表1● 併用療法の症状増悪率と再発率など

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
アルガトロバン単独	16.8 ± 23.8	13.9％	5.7％	5.5％
アルガトロバン・オザグレル併用	20.0 ± 23.0	52.6％	13.3％	6.5％
アルガトロバン・内服の抗血小板・凝固薬併用	19.8 ± 15.9	25.0％	14.3％	11.1％
アルガトロバン・アスピリン併用（自験例）	10.2 ± 13.9	30.8％	11.5％	23.1％
エダラボン単独	10.1 ± 19.3	15.3％	11.9％	24.5％
エダラボン・オザグレル併用	19.6 ± 25.9	9.8％	4.9％	0.0％
エダラボン・内服の抗血小板・凝固薬併用	23.0 ± 32.0	33.3％	0.0％	0.0％
エダラボン・アスピリン併用（自験例）	25.8 ± 37.9	2.8％	2.8％	13.9％

表2● 併用療法の臨床背景と各病型への使用頻度

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
アルガトロバン単独	434	71.5 ± 11.5	260/174	10.4％	47.7％	20.0％	18.2％	3.7％
アルガトロバン・オザグレル併用	78	70.4 ± 10.1	48/30	3.8％	55.1％	5.1％	28.2％	7.7％
アルガトロバン・内服の抗血小板・凝固薬併用	9	64.0 ± 14.0	3/6	11.1％	55.6％	0.0％	33.3％	0.0％
アルガトロバン・アスピリン併用（自験例）	26	67.9 ± 11.2	17/9	3.8％	30.8％	15.4％	50.0％	0.0％
エダラボン単独	111	75.4 ± 12.3	68/43	8.1％	11.7％	66.7％	6.3％	7.2％
エダラボン・オザグレル併用	71	70.4 ± 10.8	46/25	4.2％	25.3％	7.0％	60.6％	2.8％
エダラボン・内服の抗血小板・凝固薬併用	3	74.3 ± 7.5	1/2	0.0％	100.0％	0.0％	0.0％	0.0％
エダラボン・アスピリン併用（自験例）	36	70.8 ± 12.9	20/16	2.8％	33.3％	25.0％	30.6％	8.3％

TIA：一過性脳虚血発作，AT：アテローム血栓性梗塞，CE：心原性脳塞栓，LI：ラクナ梗塞

表3● 併用療法の評価

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
アルガトロバン単独	5.5 ± 6.9	3.8 ± 7.1*	5	2*	61.8％
アルガトロバン・オザグレル併用	3.8 ± 5.2	3.9 ± 6.5	5	3	42.3％
アルガトロバン・内服の抗血小板・凝固薬併用	1.8 ± 1.3	1.3 ± 1.5*	3	1*	88.9％
アルガトロバン・アスピリン併用（自験例）	4.1 ± 5.0	2.4 ± 3.9*	4	2*	69.2％
エダラボン単独	10.3 ± 8.2	9.7 ± 10.9	11	7	38.7％
エダラボン・オザグレル併用	2.4 ± 3.4	1.3 ± 4.1*	3	1*	80.3％
エダラボン・内服の抗血小板・凝固薬併用	1.2 ± 2.7	1.2 ± 2.7	2	2	66.7％
エダラボン・アスピリン併用（自験例）	6.1 ± 7.4	3.2 ± 6.6*	3	1*	72.2％

*は入院時に比べ有意に（$p < 0.05$）改善（JSSは対応のあるt検定，NIHSSはWilcoxonの符号付き順位検定を用いた）．

自験例の検討

筆者の所属する梶川病院では最近アルガトロバン[4]あるいはエダラボンにアスピリンを急性期より積極的に併用している．今後データバンクへの登録予定例も含め自験例の成績を表に示した．アルガトロバンとアスピリンの併用は，その半数をラクナ梗塞に使用した（表2）．JSSは入院時の4.1から退院時の2.4へと有意に改善したが（表3），出血性梗塞が23％にみられたことは注意を要する（表1）．高木らは急性期アテローム血栓性梗塞においてアルガトロバンにチクロピジンを併用した場合，高血圧を合併した高齢者においては出血時間の異常延長をきたすおそれが否定できないと報告している[5]．アスピリン併用においても，当初から併用するか，アルガトロバンが持続投与から間欠投与へ移行する3日目から併用するかなど検討する必要がある．一方，エダラボンとアスピリンの併用療法は比較的各病型に万遍なく用いられていたが（表2），入院後の症状増悪，入院中の再発も少なく（表1），JSSも入院時の6.1から3.2へと有意に改善した（表3）．アスピリンは安価であり，費用対効果を考えると今後効果を検証していく価値がある治療法と考えた．

