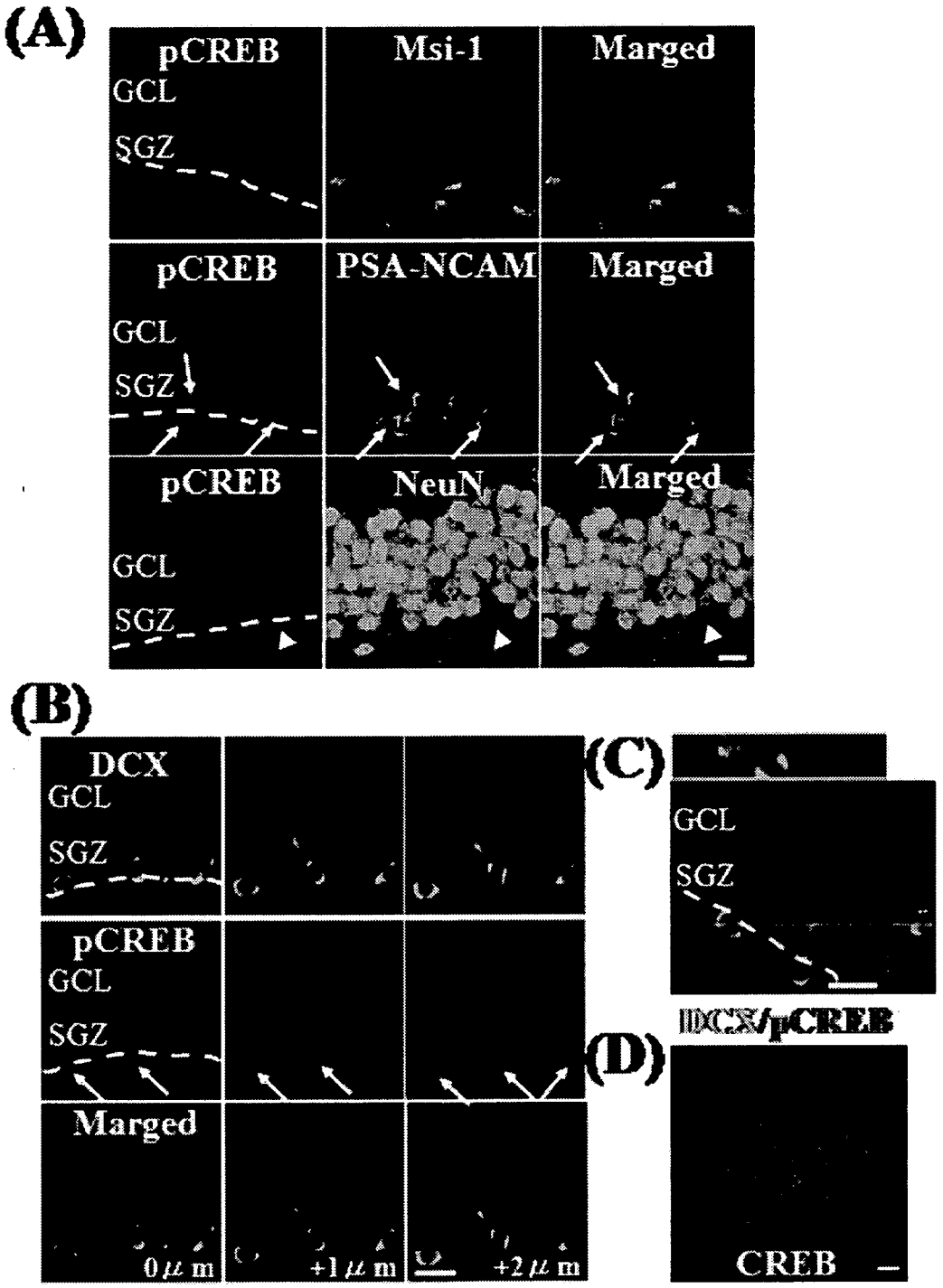

Figure 2. A, Double-immunofluorescence staining of pCREB/Msi-1, pCREB/PSA-NCAM, and pCREB/NeuN in the SGZ. Msi-1–positive cells did not stain for pCREB. PSA-NCAM–positive immature neurons showed intense pCREB immunoreactivity (arrows). NeuN-positive mature neurons showed low levels of pCREB immunostaining (arrowheads). B, Colocalization of DCX and pCREB immunostaining in the SGZ after ischemia (arrows). C, Higher-magnification views of selected individual *z* planes and a series through the section (distance is 10 μm). D, CREB immunostaining in the dentate gyrus. Bars=30 μm in A; 20 μm in B, C, and D.

(NeuN) markers (Figure 2). Msi-1–positive cells in the SGZ and GCL did not stain for pCREB (Figure 2 *A*). Most DCX- or PSA-NCAM–positive immature neurons showed colocalization of pCREB staining (Figures 2A through 2C). Most NeuN-positive mature neurons showed low levels of pCREB staining (Figure 2A). Although only cells within or near the SGZ were positive for pCREB expression (Figures 2A through 2C), most cells throughout the GCL show CREB immunoreactivity (Figure 2D).

To assess the importance of pCREB in immature newborn neurons, inhibition of the CREB-CRE cascade by CRE-decoy oligonucleotide administration was performed. To confirm the distribution of infused CRE-decoy oligonucleotide, brains were removed 24 hours after FITC-labeled CRE-decoy injec-

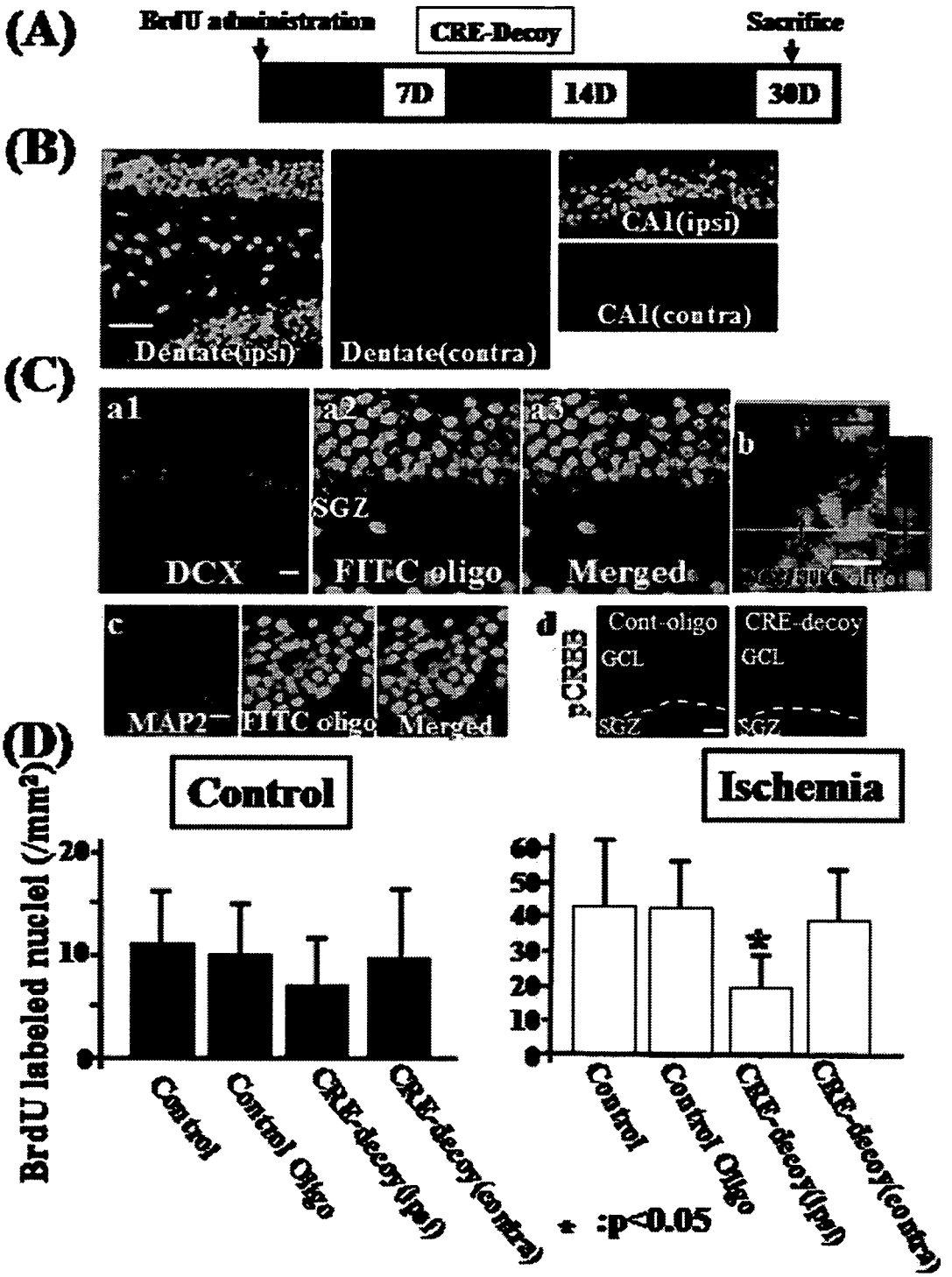

Figure 3. A, Protocol for CRE-decoy treatment. B, Distribution of FITC-labeled CRE decoy was observed throughout the ipsilateral dentate gyrus and pyramidal cell layer in CRE-decoy–injected mice but was not detected in the contralateral hippocampus. C, Expression of FITC-oligo was detected in the immature (a, b) and mature (c) neurons. pCREB immunofluorescence in control oligonucleotide or CRE-decoy oligonucleotide (d). D, Quantification of BrdU-positive newborn neurons under normal conditions and after ischemia. The number of BrdU-positive cells at 30 days after BrdU administration was counted (n=8). *$P<0.05$ vs vehicle. Bar=50 μm in B; 20 μm in C.

tion. Intense FITC immunoreactivity was observed throughout the pyramidal layer and the dentate gyrus on the infused side but not on the contralateral side, indicating that the FITC–CRE-decoy oligonucleotide did not diffused contralaterally (Figure 3B). Next, to examine the phenotype of the FITC–CRE-decoy oligonucleotide–positive cells in the dentate gyrus in the hippocampus, double immunofluorescence was performed. Most DCX-positive immature neurons and MAP2-positive mature neurons showed colocalization of FITC immunostaining (Figure 3C, a–c). Also, the infusion of CRE-decoy oligonucleotide decreased the pCREB immunoreactivities throughout the dentate gyrus (Figure 3C, d).