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9 病型別にみた脳血管狭窄性病変と重症度・予後

- TIA やラクナ梗塞では脳血管狭窄は軽度の例が多いが，アテローム血栓性梗塞では中等から高度の狭窄性病変の存在頻度が高く，心原性脳塞栓では栓子によると思われる頭蓋内主幹動脈の閉塞が高頻度に観察された．
- 脳血管の狭窄性病変の有無，程度が入院時 JSS・退院時 JSS に影響を与えていることが示唆された．
- ラクナ梗塞では，JSS で評価した症状の経過に脳血管の狭窄性病変の有無，程度が関与すると考えられた．
- 脳血管の狭窄性病変の存在が，入院後症状進行，再発，出血性脳梗塞発症の増悪因子である可能性が示唆された．

虚血性脳血管障害の分類にはNINDSの臨床病型分類が用いられることが多い．一方，MRIや超音波検査の普及により，簡便に頭蓋内外の血管の評価が可能となり，心原性脳塞栓やラクナ梗塞に分類される症例においても脳血管の狭窄病変の合併が少なからず発見されるが，臨床病型ごとにその意義について詳細に検討した報告は少ない．本稿では，脳血管の狭窄性変化と病型，重症度，入院後の症状進行，再発，出血性脳梗塞の発症についての検討を行った．

対象は脳卒中データバンク（JSSRS）で2001年度までに集積した虚血性脳血管障害の症例から，t-PA（組織プラスミノーゲンアクチベーター）などの血栓溶解療法施行例を除外し，検討項目のデータがそろっている2,254例とした．病型別の患者背景は表1に示す通りであるが，病型間で年齢，性別，高血圧・糖尿病・高脂血症有病率に有意な差があった（one-factor ANOVAおよびχ^2独立性の検定）．

臨床病型別分類と脳血管検査所見

臨床病型分類別の脳血管検査所見の頻度を示す（図1）．TIA（一過性脳虚血発作）やアテローム血栓性梗塞では頭蓋外動脈の高度狭窄や閉塞所見が比較的高頻度にみられるのに対し，ラクナ梗塞では正常～軽度硬化を高頻度に認めた．また心原性脳塞栓では他病型に比し正常～高度狭窄の頻度が低いが，頭蓋内主幹動脈閉塞の頻度が高い．それぞれの病型の成因の違いを反映した結果であると推察される．今回は，脳血管検査所見のうち正常～軽度硬化を除いた症例を狭窄率50％未満と狭窄率50％以上に分類し，以下の検討を行った．この2群間に高血圧，糖尿病，高脂血症の有病率に統計学的な有意差を認めなかった．

脳血管狭窄性病変とJSS

対象症例全体の検討では，狭窄率50％未満群よりも狭窄率50％以上群のほうが，入院時JSS・退院時JSSがともに高値であった（Student's t-test）（図2）．JSSの値は入院後2群とも改善していたが，JSSの変動に2群間で有意差を認めなかった（反復測定分散分析）．病型別の検討では，TIAでは，入院時・退院時JSSとも2群間で有意差を認めず，JSSの変動にも2群間で有意差を認めなかった．アテローム血栓性梗塞および心原性脳塞栓では，入院時・退院時JSSともに狭窄率50％未満群のほうが低値であったが，JSSの変動に差はなかった（図2）．その一方，ラクナ梗塞では，入院時JSSに差はなかったが，退院時JSSは狭窄率50％未満群のほうが低値であり，変動パターンに明らかな有意差を認めた（各病型はいずれも反復測定分散分析）（図2）．これは，症状の経過に脳血管の狭窄性病変が関与している可能性が推察され，興味深い結果であった．