Under normal conditions, the numbers of BrdU-positive cells in the dentate gyrus of control, control oligonucleotide–,

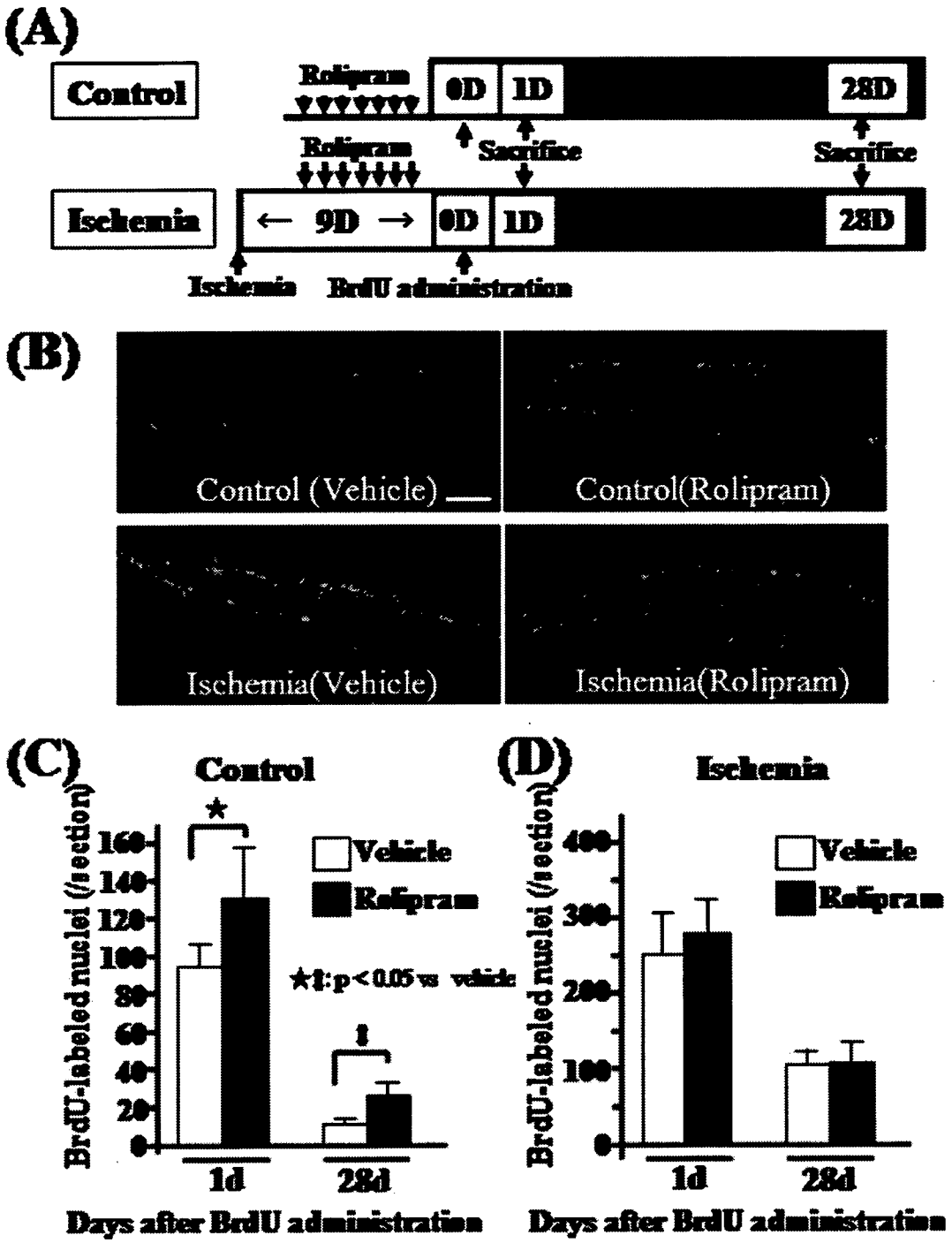

Figure 4. Effect of rolipram treatment on the proliferation of newborn cells in the adult dentate gyrus. A, Protocol for rolipram treatment. B, BrdU-positive newborn cells in the dentate gyrus 1 day after BrdU administration. C and D, Quantification of BrdU-positive newborn cells under normal (C) and ischemic (D) conditions (n=6). #*$P<0.05$ vs vehicle. Bar=400 μm in B.

CRE-decoy (ipsilateral)–, and CRE-decoy (contralateral)–treated groups were 11.9±5.3, 10.0±5.0, 6.7±4.9, and 9.6±6.7/mm^2, respectively (Figure 4C). Under ischemic conditions, mice treated with CRE-decoy oligonucleotide showed a significant decrease in the number of BrdU-positive cells in the dentate gyrus compared with control mice at 30 days after BrdU administration (44.2±23.4/mm^2 in control mice, 43.2±10.5/mm^2 in mice treated with control oligonucleotide, 19.8±8.5/mm^2 in mice treated with CRE-decoy oligonucleotide [ipsilateral], and 40.5±18.4/mm^2 in CRE-decoy oligonucleotide [contralateral], n=6). Additionally, mice treated with CRE-decoy oligonucleotide showed a significant decrease in the number of NeuN- and BrdU-double-positive cells on the infused side in the SGZ compared with control oligonucleotide–treated mice at 30 days after BrdU administration (10.0±2.3/mm^2 in the CRE-decoy oligonucleotide–treated mice on the infused side, 25.9±6.0/mm^2 in the control oligonucleotide–treated mice).

To evaluate the effect of rolipram on the proliferation of newborn cells under normal and ischemic conditions,

rolipram-treated and vehicle-treated mice were killed 1 or 28 days after BrdU administration (Figure 4). Under normal conditions, the number of BrdU-positive cells in the rolipram-treated (3 mg/kg) mice (127.6±30.1/section) was significantly increased over that in the vehicle-treated mice (97.6±11.2/section) 1 day after BrdU administration (Figures 4B and 4C). Because not many newborn cells survived, the total number of BrdU-positive newborn cells in mice treated with rolipram and vehicle decreased proportionally at this time point. However, there was still a significant increase in the number of BrdU-positive cells in rolipram-treated mice relative to vehicle (23.8±4.2/section in the rolipram group, 10.4±3.8/section in the vehicle group; Figures 4B and 4C). In contrast, under ischemic conditions, no significant differences were noted between rolipram-treated (3 mg/kg) mice (274.6±45.4/section) and vehicle-treated mice (252.5±55.4/section) 1 day after BrdU administration (Figures 4B and 4C). At 28 days after BrdU administration, the number of BrdU-positive cells was 115.4±29.2/section in the rolipram-treated mice and 108.2±15.0/section in the vehicle-treated mice.

Next, to evaluate the implication of pCREB expression in immature neurons, we examined the effect of rolipram on the survival of newborn cells. Treatment with 3 mg/kg rolipram from 7 to 28 days after BrdU administration significantly increased the numbers of both BrdU-positive cells (35.1±11.2/mm^2 in the vehicle mice, 42.2±12.1/mm^2 in 1 mg/kg rolipram-treated mice, 56.9±18.2/mm^2 in 3 mg/kg rolipram-treated mice) and BrdU- and NeuN-double-positive newborn cells (18.9±6.7/mm^2 in the vehicle-treated group, 27.3±7.3/mm^2 in mice treated with 1 mg/kg rolipram, 31.5±10.0/mm^2 in mice treated with 3 mg/kg rolipram; Figure 5B). Consistent with these results, daily injection of 3 mg/kg rolipram for 3 weeks significantly increased pCREB staining in DCX-positive immature neurons (55.5±8.2% in the vehicle mice, 75.0±10.4% in 3 mg/kg rolipram-treated mice; Figure 5C). To assess the contribution of apoptotic cell death to the progressive reduction, we used double immunolabeling with anti-BrdU antibody and TUNEL staining. Under ischemic condition, the number of BrdU/TUNEL double-positive cells in the rolipram-treated mice (0.4±0.3/section, n=9) was significantly lower than that in the vehicle group (1.6±0.8/section, n=9).

Discussion

The present findings provide insight into the role of CREB in adult neurogenesis after brain ischemia. Previous studies have shown that running exercise and enriched environment promote the survival of newly generated neurons. An enriched environment has also been shown to enhance pCREB expression in immature neurons of the adult hippocampus.[16]

Previous experiments have suggested that, under physiologic conditions, cAMP-CREB signaling plays an important role in the dentate gyrus in the hippocampus, and activation of cAMP-CREB signaling influences multiple aspects of neurogenesis, including the proliferation, survival, differentiation, and maturation of newborn neurons.[6,7,17] In agreement with reports under physiologic conditions, we found that rolipram treatment enhanced the survival of newborn cells in the hippocampal dentate gyrus. However, after ischemia, in contrast to the enhancing effect of rolipram on the survival of new neurons, rolipram had no significant effect on the proliferation of newborn cells in the hippocampal dentate gyrus. The discrepancy between results under physiologic conditions[18] and those under ischemic conditions[19] has been also reported for the effect of brain-derived neurotrophic factor. These findings suggest that cAMP-CREB signaling has different modulatory actions on physiologic versus ischemia-induced neurogenesis in the hippocampal dentate gyrus.

In adult hippocampal neurogenesis, newborn cells show a progressive decrease in number within the first weeks, which stabilizes after 4 weeks.[20] The time course of colocalization of pCREB and DCX in newborn immature neurons after ischemia was similar to that under normal conditions. We also found that ischemia simultaneously increased neurogenesis and neuronal elimination within a few weeks.[10] In this study, we demonstrated that cAMP-CREB signaling was involved in the survival of newborn neurons after ischemia.

To activate the promoter of CREB target genes possessing the CRE sequence, phosphorylation of CREB at Ser 133 is crucial.[5] Phosphorylation of CREB at this site is thought to recruit the coactivator CREB-binding protein (CBP), which functions by facilitating the interaction of CREB with the basal transcription machinery and by catalyzing the acetylation of chromatin of histone acetyltransferase activity. This activates transcription of genes containing the CRE sequence in their promoter.[21] Therapeutic pharmacological approaches for targeting cAMP-CREB-CBP signaling include PDE4 inhibitors and histone deacetylase inhibitors.[22] PDE hydrolyzes cAMP to AMP. Therefore, inhibition of PDE enhances cAMP-dependent CREB-CBP signaling. Among the PDE subfamily, PDE4 represents 70% to 80% of PDE activity in neuronal tissue.[22] In neuron cultures, PDE4 tightly regulates cAMP formed by stimulation of *N*-methyl-D-aspartate receptors.[23] The PDE inhibitor rolipram readily crosses the blood-brain barrier.[24] Pretreatment with rolipram (3 mg/kg) decreases ischemic neuronal damage.[25] In addition, administration of 1 mg/kg rolipram significantly enhanced hippocampal neurogenesis under normal conditions. Therefore, further studies will be needed to provide insight into the maximal effect of rolipram on neuronal protection and neurogenesis.

It was recently shown that rolipram treatment promotes axonal regeneration, attenuates glial scar formation, and enhances functional recovery after spinal cord injury.[24] Rolipram also has been shown to improve synaptic and cognitive functions in the Alzheimer mouse model.[26] Thus, activation of the cAMP-CREB pathway by rolipram may be an effective therapy for poststroke complications.