脳血管狭窄性病変と入院後症状進行，再発，出血性脳梗塞発症

入院後に出血性梗塞をきたした症例，入院後症状の進行を認めた症例，入院後再発をきたした症例は，いずれも狭窄率50％未満群よりも狭窄率50％以上群において有意に高頻度であり（表2），脳血管の狭窄性病変が発症後の増悪因子となっていると思われる．これを臨床病型別に検討すると，TIA（表3）では，症例数は少ないものの入院後再発は，狭窄率50％未満群よりも狭窄率50％以上群において有意に高頻度に認めた．ラクナ梗塞（表4）では，出血性梗塞，入院後再発は少なく，入院後の症状進行は11.7％と比較的高頻度であったが，その頻度は2群間で差がなかった．アテローム血栓性梗塞（表5）では，入院後再発は狭窄率50％未満群よりも狭窄率50％以上群において有意に高頻度であり，出血性梗塞や入院後の症状進行もその傾向があった．心原性脳塞栓（表6）では，入院後再発には2群間に有意差がなかったが，出血性梗塞，入院後の症状進行は狭窄率50％未満群よりも狭窄率50％以上群において有意に高頻度であった（表2～6はいずれもχ^2独立性の検定）．これらの臨床病型別の検討からは，ラクナ梗塞を除く脳卒中病型において，脳血管の狭窄性病変が脳梗塞発症後の増悪因子となっている可能性が示唆された．

表1● 虚血性脳血管障害病型別の患者背景

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
TIA	66.6 ± 13.1	1.57	54.9	23.9	33.8
アテローム血栓性梗塞	70.0 ± 10.6	1.79	69.3	32.7	28.8
ラクナ梗塞	68.7 ± 10.7	1.42	70.8	31.7	31.7
心原性脳塞栓	72.5 ± 11.9	1.46	54.6	18.1	16.1
脳梗塞（その他）	67.7 ± 14.4	1.37	56.3	20.8	22.4

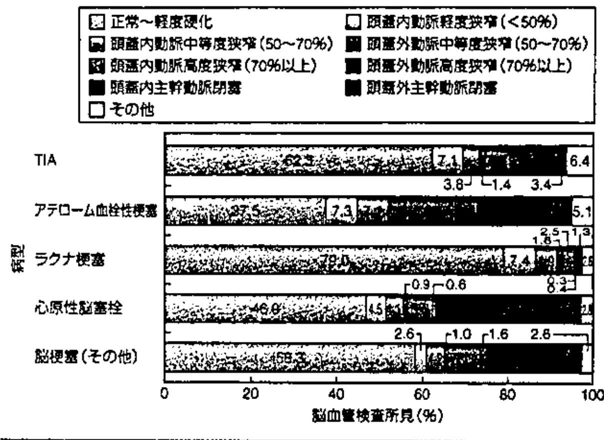

図1● 虚血性脳血管障害病型別の脳血管狭窄頻度

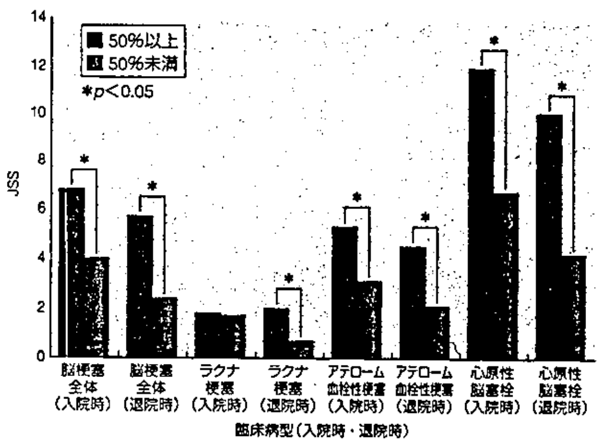

図2● 脳血管狭窄率と脳卒中重症度（JSS）

表2● 虚血性脳血管障害全体における脳血管狭窄率別にみた入院後再発，進行，出血性梗塞の頻度

[illegible]	[illegible]	[illegible]
入院後再発	6／226 (2.7%)	64／771 (8.3%) *
入院後進行	28／226 (12.4%)	148／771 (19.2%) *
出血性梗塞	12／226 (5.3%)	103／771 (13.3%) *

*$p < 0.05$

表3● TIAにおける脳血管狭窄率別にみた入院後再発，進行，出血性梗塞の頻度

[illegible]	[illegible]	[illegible]
入院後再発	6/157 (3.8%)	7/55 (12.7%) *
入院後進行	3/157 (1.9%)	0/55 (0%)
出血性梗塞	1/157 (0.6%)	0/55 (0%)