In this study, CRE-decoy treatment and rolipram might have indirectly influenced the cAMP-CREB cascade via expression of growth factors that are released from granule cells that surround pCREB-positive newborn neurons[6,7]. Neurotrophins play crucial roles in adult neurogenesis after ischemia as well as under normal conditions. In particular, brain-derived neurotrophic factor and insulinlike growth fac-

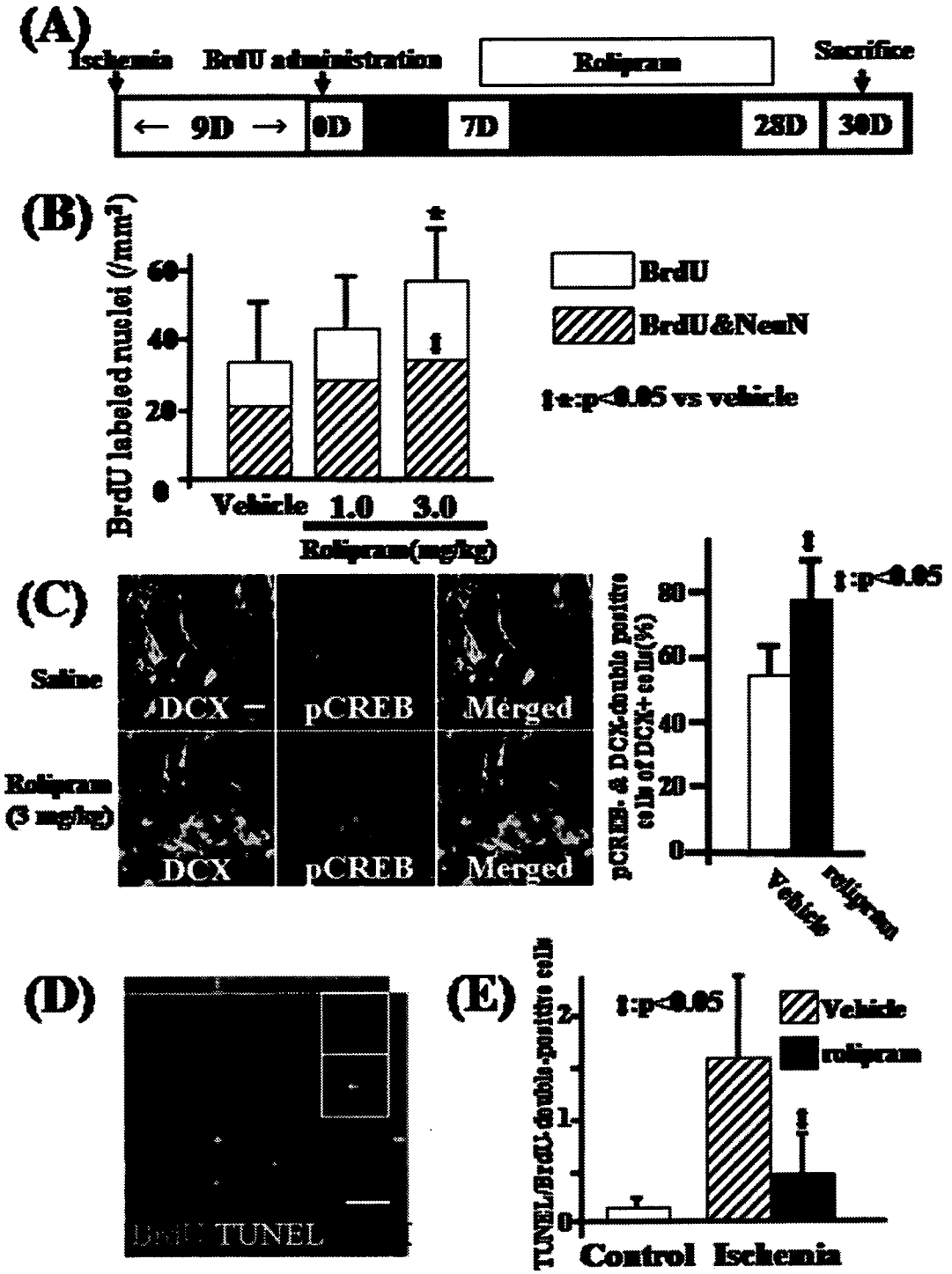

Figure 5. Effect of rolipram treatment on the survival of newborn neurons in the ischemic adult mouse dentate gyrus. A, Protocol for rolipram treatment. B, Number of NeuN- and BrdU-double-positive cells and the total number of BrdU-positive cells at 30 days after BrdU administration (n=8). *$P<0.05$ vs vehicle. C, DCX and pCREB fluorescence in vehicle- or 3 mg/kg rolipram–treated mice starting 7 days after BrdU labeling and daily for 21 consecutive days. D, Colocalization of TUNEL staining and BrdU after ischemia is shown. E, Quantification of apoptosis of newborn neurons after rolipram and vehicle treatment. The number of BrdU/TUNEL double-positive cells at 21 days after BrdU administration was counted (n=8). #$P<0.05$ vs vehicle. Bar=50 μm in C; 30 μm in E.

tor promote adult neurogenesis,[27,28] and both growth factors possess the CRE sequence in their promoters.

In summary, we have shown that pCREB expression is involved in the survival of newborn immature neurons in the adult hippocampus after ischemia. Pharmacological activation of cAMP-CREB signaling after ischemia by administration of the PDE inhibitor rolipram enhanced the survival of newborn neurons. These results indicate that pharmacological activation of cAMP-CREB signaling may provide a therapeutic approach for the treatment of stroke and poststroke complications.

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Disclosures

None.

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Radiological Findings, Clinical Course, and Outcome in Asymptomatic Moyamoya Disease

Results of Multicenter Survey in Japan

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Background and Purpose—Although the development of a noninvasive MR examination has increased the opportunity to identify asymptomatic patients with moyamoya disease who have experienced no stroke episodes, their clinical features are still unclear. This was the first multicenter, nation-wide survey focused on asymptomatic moyamoya disease in Japan and was designed to clarify their clinical features.

Methods—A clinical database of asymptomatic patients with moyamoya disease was collected from 12 participating hospitals in Japan between 2003 and 2006. In total, 40 patients were enrolled in this historical prospective cohort study. Of these, 6 underwent surgical revascularization, including superficial temporal artery to middle cerebral artery anastomosis and/or pial synangiosis. Their demographic and radiological findings as well as outcome were evaluated.

Results—On initial evaluation, cerebral infarction and disturbed cerebral hemodynamics were detected in ≈20% and 40% of the involved hemispheres, respectively. Angiographical stage was more advanced in more elderly patients. Of 34 nonsurgically treated patients, 7 experienced transient ischemic attack (n=3), ischemic stroke (n=1), or intracranial bleeding (n=3) during follow-up periods (mean, 43.7 months). The annual risk for any stroke was 3.2%. Disease progression was associated with ischemic events or silent infarction in 4 of 5 patients. No cerebrovascular event occurred in the 6 patients who underwent surgical revascularization.

Conclusions—The findings revealed that asymptomatic moyamoya disease is not a silent disorder and may potentially cause ischemic or hemorrhagic stroke. Asymptomatic patients with moyamoya disease should be carefully followed-up to further clarify their outcome and to establish the management guideline for them. (***Stroke.* 2007;38:1430-1435.)**

Key Words: cerebral infarction ■ disease progression ■ intracranial bleeding ■ moyamoya disease ■ ■ prognosis

Moyamoya disease is characterized by progressive stenosis of the terminal portion of the bilateral internal carotid arteries and is associated with an abnormal vascular network, called moyamoya vessels.[1] The etiology of the disease is still unknown; however, several epidemiological studies have suggested the involvement of some genetic factors in its pathogenesis. The potential contribution of infections has also been pointed out, although specific pathogens have not been identified.[2]

It is well known that moyamoya disease causes transient ischemic attacks (TIAs), cerebral infarction, or intracranial bleeding in both children and adults. Intracranial bleeding, in particular, often results in a poor outcome.[3,4] Cerebral revascularization surgery is believed to reduce the incidence and improve the long-term prognosis in patients with moyamoya disease.[3,5,6] The recent development of noninvasive diagnostic modalities, including MRI and MRA, has led to the realization that the incidence of asymptomatic moyamoya disease may be higher than previously thought.[7–9] "Asymptomatic" patients with moyamoya disease have previously been defined as those who have experienced neither ischemic nor hemorrhagic episode, although the definition is not determined.[7–9] However, even in Japan, their epidemiology is still obscure, and guidelines for the management of asymptomatic moyamoya disease have not yet been established. Thus, it is essential to elucidate their clinical features and natural course so that guidelines for the management of asymptomatic patients can be established. As a preliminary study, we have previously analyzed the clinical data of 10 asymptomatic patients whose diagnoses were made at Hokkaido University Hospital as moyamoya disease. However, the results were limited in their usefulness because of small patient numbers and short follow-up periods.[8]

Based on these considerations, we conducted the first multicenter, nation-wide survey focused on asymptomatic patients

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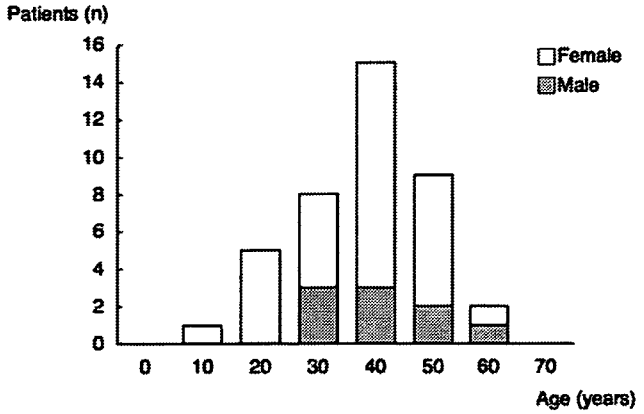

Figure 1. Distribution of age and gender in 40 patients enrolled in this study because of asymptomatic moyamoya disease.

with moyamoya disease to clarify clinical characteristics, radiological findings, and outcome. We believe that the accumulation of this clinical data will be valuable for the establishment of management guidelines for moyamoya disease.[10]

Materials and Methods

Participating Centers and Hospitals

In August 2003, we sent an invitation to participate to the members of the Research Committee on Moyamoya Disease of the Japan of Ministry of Health, Labor and Welfare of Japan, in 16 hospitals. Of these, 12 hospitals accepted our invitation, and a total of 40 asymptomatic patients were enrolled in this historical prospective cohort study (see Appendix). Follow-up data were collected from the 12 participating hospitals in March 2006.

Patients

All patients were Japanese and met the guidelines for the diagnosis of moyamoya disease set by the Research Committee on Moyamoya Disease of the Ministry of Health and Welfare of Japan. All of them previously had no ischemic or hemorrhagic episode and were neurologically free. Patients who experienced any episode suggestive of TIA, cerebral infarction, intracranial bleeding, seizure, or involuntary movement caused by moyamoya disease were excluded. MRI and MRA were performed in all patients, using a 1.5-T whole-body magnetic resonance imager. Cerebral angiography was performed in 37 of 40 patients. Using xenon CT, single photon emission tomography, or PET, cerebral blood flow and cerebrovascular reactivity to acetazolamide were determined in 35 of 40 patients.

In this study, patient demographical data, radiological findings, medical and surgical treatment, and outcome were precisely analyzed.

Statistical Analysis

Continuous variables were expressed as percentage or as mean±SD. Statistical analysis was performed using χ^2 test and Kruskal-Wallis test as appropriate. The statistical level of significance was set at $P<0.05$. Statistical analysis was completed with StatView version 5.0 (SAS Institute, Inc).

Results

Demographic Features

Of 40 asymptomatic patients with moyamoya disease, there were 13 males and 27 females. Thus, the female-to-male ratio was 2.1. Their mean age at diagnosis was 41.4±12.6 years, ranging from 13 to 67 years (Figure 1). Thirty-seven patients had typical "bilateral" moyamoya disease (definite cases) diagnosed, and the remaining 3 had "unilateral" moyamoya disease diagnosed (probable cases). Therefore, the total number of involved hemispheres was 77.

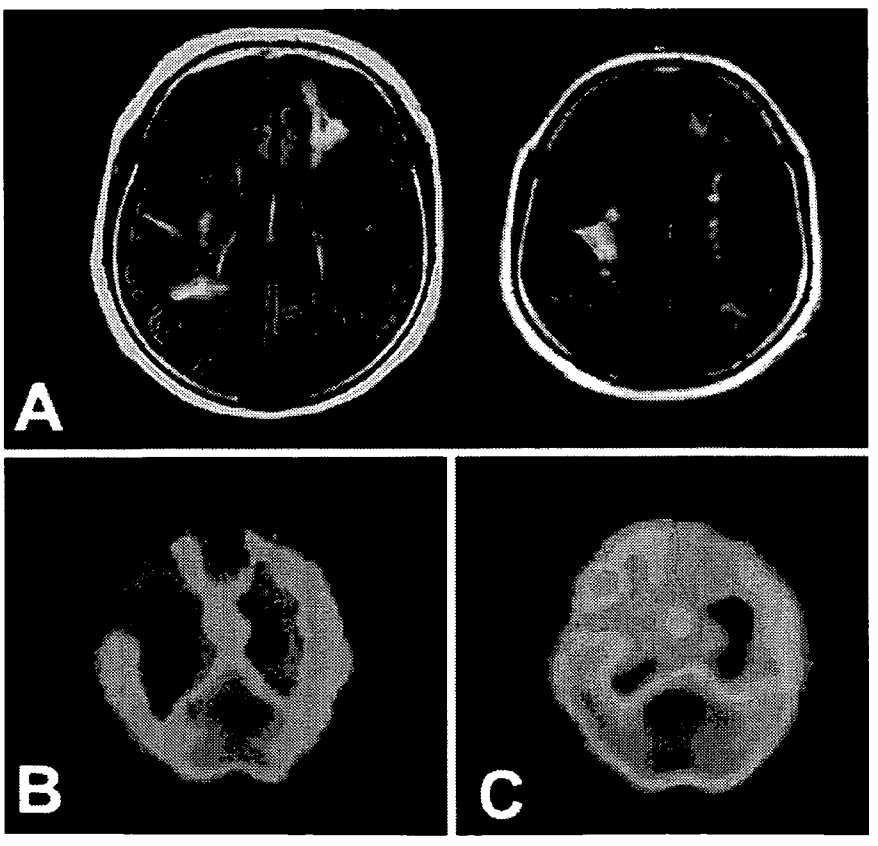

Figure 2. Radiological findings of a 65-year-old woman with asymptomatic moyamoya disease. Note multiple cerebral infarctions in both hemispheres on MRI (A) and reduction of cerebral blood flow and its reactivity to acetazolamide on single photon emission tomography before (B) and after intravenous injection of acetazolamide (C).

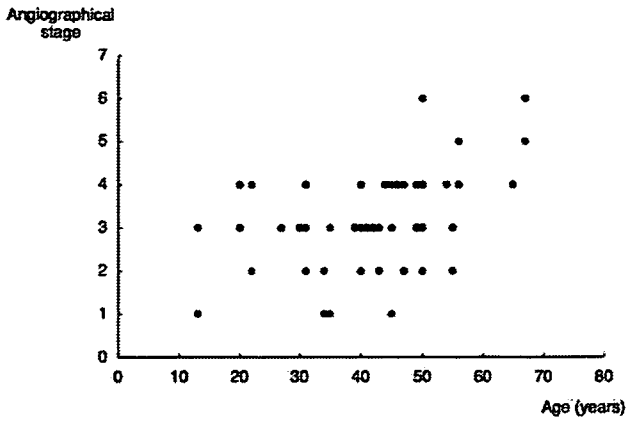

Figure 3. Relationship between age and angiographical stage in asymptomatic moyamoya disease.

Clues to the diagnosis were tension-type headache in 14 patients, dizziness in 5, and head trauma in 4. Five patients were incidentally diagnosed on MRI and MRA performed for a brain health check-up. Five diagnoses were made on MRI and MRA performed for screening, because a member of their family had moyamoya disease diagnosed. They were siblings in 2 and offspring in 3. The remaining 7 cases were diagnosed on MRI and MRA performed because of an unrelated disease in other organs.

Radiological Findings

MRI detected cerebral infarction in 16 (20.8%) of 77 involved hemispheres, or in 12 (30%) of 40 patients (Figure 2). However, there was no cerebral infarction in the uninvolved hemispheres or in the vertebrobasilar territories. No intracranial bleeding was noted. Disease stage, as determined by cerebral angiography, varied widely. Of 72 examined hemispheres, four (5.6%) were graded as stage 1, 10 (13.9%) as stage 2, 33 (45.8%) as stage 3, 21 (29.2%) as stage 4, 2 (2.8%) as stage 5, and 2 (2.8%) as stage 6. Thus ≈75% of the hemispheres were graded as stage 3 or stage 4. Correlation analysis revealed that older patients had significantly more advanced disease stage (P=0.0134; Figure 3).

Cerebral blood flow studies showed that 39 (55.7%) of 70 examined hemispheres had normal cerebral blood flow and cerebrovascular reactivity to acetazolamide. However, 24 hemispheres (34.3%) had moderate impairment of cerebral hemodynamics, ie, normal cerebral blood flow but reduced cerebrovascular reactivity to acetazolamide. Seven (10%) had reduced cerebral blood flow and cerebrovascular reactivity, suggesting a marked reduction of cerebral perfusion pressure (Figure 2).[11]

Treatments and Outcome

Of 40 subjects, 6 underwent bypass surgery, including superficial temporal artery to middle cerebral artery anastomosis, on one or both hemispheres. Eleven patients were medically treated with anticonvulsants, antiplatelet agent, or other pharmacological agents. The remaining 24 patients were conservatively followed-up as outpatients. All patients were followed-up for a mean period of 43.7 months, with a range of 1 to 150 months.

Of 6 patients who underwent bypass surgery, none experienced any ischemic or hemorrhagic episodes during follow-up periods.

TABLE 1. Summary of Clinical Data in 10 Asymptomatic Patients Who Developed Cerebrovascular Events or Showed Silent Radiological Changes During Follow-Up Periods

Case	Age	Gender	Cerebral Infarction	Angiographical Stage		CBF Study	Bypass Surgery	Cerebrovascular Event	Radiological Change	Follow-Up Period (months)
				Rt	Lt					
Symptomatic Transition										
1	22	F	None	4	2	CBF/CVR decrease (Rt)	None	TIA (Rt)	Cerebral infarction (Rt), disease progression (both)	36
2	49	M	None	3	3	CVR decrease (both)	None	TIA (Lt)	None	93
3	33	F	None	4	3	Normal	None	TIA (Lt)	Disease progression (Lt)	17
4	62	M	None	2	3	CVR decrease (Rt)	None	Ischemic stroke (Lt)	Cerebral infarction (Lt), disease progression (both)	45
5	31	M	None	4	4	Not done	None	Hemorrhagic stroke (Lt)	ICH (Lt)	1
6	51	F	None	4	4	Normal	None	Hemorrhagic stroke (Lt)	ICH (Lt)	54
7	33	F	Lt (+)	3	3	CVR decrease (both)	None	Hemorrhagic stroke (Rt)	ICH (Rt)	42
Silent Radiological Changes										
8	47	M	None	4	2	CVR decrease (Rt)	None	None	Cerebral infarction (Lt), disease progression (both)	14
9	56	F	None	3	2	Normal	None	None	Disease progression (Lt)	60
10	51	F	Rt (+)	4	3	CVR decrease (Rt)	None	None	Microbleeds (Rt)	8

CBF, cerebral blood flow; CVR, cerebrovascular reactivity; ICH, intracerebral hemorrhage.

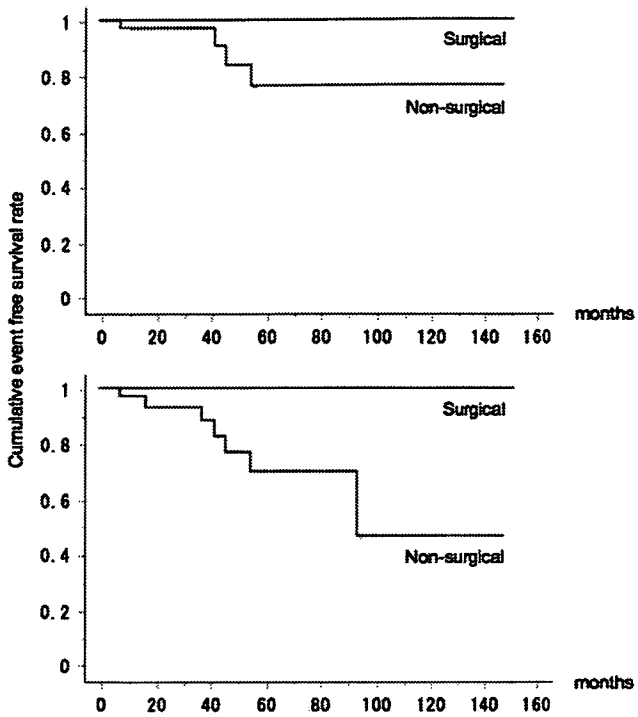

Figure 4. Kaplan–Meier cumulative event-free survival rate curves for stroke (top) and for all cerebrovascular event including TIA and stroke (bottom). Data for nonsurgically treated patients are shown in red; data for surgically treated patients are shown in blue.

Of other 34 nonsurgically treated patients, 7 experienced cerebrovascular events. Of these, 3 patients had TIA, 1 had ischemic stroke, and 3 had intracranial bleeding. Thus, 4 patients had ischemic or hemorrhagic stroke during follow-up periods (Table 1). The annual risk for any stroke was 3.2%. Figure 4 shows a Kaplan-Meier curve to demonstrate the time to cerebrovascular event.