*$p < 0.05$

表4● ラクナ梗塞における脳血管狭窄率別にみた入院後再発，進行，出血性梗塞の頻度

[illegible]	[illegible]	[illegible]
入院後再発	7/573 (1.2%)	1/64 (1.6%)
入院後進行	67/573 (11.7%)	8/64 (12.5%)
出血性梗塞	7/573 (1.2%)	0/64 (0%)

表5● アテローム血栓性梗塞における脳血管狭窄率別にみた入院後再発，進行，出血性梗塞の頻度

[illegible]	[illegible]	[illegible]
入院後再発	12/342 (3.5%)	32/346 (9.2%) *
入院後進行	64/342 (18.7%)	85/346 (24.6%)
出血性梗塞	12/342 (3.5%)	23/346 (6.6%)

*$p < 0.05$

表6● 心原性脳塞栓における脳血管狭窄率別にみた入院後再発，進行，出血性梗塞の頻度

[illegible]	[illegible]	[illegible]
入院後再発	51/278 (18.3%)	73/236 (30.9%)
入院後進行	15/278 (5.4%)	41/236 (17.4%) *
出血性梗塞	12/278 (4.3%)	19/236 (8.1%) *

*$p < 0.05$

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Ⅲ．研究成果の刊行物・別刷

書　　　　籍

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CRE-mediated gene expression in cerebral ischemia

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Abstract

Although accumulating evidence indicates that cAMP response element binding protein (CREB) phosphorylation is upregulated after cerebral ischemia, it remains uncertain whether CREB phosphorylation induced after ischemia leads to CRE-mediated gene transcription and is involved in cell survival or not. Using CRE-LacZ transgenic mice, we demonstrated that CRE-mediated gene expression was found in a subset of pCREB-positive neurons after transient global and focal cerebral ischemia and in cultured neurons after exposure to glutamate. Treatment with CRE-decoy oligonucleotide suppressed upregulation of BCL-2 expression and accelerated neuronal damage after exposure to glutamate. CRE-mediated gene expression occurs in neurons after metabolic stresses and exerts its neuroprotective action through production of survival-promoting molecules such as anti-apoptotic protein BCL-2.
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Keywords: CREB; Ischemia; BCL-2; Glutamate; CRE-decoy oligonucleotide

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1. Introduction

Nuclear transcription factor cAMP response element binding protein (CREB) belongs to the b ZIP superfamily. CREB and the related factors, CREM (cAMP response element modulator) and activating transcription factor 1 (ATF-1) form both homo- and heterodimers, and that each can bind to the same cis-regulatory element, cAMP response element (CRE). A wide range of extracellular stimuli is capable of activating CREB family members through phosphorylation of CREB, and CREB-mediated gene expression has been implicated in circadian entrainment, synaptic plasticity, neuron growth and survival (Fig. 1) [1].

Ischemic stresses induce several genes including c-*fos* [2] and brain derived neurotrophic factor (BDNF) [3]. Because they have a consensus CRE (TGACGTCA) in the promoter region, it has been speculated that the activation of CRE binding protein (CREB) and CRE-mediated gene expression occurs after ischemia. Previous studies examined CREB activation in the ischemic brain by detecting CREB phophorylation [4–6]. However, phosphorylation of the Ser-133 residue is necessary but not sufficient for activation of CRE-mediated gene transcription [7]. In this study, we demonstrated CRE-mediated gene expression in the ischemic brain and suggested the functional role of CREB activation in cultured neurons after metabolic stress.

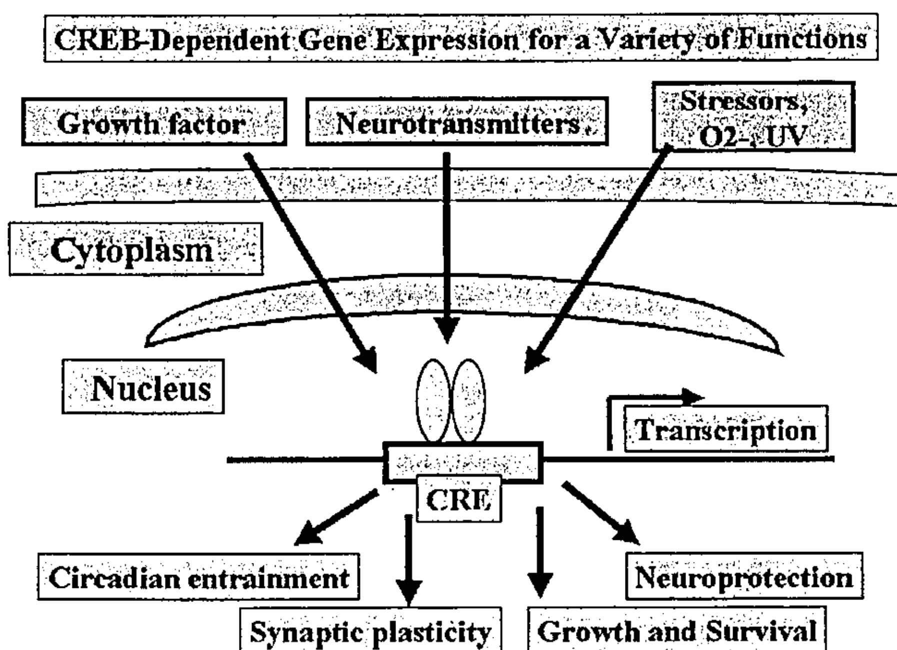

Fig. 1. CRE-dependent gene expression for a variety of functions. External stimuli such as growth factor receptor, neurotransmitter and stressors promote CREB phosphorylation and CREB-mediated gene expression shown in the upper half. CREB activation leads to physiological and pathological consequences such as circadian entrainment, synaptic plasticity, growth and survival.

2. Material and methods

2.1. Ischemia model

For Western blot analysis, adult Mongolian gerbils were used. Transient forebrain ischemia was produced by bilateral occlusion of the common carotid artery with aneurysm clips for 5 min under anesthesia with 2% halothane. We also used male CRE-LacZ transgenic mice [8], age 12–16 weeks, to confirm that CREB phosphorylation after ischemia induces CRE-mediated transcription. Transient forebrain ischemia was produced in CRE-LacZ transgenic mice as previously described [9]. Both common carotid arteries were then exposed at the neck, occluded with microaneurysmal clips for 15 min and then reperfused. CRE-LacZ transgenic mice were used to examine CRE-mediated gene expression in cortical area after permanent occlusion of middle cerebral artery. Under halothane anesthesia, left middle cerebral artery (MCA) was occluded with electrocoagulation.

2.2. Neuronal cell culture

Primary cultures of the rat or mouse hippocampal neurons were obtained. Cells were treated with glutamate (100 μM) for 15 min after 6 or 7 days in vitro. Glutamate and all other chemicals were added directly to the medium. Quantitative assessments of neuronal injury were accomplished by measuring the lactate dehydrogenase (LDH) activity in the media 24 h after exposure to glutamate with the cytotoxicity detection kit. Treatment with CRE-decoy oligonucleotide was performed as described recently with some modifications [10]. Sequences of CRE-decoy and control oligonucleotides are as follows: CRE-decoy, 5′-TGACGTCATGACGTCATGACGTCA-3′; nonsense-sequence control, 5′-CTAGCTAGCTAGCTAGCTAGCTAG-3′. CRE-decoy and control oligonucleotides were added to the cells in the presence of DOTAP. At 24 h of incubation, glutamate was added directly to the medium.

2.3. Western blot analysis

After extraction with 2% sodium dodecyl sulfate (SDS), an equal amount of protein for each sample was separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblot with polyclonal anti-CREB, anti-phosphorylated CREB (anti-pCREB) or anti-BCL-2 antibody.

2.4. X-gal staining, immunohistochemistry and immunocytochemistry

For X-gal staining, frozen sections after fixation with Zamboni's solution were incubated in the reaction solution consisting of 1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) in PBS. After reaction with X-gal, the sections were immunostained with anti-pCREB, anti-NeuN, anti-glial fibrillary acidic protein, anti-MAC2 and anti-CD31 antibody and finally visualized with the 3-amino-9-ethylcarbazole (AEC) solution.

3. Results

3.1. CRE-mediated gene expression after ischemia and exposure to glutamate

After transient global ischemia, total CREB levels were unchanged in the non-ischemic animals and during ischemia and reperfusion. In contrast, pCREB levels decreased during ischemia, but increased during reperfusion peaking at 15 min, and then declined (Fig. 2, top panel). In cultured neurons, glutamate induced phosphorylation of Ser^{133} in CREB. CREB phosphorylation peaked at 10 min and returned to the baseline level by 180 min (Fig. 2, bottom panel). The pattern with enhancement of CREB phosphorylation was similar to that observed after global ischemia.