Table 2 shows the relationship between cerebral hemodynamics at initial diagnosis and cerebrovascular events in nonsurgically treated patients. Disturbed hemodynamics was significantly linked to ischemic episodes ($P<0.05$). Disease progression caused TIA and ischemic stroke in both patients who showed normal hemodynamics on initial evaluation.

No death was observed during the patient follow-up periods. The outcome in March 2006 was categorized based on a modified Rankin scale score of 0 (n=38), 1 (n=1, ischemic stroke) and 4 (n=1, intracranial bleeding). Thus, favorable outcome (defined as modified Rankin scale ≤2) was observed in 39 (97.5%) of the 40 patients.

TABLE 2. Relationship Between Cerebral Hemodynamics at Initial Diagnosis and Cerebrovascular Events During Follow-Up Periods in Patients Who Were Medically Treated

	Cerebrovascular Event		
	None	TIA/Infarct	Bleeding
Normal	36	2*	1
Moderate ischemia	15	1	1
Severe ischemia	5	1	0
Not examined	6	0	1

*Cerebrovascular events were closely related to disease progression during follow-up periods.

Follow-Up MRI and MRA

None of 6 surgically treated patients had any new cerebral infarction and intracranial bleeding on follow-up MRI.

Of other 34 nonsurgically treated patients, 7 had new lesions on follow-up MRI (Table 1). Of these, 3 had new cerebral infarction, asymptomatic in 1 patient and asymptomatic in 2. Other 4 patients had new intracerebral hemorrhage, asymptomatic in 1 patient and symptomatic in 3. Of 34 nonsurgically treated patients, 5 showed progression of disease stage on follow-up MRA or cerebral angiography (Figure 5). Disease progression was asymptomatic in 1 patient, but caused silent cerebral infarction in 1, TIA in 2, and ischemic stroke in another (Table 1).

Discussion

This study is the first multicenter, nation-wide survey focused on asymptomatic patients with moyamoya disease and has important implications for defining its clinical features, radiological findings, and prognosis. Our findings are summarized as follows. Cerebral infarction and disturbed cerebral hemodynamics were detected in ≈20% and 40% of the involved hemispheres, respectively. Angiographical stage was more advanced in more elderly patients. Of 34 nonsurgically treated patients, 7 experienced TIA, ischemic stroke or intracranial bleeding during a mean follow-up period of 43.7 months. Cerebral infarction or intracerebral hemorrhage did not occur in 6 patients who underwent surgical revascularization.

Epidemiology of Asymptomatic Moyamoya Disease

Previously, asymptomatic cases of moyamoya disease have only been sporadically reported.[7,9] Screening of family members with moyamoya disease has also identified small numbers of asymptomatic patients.[12,13] Therefore, the incidence of asymptomatic moyamoya disease had been considered to be very low. However, Yamada et al[14] reported the results of a nation-wide questionnaire conducted in 1994 and identified 33 asymptomatic patients (1.5%) out of a total of 2193 patients. Recently, Nanba et al[8] (2003) reviewed their single-center experiences and precisely reported the clinical features of 10 asymptomatic patients with moyamoya disease. Therefore, although an accurate prevalence of asymptomatic moyamoya disease is still unknown, it may be much higher than considered before. The female-to-male ratio and mean age of the patients in these studies were similar to those of moyamoya disease as a whole.[15]

Of the 40 patients, 23 had moyamoya disease diagnosed when they visited hospitals for treatment of complaints unrelated to moyamoya disease. Although it is known that moyamoya disease is sometimes associated with migraine,[16] the headaches of these patients were considered to be tension-type. The remaining 17 patients had no symptoms and had moyamoya disease diagnosed incidentally during screening examinations using MRI and MRA. Thus, noninvasive MR examination would increase the opportunity to detect asymptomatic moyamoya disease in future.[17]

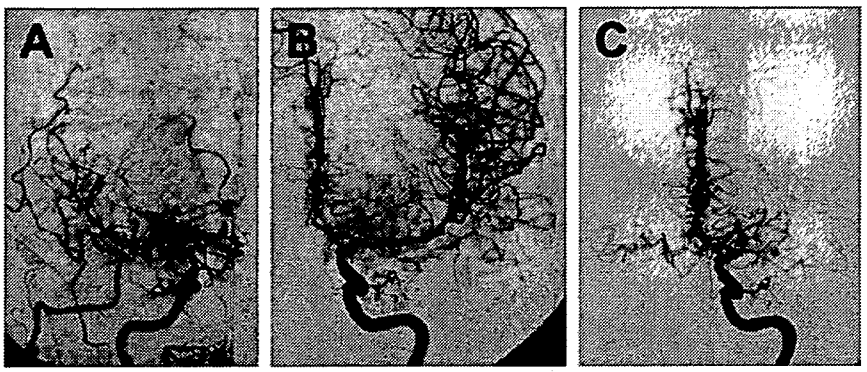

Figure 5. Internal carotid angiograms of a 51-year-old man who experienced ischemic stroke 4 years after initial diagnosis. Right (A) and left (B) internal carotid angiograms on initial diagnosis, and left internal carotid angiogram (C) 4 years later.

Silent Radiological Findings

Silent cerebral infarction was noted in ≈20% of the involved hemispheres but was not detected in other territories. The incidence was almost same as that of silent cerebral infarction (21%) in 1015 elderly patients aged 60 to 90 years (mean, 72 years).[18] According to a population-based consecutive autopsy study in Japan, the incidence of silent cerebral infarction was 4.4% in 40- to 59-year-old population.[19] Therefore, moyamoya disease may be related to the development of silent cerebral infarction even in asymptomatic patients. Although adults with moyamoya disease have also been known to have intracranial bleeding, no intracranial bleeding was observed in this study. However, very recent studies have shown that T2*-weighted MRI can detect microbleeds in a certain subgroup of patients with moyamoya disease.[20,21] With this technique, however, further study would be necessary to predict their risk for intracranial bleeding by assessing for the presence of microbleeds.

In this study, cerebral blood flow measurements demonstrated that ≈40% of the involved hemispheres had a moderate or severe reduction of cerebral perfusion reserve, despite the fact that patients remained asymptomatic. Recent studies have proven that both increased oxygen extraction fraction and impaired reactivity to acetazolamide can be independent predictors for subsequent ischemic stroke in patients with occlusive carotid artery diseases.[11,22–24] Therefore, critical follow-up would be essential for patients found to have increased oxygen extraction fraction or impaired cerebrovascular reactivity.

Clinical and Radiological Course

This study clearly demonstrates that disease stage is more advanced in older patients. Although disease progression in adult moyamoya disease was believed to be very rare before, a recent study has shown that disease progression occurs in ≈20% of patients during a mean follow-up period of 6 years.[25] Occlusive arterial lesions progress in both anterior and posterior circulation, in both bilateral and unilateral types, and in both symptomatic and asymptomatic patients. Multivariate analysis has revealed that female gender is an independent risk factor for disease progression.[25] Therefore, it should be emphasized that disease progression may occur silently and cause ischemic or hemorrhagic stroke even in asymptomatic patients. Aging-related atherosclerosis may also be involved in disease progression in elderly patients. Indeed, this study demonstrates that disease progression occurred in 5 asymptomatic patients, caused TIA or ischemic stroke in 3 patients, and resulted in silent cerebral infarction in one. The findings strongly suggest that it is quite important to repeat MRI and MRA at regular intervals when asymptomatic patients are conservatively followed-up to detect disease progression before ischemic stroke occurs.

There is no guideline to direct how asymptomatic patients with moyamoya disease should be managed.[8] In this study, of the 34 nonsurgically treated patients, 7 patients experienced cerebrovascular episodes, including TIA. The annual risk for ischemic or hemorrhagic stroke was 3.2%. Disease progression was closely related to the onset of ischemic episodes. Nanba et al[8] reported that 1 of 10 asymptomatic patients experienced ischemic stroke during follow-up periods. Yamada et al[14] also evaluated the natural course of 33 asymptomatic patients with moyamoya disease; they reported that 2 patients died from intracranial bleeding, and 4 patients experienced TIA during a mean follow-up period of 44 months.

Of note, there is a peculiarity common to the present study and the report by Yamada et al.[14] None of patients who underwent surgical revascularization had any cerebrovascular event during follow-up periods, except for surgical morbidity.[14] These findings may suggest that asymptomatic moyamoya disease is not a silent disorder and readily progresses to cause ischemic or hemorrhagic stroke. Surgical revascularization may be indicated, at least, in patients who have disturbed cerebral hemodynamics if surgical morbidity is low enough, because the procedure is considered effective for improving cerebral blood flow and metabolism and preventing ischemic stroke.[26,27] It is still unclear whether surgical revascularization could reduce the incidence of intracranial bleeding caused by moyamoya disease, although a randomized clinical trial in Japan is ongoing.[28] Even if patients are conservatively followed-up, precise and regular MRI/MRA examinations could be essential for improving long-term outcome by predicting subsequent ischemic and hemorrhagic stroke, because repeated MRI/MRA have the ability to detect disease progression and silent microbleeds before the onset of ischemic or hemorrhagic stroke.[20,21,25]

Limitation of This Study

There are certain limitations to this study that should be noted. This study is a historical prospective cohort study and not a prospective cohort study. The subjects included in this study were collected from 12 hospitals. These hospitals are considered representative of the major institutions in Japan responsible for the management of moyamoya disease. Ide-

ally, however, a prospective cohort or randomized study should be performed on the basis of a larger population of asymptomatic patients to build the accurate evidence on the clinical features and outcome of this disease.

Conclusions

This multicenter, nation-wide survey reveals that the prevalence of asymptomatic patients with moyamoya disease may be higher than previously thought. Although these patients are still "asymptomatic," their radiological findings are not always normal. A certain subgroup has silent cerebral infarction, advanced arterial lesions, and impaired cerebral hemodynamics. Of 34 nonsurgically treated patients, 7 transitioned to become "symptomatic" patients during follow-up periods. Silent radiological findings were added in 3 other patients. None of patients who underwent surgical revascularization experienced any cerebrovascular event during follow-up periods. Careful and long-term neurological and radiological follow-up would be essential to improve the outcome of these patients by preventing ischemic and hemorrhagic stroke. Further prospective studies may be necessary to finalize the management guideline for asymptomatic patients with moyamoya disease.

Appendix

All of clinical data in this study were collected from Department of Neurosurgery, Iwate Medical University; Department of Neurology, Keio University; Department of Neurosurgery, Chugoku Rosai Hospital; Department of Neurosurgery, Nara Medical University; Department of Neurosurgery, Nagasaki University; Departments of Neurology and Neurosurgery, Kyushu Medical Center; Department of Neurosurgery, Nagaoka Central Hospitals; Department of Neurosurgery, Nagoya City University; Departments of Neurology and Neurosurgery, Kitasato University; Department of Neurosurgery, Gifu University; Department of Neurosurgery, Sapporo Medical University; and Department of Neurosurgery, Hokkaido University.