In brain sections from sham-operated CRE-LacZ transgenic mice, there were only few pCREB-positive or X-gal-positive cells. After reperfusion for 60 min, most pyramidal neurons in the hippocampus were pCREB-positive. X-gal-positive cells were also observed, but the number was smaller than that of pCREB-positive cells (Fig. 3, top panel). In cultured neurons prepared from control transgenic mice, there were only few pCREB- or β-galactosidase-positive cells. However, the number of pCREB- and β-galactosidase-positive cells increased markedly 30 min after exposure to glutamate (100 μM). A subset of pCREB-positive neurons was also β-galactosidase-positive (Fig. 3, bottom panel).

3.2. Functional role of CRE-mediated gene expression following exposure to glutamate

To determine the role of CREB in neuroprotection and survival in cultured neurons, we examined the effect of CRE-decoy oligonucleotide on CREB phosphorylation, BCL-2 production and cell viability after glutamate exposure. CREB phosphorylation at 10 min after exposure to glutamate was not inhibited by CRE-decoy or control oligonucleotide; however, BCL-2 production at 6 h after exposure to glutamate for

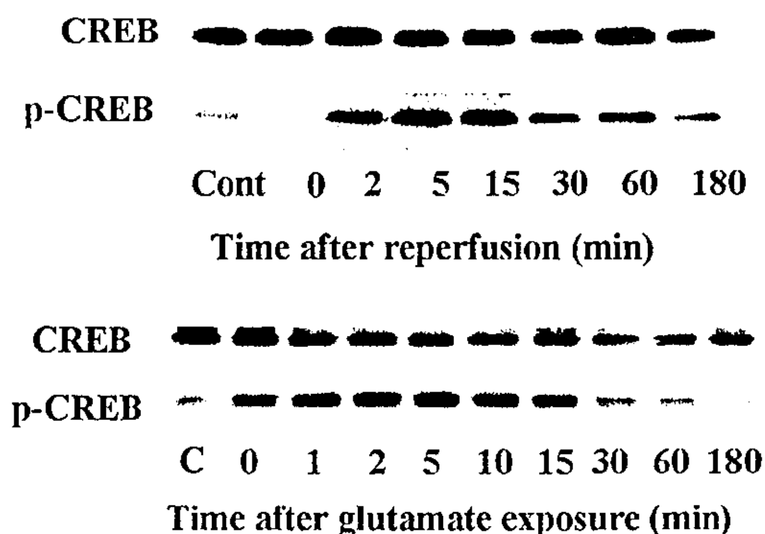

Fig. 2. CREB phosphorylation at Ser^{133} in gerbil hippocampus after transient forebrain ischemia (top panel) and by exposure to glutamate in cultured neurons (bottom panel).

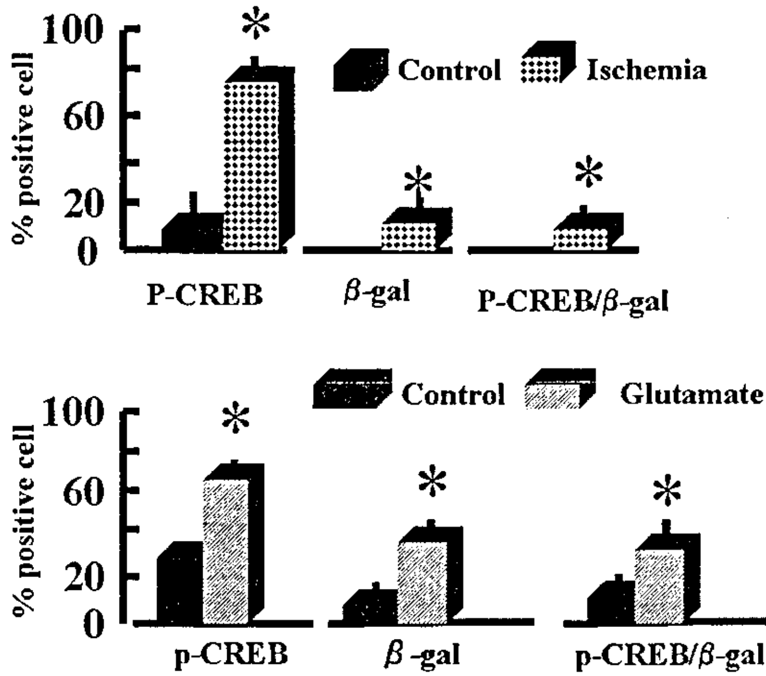

Fig. 3. CRE-mediated LacZ transcription in the ischemic hippocampus and in cultured neurons after exposure to glutamate. $*p<0.05$ vs. control.

15 min was suppressed by CRE-decoy oligonucleotide (Fig. 4). Pretreatment with CRE-decoy oligonucleotide also significantly increased the level of neuronal damage compared to that in controls. Pretreatment with control oligonucleotide did not suppress

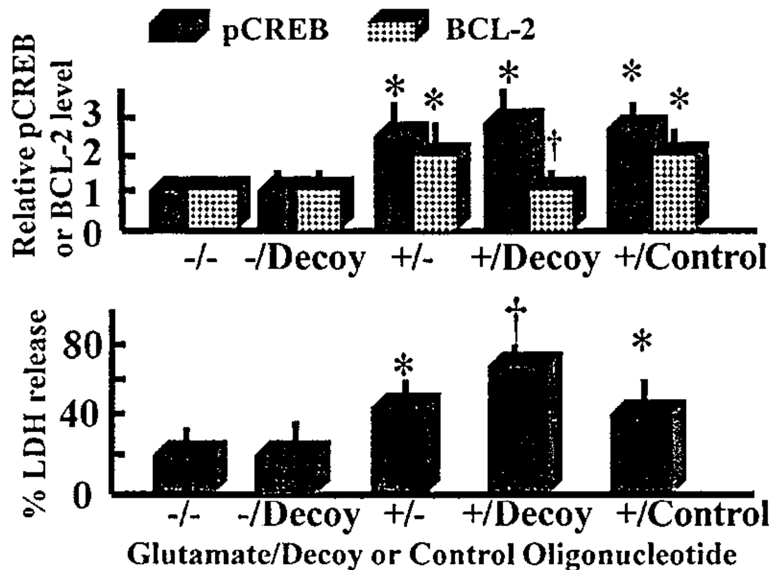

Fig. 4. Pretreatment with CRE-decoy oligonucleotide inhibits glutamate-induced BCL-2 production (top panel) and exacerbates the neuronal cell damage observed after exposure to glutamate (bottom panel). Pretreatment with CRE-decoy or control oligonucleotide was indicated with "+Decoy Oligo" and "+Control Oligo" in the figures. The data in the bar graph are presented as fold increase in pCREB and BCL-2 levels. $*p<0.05$ vs. controls. $^{\dagger}p<0.05$ vs. glutamate treatment without oligonucleotide.

Table 1
CRE-mediated gene expression after MCA occlusion

Percentage of positive cells in X-gal-positive cells	
NeuN	55.9 ± 12.3
GFAP	9.5 ± 9.9
MAC2	11.0 ± 2.8
CD31	2.9 ± 3.4

BCL-2 production or increase the level of cell damage compared to that in controls (Fig. 4).

3.3. CRE-mediated gene expression after focal cerebral ischemia

In the control normal brain of CRE-LacZ transgenic mice, few β-gal-positive cells were observed. After MCA occlusion, scattered X-gal-positive cells were observed in the peripheral area of the MCA territory. pCREB-positive cells were abundant in the area, and a subset of pCREB-positive cells was X-gal-positive. More than half of X-gal-positive cells were NeuN-positive, and 5–10% of X-gal-positive cells was GFAP-positive, MAC2-positive and CD31-positive (Table 1). In the ischemic core and in the contralateral hemisphere, only few X-gal-positive cells were observed.

4. Discussion

The present study demonstrated that CRE-mediated gene transcription occurred after exposure to glutamate in cultured neurons and after both global and focal ischemia. The experiment with CRE-decoy oligonucleotide also supported the neuroprotective role of CRE-mediated gene expression which follows CREB phosphorylation in exposure to glutamate. Recently, the BCL-2 gene was found to have a CRE in the 5′ promoter region [11]. Accumulating evidence indicates that overexpression of BCL-2 provides protection against apoptosis [12] and ischemic neuronal death [9]. Thus, the protective effect of CREB phosphorylation against glutamate- or ischemia-induced neuronal degeneration may be due to increased expression of BCL-2. In the present study, we observed increased BCL-2 expression after exposure to glutamate, which was inhibited by pretreatment with CRE-decoy oligonucleotide, suggesting that BCL-2 expression was induced by CREB activation and involved in the neuroprotective role of CREB. CREB phosphorylation has been examined in experimental stroke models. Walton et al. [4] showed that ischemia-resistant granule neurons produced an increase in pCREB immunoreactivity following a unilateral hypoxic-ischemic injury. Hu et al. [5] demonstrated that CREB phosphorylation was induced in the adult rat hippocampus, mainly in the resistant dentate granule cells after transient global ischemia. Recently, it was demonstrated that in the focal ischemia model, CREB phosphorylation was marked in the peri-infarct area [6]. Those results strongly suggested that CRE-mediated gene expression occurred after ischemia; however, direct evidence for CRE-mediated transcription has not been shown. In the present study, we