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Disclosures

None.

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EXPRESSION OF HYPOXIA-INDUCING FACTOR-1α AND ENDOGLIN IN INTIMAL HYPERPLASIA OF THE MIDDLE CEREBRAL ARTERY OF PATIENTS WITH MOYAMOYA DISEASE

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OBJECTIVE: Moyamoya disease (MMD) is a cerebrovascular occlusive disease characterized by progressive stenosis or occlusion at the distal ends of the bilateral internal arteries. In MMD, intimal hyperplasia was previously reported to be found in autopsy samples. In this study focusing on the mechanism of remodeling of the intracranial arterial walls of patients with MMD, we surgically collected tiny pieces of the wall of the middle cerebral artery (MCA) from patients with MMD and analyzed them using histological and immunohistochemical methods.

METHODS: Twelve patients underwent surgical procedures for treatment of standard indications of MMD at Kyoto University Hospital. Specimens of MCA were obtained from MMD patients during the surgical procedures. Nine MCA samples were also obtained in the same way from control patients. The samples were analyzed by immunohistochemical methods.

RESULTS: MCA specimens from MMD patients had a thicker intima than those from the control group. In MMD samples, the immunoreactivity indicating hypoxia-inducing factor-1α was higher in the endothelium and intima; endoglin expression was also higher in the endothelium. No vascular endothelial growth factor immunoreactivity was detectable in the MMD samples. In addition, transforming growth factor-β3 immunoreactivity was also detected and was co-localized with that of hypoxia-inducing factor-1α and endoglin, mainly in the endothelium.

CONCLUSION: Our results indicate that the MCA specimens from MMD patients had thicker intimal walls than the specimens from control patients. In addition, hypoxia-inducing factor-1α and endoglin were overexpressed in the intima of the MCA of MMD patients.

KEY WORDS: Endoglin, Hypoxia-inducing factor-1α, Intimal hyperplasia, Moyamoya disease

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Moyamoya disease (MMD) is a cerebrovascular occlusive disease characterized by progressive stenosis or occlusion at the distal ends of bilateral internal arteries (29). The unusual vascular network at the base of the brain (moyamoya vessels) is considered to represent collateral channels formed as a result of progressive ischemic changes in the brain (29). The etiology of the disease is undefined. The findings that the incidence of the disease is highest in, but not confined to, the Japanese population and that the condition is often familial suggest the involvement of a genetic factor in its pathogenesis (29).

Histopathological investigations on autopsy subjects have demonstrated that the main vascular lesion in moyamoya disease is stenosis or occlusion caused by a fibrocellular intimal thickening with a multilayered elastic lamina and some lipid deposits (11, 29, 33). In addition, a decrease in the number of medial smooth muscle cells was found (11, 33). As for the treatment of MMD, superficial temporal artery-middle cerebral artery (STA-MCA) bypass surgery or indirect revascularization is usually performed (12, 14, 17). During this surgery, the middle cerebral artery (MCA) of MMD patients is observed to have a type of

arterial wall different from that bearing atherosclerotic lesions (17, 34).

Several studies on extracranial vessels have been reported previously (2, 4, 8, 10, 28). However, few reports are available on intracranial arteries from MMD patients other than those based on autopsy specimens (11, 33).

In this study, we collected tiny pieces of MCA walls from patients with MMD and analyzed their histopathological features. In addition, we analyzed these vessel walls immunohistochemically to explore the mechanism of remodeling of the intracranial arterial walls in the MMD patients.

PATIENTS AND METHODS

Patients

Twelve patients underwent surgical procedures for treatment of standard indications of moyamoya disease at Kyoto University Hospital in Kyoto, Japan. Specimens were obtained from the patients during the surgical procedures (*Fig. 1*). Clinical data on the patients are summarized in *Table 1*. Twelve control MCA samples were also obtained in the same manner from the control subjects described in *Table 1*. Nine patients with internal carotid artery or MCA occlusion underwent STA-MCA bypass surgery in the chronic stage. Three patients with aneurysms also underwent STA-MCA bypass surgery when the parent artery was occluded. This study was performed under the guidelines provided by the ethics committee of the Kyoto University School of Medicine.

Sample Preparation

During STA-MCA bypass surgery, an 11–0 nylon monofilament was passed around the wall of the recipient artery. The vessel was then pulled up by lifting the monofilament with forceps, and the operator (YT or KK) performed arteriotomy with microscissors (*Fig. 1*). Then, STA-MCA end-to-side anastomosis was performed. In some experiments, a control sample was obtained from a 27-year-old woman with an arteriovenous malformation.

All specimens were fixed in 10% formalin overnight and embedded in paraffin the next day. The specimens were stored at room temperature. In each case, multiple, sequential, 6-μm thick tissue sections cut from paraffin blocks were deparaffinized in xylene, rehydrated, and prepared for immunohistochemical studies.

Antibodies

Anti-hypoxia-inducible factor-1α (HIF-1α; 1:100; NeoMarkers, Fremont, CA), anti-endoglin, (CD105; 1:30; DAKO, Glostrup, Denmark), anti-vascular endothelial growth factor (VEGF; 1:100; NeoMarkers), and anti-transforming growth factor (TGF)-β3 (1:50; SantaCruz Biotechnology, Santa Cruz, CA) were used as the primary antibodies in this study. As secondary antibodies, fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G and cyanogen3-conjugated anti-rabbit immunoglobulin G (1:200; Jackson Immunoresearch, West Grove, PA) were used.

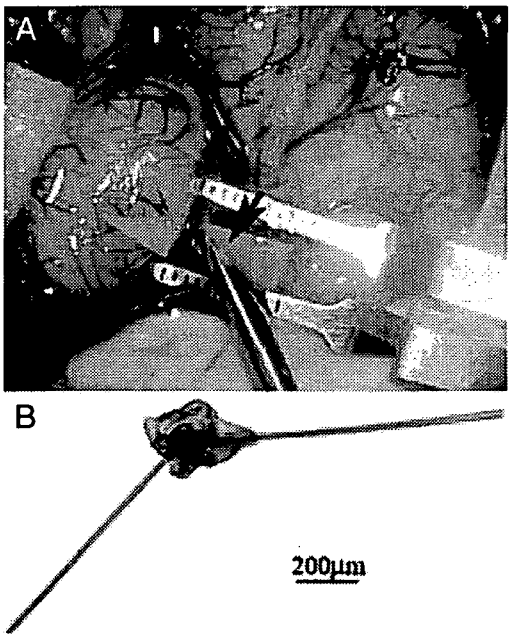

FIGURE 1. A, *surgical view of STA-MCA bypass for the treatment of MMD. The operator (YT or KK) performed arteriotomy with microscissors* (arrow *indicates collected tissue).* B, *samples were fixed in 10% formalin and viewed under a microscope.*

TABLE 1. Summary of patients[a]

	MMD	Control
No. of patients	12	12
Age (yr)	37 ± 8	55 ± 20
Sex (men/women)	2/10	10/2
Type of onset		
TIA	11	7
Infarction	1	4
Hemorrhage	0	0
Mass effect	0	1
Disease		ICA-O 8
		MCA-O 1
		Aneurysm 3
Samples		
M4	12	10
M2	0	2

[a] MMD, moyamoya disease; TIA, transient ischemic attack; ICA-O, internal cerebral artery occlusion; MCA-O, middle cerebral artery occlusion. Data are expressed as mean ± standard deviation.

Immunohistochemical Analysis

The sections were washed for 5 minutes with 0.01 mol/L phosphate-buffered saline (PBS; pH, 7.2), followed by a 15-minute incubation with 10 μg/ml proteinase K. After being blocked with 3% H_2O_2, the sections were preincubated with normal goat serum (diluted 1:50) and then incubated overnight at 4°C with the desired primary antibody. After three 15-minute

rinses with PBS, the sections were incubated for 30 minutes with the anti-mouse Envision/horseradish peroxidase system (Dako). After three more rinses with PBS, the sections underwent color development for 5 minutes at room temperature in a substrate medium containing 0.05% 3,3-diaminobenzidine and 0.02% H_2O_2 in Tris-hydrochloride buffer (pH, 7.6). The specificity of the staining was confirmed by the absence of specific staining when nonimmune rabbit immunoglobulin G was substituted for the primary antibody. The sections were analyzed under a BX51 fluorescent microscope (Olympus Optical Co., Tokyo, Japan) or a Fluoview FV300 laser confocal microscope (Olympus Optical Co.).

Immunofluorescence Double-staining

The sections were washed for 5 minutes with 0.01 mol/L PBS (pH, 7.2) and then incubated for 15 minutes with 10 μg/ml proteinase K. The sections were preincubated with skim milk (diluted 1:50) and were then incubated overnight at 4°C with the desired primary antibodies. After three 15-minute rinses with PBS, they were incubated for 30 minutes with the fluorescent secondary antibodies. After three additional rinses with PBS, the sections were observed under a BX51 fluorescence microscope (Olympus Optical Co.).

Immunohistochemical Analysis

Under a BX51 fluorescence microscope (Olympus Optical Co.), the histological images were captured with a computer. Then, intimal thickness and the number of immunopositive cells were analyzed by use of an Image-Pro image-analyzing system (Media Cybernetics, Silver Spring, MD). Using an image analysis system, the immunoreactivity was assessed by two observers (YT, KK) in a blind manner. The two observers set their own result about the percentage of immunopositive cells in each sample, and the mean score was recorded.

Single-photon Emission Computed Tomography

Cerebral blood flow (CBF) and cerebrovascular reserve (CVR) were assessed as previously described (35). The entire territory of the MCA was the region of interest for evaluation of the regional CBF (rCBF).

Statistical Analysis

As for the results including intimal thickness, the percentage of immunopositive cells, rCBF, and regional CVR (rCVR), the Mann-Whitney and Fisher's exact tests were used for statistical analysis (StatView; SAS Institute, Cary, NC). A P value less than 0.05 was considered statistically significant.

RESULTS

Thickness of the Intima

Using the collected specimens, we measured the thickness of the intima in hematoxylin and eosin-stained sections (*Fig. 2*). The mean thickness of the MMD intima was significantly greater than that of the control specimens (MMD, 38 ± 19 μm; control, 11 ± 5 μm; all data are expressed as the mean ± standard deviation; $P = 0.0006$) (*Fig. 2, Table 2*). The mean values of rCBF and rCVR in the MMD patients were 35 ± 5 ml/100g/minute and −9 ± 9%, respectively. In control patients with internal carotid artery or MCA occlusion (n = 9), those values were 26 ± 4 ml/100g/minute and −8 ± 7%, respectively.

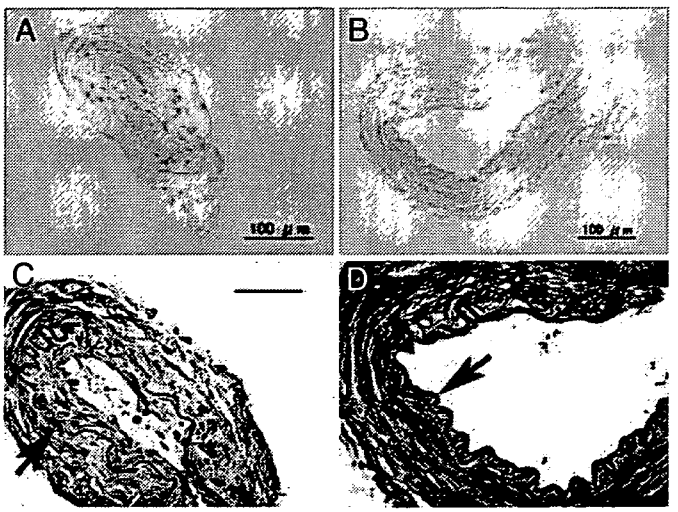

FIGURE 2. *A and B, hematoxylin and eosin-stained specimens from the MCA of a patient with MMD (A) and a control patient (B). C and D, histological findings assessed by interference differential microscopy of the MCA of a patient with MMD (C) and a control patient (D). The* arrows *indicate the internal elastic lamina. Scale bar, 150 μm (C); original magnification, ×100 (A and B) and ×200 (C and D).*

TABLE 2. Intimal hyperplasia of middle cerebral arteries of patients with moyamoya disease[a]

	MMD	Control
No. of patients	12	12
Intimal thickness (μm)	38 ± 19[b]	11 ± 5

[a] MMD, moyamoya disease. Data are expressed as mean ± standard deviation.
[b] $P = 0.0006$.

Expression of HIF-1α Immunoreactivity

Next, we performed immunohistochemical studies on the MCA from MMD patients by using antibody specific for HIF-1α. For the MMD specimens, 66 ± 22% of the endothelial and 33 ± 17% of the intimal cells demonstrated anti-HIF-1α immunoreactivity (*Fig. 3, Table 3*). On the contrary, for control samples, the respective values were 21 ± 12% and 14 ± 7% (*Fig. 3, Table 3*). (All data are expressed as the mean ± standard deviation; $P < 0.0001$ and 0.006, respectively.)

Expression of Endoglin Immunoreactivity

Next, we performed immunohistochemical analysis of MMD specimens using antibodies specifically recognizing VEGF and endoglin. VEGF immunoreactivity was not

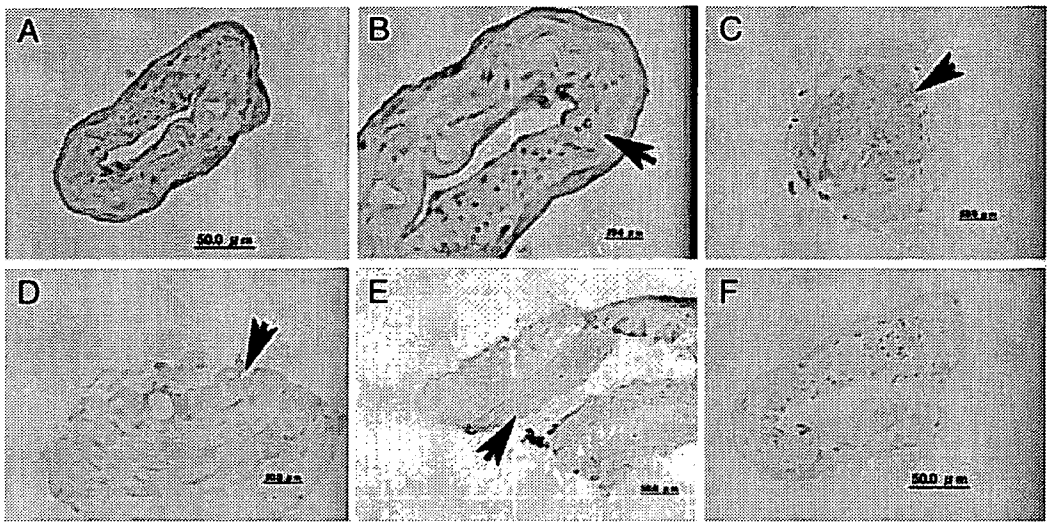

FIGURE 3. *Immunohistochemical analysis of HIF-1α.* A–C, *cells that tested immunopositive for HIF-1α are detected in the endothelium and intima of the MCA specimens of patients with MMD.* Arrows *indicate the internal elastic lamina.* D *and* E, *in this control specimen, a smaller number of such cells are detected.* Arrows *indicate the internal elastic lamina.* F, *no positive staining is seen in the negative control samples in which case the primary antibody was omitted.*

TABLE 3. Intimal expression of hypoxia-inducing factor-1α and endoglin in middle cerebral arteries with moyamoya disease[a]

	HIF-1α		Endoglin	
	Endothelium	Intima	Endothelium	Intima
MMD (n = 12)	66 ± 22%	33 ± 17%	67 ± 27%	5 ± 8%
Control (n = 12)	21 ± 12%	14 ± 7%	23 ± 15%	3 ± 5%
P value	>0.0001[b]	0.006[b]	0.008[b]	0.6

[a] HIF-1α, hypoxia-inducing factor 1 α; MMD, moyamoya disease. Data are expressed as mean ± standard deviation.
[b] *P* values less than 0.05 were considered significant.

detected in MMD samples (*Fig. 4*). Immunoreactivity indicating endoglin was detected in 67 ± 27% of the endothelial cells in the MMD specimens (*Fig. 4, Table 3*). For the control specimens, this value was 23 ± 15% (*Fig. 4, Table 3*; $P = 0.008$). On the contrary, 5 ± 8% of the intimal cells in the MMD specimens and 3 ± 5% in the control samples showed endoglin immunoreactivity (*Table 3*; $P = 0.6$).

Colocalization of TGF-β3 Immunoreactivity with HIF-1α and Endoglin Immunoreactivities

To identify the nature of the cells immunoreactive with anti-HIF-1α and anti-endoglin antibodies, we performed double-staining and observed the cells under a fluorescence microscope. The results of double-staining for HIF-1α and TGF-B3 indicated that the double-labeled cells were detected mainly in the endothelium (*Fig. 5, A–C* and *G–I*). Moreover, we also performed double-staining for endoglin and TGF-B3. The cells in the endothelium with immunoreactivity indicating endoglin were also labeled with anti-TGF-B3 (*Fig. 5, D–F* and *J–L*).

Correlation of the Results of the CBF Study with HIF-1α and Endoglin Expression in the MMD Patients

To analyze a correlation of CBF and CVR with immunohistochemical results, we assessed rCBF and rCVR in the MMD

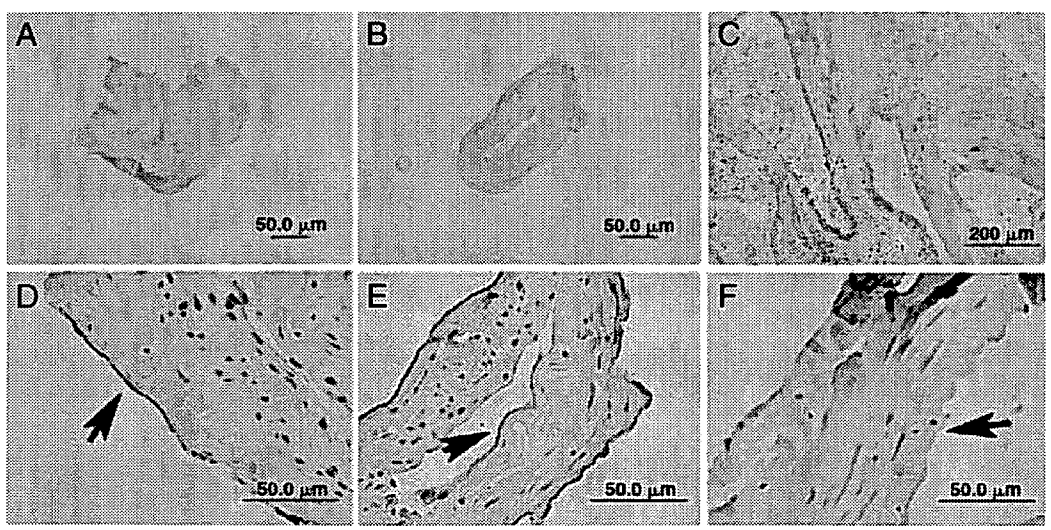

FIGURE 4. *Immunohistochemical analysis of VEGF and endoglin.* A *and* B, *immunoreactivity indicating that VEGF is not detected in the intima and the media of the MCA of patients with MMD.* C, *in a positive control specimen (cerebral arteriovenous malformation), positive immunoreactions for VEGF are detected.* D *and* E, *immunoreactivity indicating that endoglin is detected in the endothelium of the MCA from the patients with MMD.* F, *in this control specimen, no such cells are detected in the endothelium.* Arrows *indicate the endothelium. Original magnification,* ×100 (A–C) *and* ×200 (D–F).

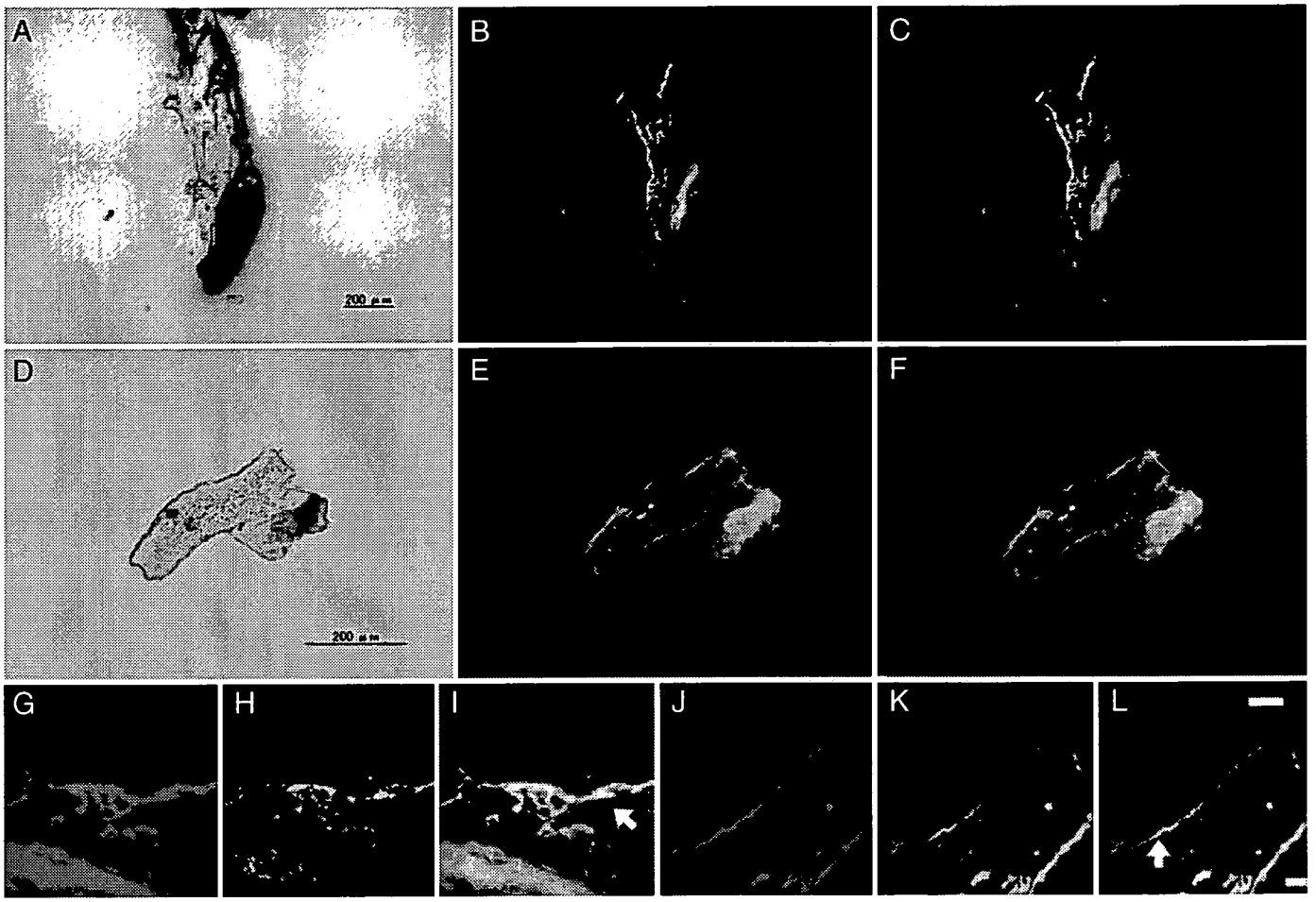

FIGURE 5. *Fluorescent microscopical assessment by double immunofluorescence staining to characterize the cells immunoreactive for HIF-1α, endoglin, and TGF-β3 in MMD specimens.* A–C, *results for double-staining for HIF-1α* (A, red) *and TGF-β3* (B, green). *HIF-1α-containing cells are also positive for TGF-β3* (C, yellow). D–F, *results for double-staining for endoglin* (D, red) *and TGF-β3* (E, green). *Endoglin is co-localized with TGF-β3, as indicated in the merged image* (F, yellow). G–I, *results for double staining for HIF-1α* (G, red) *and TGF-β3* (H, green). *HIF-1α-containing cells are also positive for TGF-β3* (I, yellow; arrow *indicates a co-localized cell*). J–L, *results for double-staining for endoglin* (J, red) *and TGF-β3* (K, green). *Endoglin is co-localized with TGF-β3, as indicated in the merged image* (L, yellow; arrow *indicates a co-localized cell*). Scale bar, *50 μm* (L). *Original magnification, ×100* (D–F), *×200* (A–C), *and ×400* (G–L).

patients (*Table 4*). The patients with more than 60% positive HIF-1α immunoreactivity in the intima showed significant rCVR reduction compared with those with less than 60% immunoreactivity (–14 ± 7% and 1 ± 7%, respectively; $P = 0.02$).

DISCUSSION

In the present study, we obtained data on surgically sampled MCA specimens from patients with MMD. On histopathological observations, the MMD specimens had a thicker intima than the controls. Furthermore, the immunoreactivity indicating the presence of HIF-1α and endoglin was higher in MMD specimens than in the controls.

MMD is characterized by angiographic findings of intracranial carotid artery stenosis and occlusions as well as a fine network of vessels at the base of the brain (29). As concluded

TABLE 4. Cerebral blood flow studies and up-regulation of hypoxia-inducing factor-1α and endoglin in moyamoya disease[a]

	HIF-1α		Endoglin	
	rCBF	rCVR (%)	rCBF	rCVR (%)
% Positive				
≤60	34 ± 7	−14 ± 7	32 ± 5	−5 ± 8
>60	37 ± 4	1 ± 7	33 ± 5	−10 ± 8
P value	0.4	0.02[b]	0.9	0.5

[a] HIF-1α, hypoxia-inducing factor 1 α; rCBF, regional cerebral blood flow; rCVR, regional cerebrovascular reserve.
[b] *P* values less than 0.05 were considered significant.

from inspection of postmortem specimens, the pathological changes in the stenotic and occluded vessels are intimal thickening, hyperplasia, and irregularities in the internal elastic lamina (11, 13). These changes in intracranial arteries and secondary angiogenesis are considered characteristic of MMD (9). The molecular mechanism underlying MMD has been investigated previously but has not been fully clarified. Our group and others reported basic fibroblast growth factor to be involved in the pathogenesis of MMD, as basic fibroblast growth factor expression was elevated in the cerebrospinal fluid and superficial temporal artery (STA) of MMD patients (10, 26, 32). In addition, Hojo et al. (8) recognized an elevated level of TGF-β in the cerebrospinal fluid, as well as TGF-β expression in the STA, of patients with MMD. Yamamoto et al. (37) also showed TGF-β to play a role in MMD by elastin synthesis. These results indicate the accumulation of elastin via the TGF-B pathway in the intimal thickening and suggest that this accumulation is responsible for the intimal thickening seen in MMD (37). Besides basic fibroblast growth factor, other growth factors such as platelet-derived growth factor and hepatocyte growth factor also play a role in MMD (2, 24, 32). Moreover, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and elastin levels were previously reported to be increased in the cerebrospinal fluid of MMD patients (28).

In this study, we also analyzed the expression of HIF-1α and endoglin using immunohistochemistry. Our group studied the role of TGF-β in MMD (8). We have searched for the up- and downstream molecules of TGF-β. Recently, evidence has been obtained indicating that HIF-1 regulates TGF-β3 transcription (19, 21). In MMD, a reduction in CBF and hypoxia was often observed. HIF-1α is one of the major factors involved tissue-oxygen homeostasis; thus, we selected HIF-1α in this study. Endoglin is known to modulate cellular responses to TGF-β. As a downstream molecule of TGF-β16, we analyzed endoglin. Thus, we selected HIF-1α and endoglin, as they are molecules associated with TGF-β. We analyzed the expression of a novel member of the TGF-β family, TGF-β3, in MMD. HIF-1α, which is a transcriptional activator involved in tissue-oxygen homeostasis (15, 22), is a heterodimeric protein formed by inducible HIF-1α and constitutively expressed HIF-1α proteins. It binds to hypoxia-responsive elements to activate transcription of genes related to iron, energy, and matrix metabolism, to vascular regulation, and to cell survival (15, 22). These types of genes include VEGF and VEGF receptor 1, TGF-β isoforms, heme oxygenase-1, inducible nitric oxide synthase, and plasminogen activator inhibitor-1 (15, 22). Among them, VEGF is one of main target molecules of HIF-1α (15). Thus, we first analyzed the expression of VEGF in MMD specimens but did not detect any VEGF expression in them. Endoglin is a component of the TGF-β receptor complex expressed mainly on the surface of endothelial cells (7, 16, 20). Endoglin binds to members of the TGF-β superfamily, including TGF-β1, TGF-β3, activin A, bone morphogenic protein-2, and bone morphogenic protein-7, in the presence of the signaling receptors and modulates cellular responses to TGF-β1 (7, 16, 20). An important role for endoglin in cardiovascular development and vascular remodeling has been learned from several experimental approaches. The function of endoglin in vascular morphogenesis was demonstrated in knockout mice, the embryos of which die at 10 to 11.5 days because of vascular and cardiac abnormalities (1, 7, 20). In the central nervous system, endoglin expression is upregulated in arteriovenous malformations and cavernous hemangiomas (6, 27).

Recently, evidence has been obtained indicating that HIF-1α also seems to regulate TGF-β3 transcription (9, 22). Two other isoforms of TGF-β, TGF-β1 and TGF-β2, have also been identified. Although TGF-β isoforms are structurally similar, they differ in their ability to bind receptors (9, 22). In human umbilical cord vein endothelial cells, the dimeric membrane glycoprotein endoglin coexists with TGF-β receptors I and II and binds TGF-β1 and -β3 with high affinity but does not bind TGF-β2 (9, 22). The role of TGF-β family members in vascular development is undoubtedly complex, as is the cascade of events that lead to the development of a blood vessel. One might conclude that the ratio of TGF-β signals via these receptors determines whether or not TGF-β stimulates or inhibits angiogenesis (8, 19).

As for the significance of this study, we emphasize that ours is the first to study use of MCA samples collected during surgery. As formerly mentioned, studies on growth factors in cerebrospinal fluid and STA samples have already been published (2, 3, 4, 8, 10, 18, 26, 28, 32, 36). However, patients with MMD present systemic arterial lesions, including those of the STA or renal artery (4, 31). Considering that the main characteristic of MMD is intracranial stenosis, studies using intracranial arterial samples are the most important. In this study, the control patients are significantly older than the MMD patients. In carotid arteries, intimal hyperplasia often occurs in older patients with arteriosclerosis. In the present study, intimal hyperplasia is more obvious in the MMD patients. Intimal hyperplasia in these patients may not be owing only to age.

Considering the differences in angiogenic markers between control and MMD patients, CBF reduction is not the only cause of this difference because the mean rCBF value was lower in the control specimens than in the MMD patients. As shown in *Table* 4, CVR reduction in MMD is one of the origins for HIF-1α induction. In addition, growth factors such as fibroblast growth factor are reported to be elevated in the cerebrospinal fluid of MMD patients (10). With the presence of fibroblast growth factor, HIF-1α promotes the proliferation of smooth muscle cells (25, 30). In addition, hepatocyte growth factor, which is also elevated in the cerebrospinal fluid of these patients, induces HIF-1α (5, 18). These growth factors may play a role in HIF-1α induction and proliferative responses in MMD (25).

In this study, we used M4 samples. In MMD disease, progressive occlusion occurs in the circle of Willis arteries, but is thought to occur rarely in the cortical M3 and M4. However, according to previous reports using autopsy specimens, cortical MCA branches also showed intimal hyperplasia (33). These reports, as well as our study, indicated that MMD affects not only the area around the terminal portion of internal carotid artery, but also the cortical branches of the MCA.

Ultimately, a mechanistic insight into MMD pathophysiology may have to wait for the results of genetics studies. We know the importance of genetic study and recognize that our study is limited. Descriptive studies such as ours are open to bias, and the molecules studied are still somewhat arbitrary. Our group has an ongoing project to study the genetics of MMD. However, only 10 to 20% of MMD patients have a defined genetic background. Thus, genetic approach cannot completely clarify the pathophysiology of MMD and, therefore, observational studies are also important.

In summary, we showed that intimal hyperplasia occurred in the MCA intima of patients with MMD. In addition, HIF-1α and endoglin were overexpressed in the intima. This study and future analysis using intracranial MCA specimens have the possibility to clarify the etiology of MMD.

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