used CRE-LacZ transgenic mice to examine CRE-mediated transcription after ischemia. In the hippocampus after transient global ischemia, not all but a subset of pCREB-positive cells expressed CRE-mediated transcription. Furthermore, NeuN-positive neurons showed CRE-mediated transcription in the peripheral area of the MCA territory after permanent MCA occlusion. Those results suggested that surviving neurons after ischemia exhibited CRE-mediated gene expression which would be potentially neuroprotective. Although protective role of CRE-mediated transcription is supported based on the finding that CRE-decoy oligonucleotides suppressed survival in cultured neurons after glutamate exposure, functional role of CRE-mediated gene expression in cerebral ischemia has to be investigated in future studies.

In conclusion, we demonstrated CRE-mediated transcription in neurons after glutamate exposure and after both global and focal ischemia. Activation of CREB may be one of the most important defense mechanisms in the neurons against metabolic stress such as ischemia and excitotoxicity.

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Cyclooxygenase-2 is involved in increased proliferation of neuronal progenitor cells in the ischemic mouse hippocampus

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Abstract

Global ischemia promotes neurogenesis in the dentate gyrus of the adult mouse hippocampus. Cyclooxygenase (COX)-2, the principal isoenzyme in the brain, modulates inflammation, glutamate-mediated cytotoxicity, and synaptic plasticity. The enhancement of dentate gyrus neurogenesis following ischemia was attenuated by COX inhibitors and in COX-2 knockout mice. These results demonstrate that COX-2 may modulate the enhancement of neurogenesis following ischemia.
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Keywords: Neurogenesis; Hippocampus; COX-2; Neuronal progenitor; Musashi-1

1. Introduction

It is well established in several rodent models studies that brain ischemia stimulates neurogenesis [1–5], however, the molecular and cellular mechanisms underlying postischemic enhancement of neurogenesis are unknown.

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Cyclooxygenase (COX), a rate-limiting enzyme in synthesis of prostaglandins (PGs) from arachidonic acid, produces PGH2, which in subsequent steps gives rise to PGs with varied physiologic functions [6–8]. Two Cox isoenzymes exist, COX-1 and COX-2, and COX-2 is expressed constitutively at relatively high levels in brain [9]. In addition to the role of COX-2 in proinflammatory actions in the central nervous system (CNS), recent studies have found COX-2 to be a multifunctional neural modulator [10,11]. COX-2 also acts importantly in cell proliferation, having been implicated in growth and progression of a variety of tumor types [12,13].

The quantities of circulating adrenal steroids negatively regulate neurogenesis in the dentate gyrus throughout life [14,15]. Glucocorticoids selectively inhibit expression of COX-2 without affecting COX-1. Thus, to determine whether COX-2 influences hippocampal neurogenesis in adult mice, we examined postischemic proliferation of progenitor cells in the SGZ after administering COX inhibitors and in COX-2 knockout mice.

2. Materials and methods

2.1. Animals

Adult male C57Black/6 mice (11 to 12 weeks old) were used in this study. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Medicine. Animal care was given according to the guidelines of Animal Center of Osaka University Graduate School of Medicine.

Cyclooxygenase-2 knockout mice [16] were obtained from Jackson laboratories (Bar Harbor, Maine). Mice were backcrossed to C57Black/6 mice five to seven times, and were studied at age of 10 to 12 weeks. Experiments were performed in age-matched littermates, including those homozygous for the knockout (−/−), heterozygous (+/−), and also wild type (+/+). Genotypes of all COX-2 knockout mice were determined by polymerase chain reaction analysis.

2.2. Transient forebrain ischemia

General anesthesia was introduced with 4% halothane and maintained with 1% halothane by means of an open facemask. A polyacrylamid column for measurement of cortical microperfusion by laser-Doppler flowmetry (LDF; Advanced Laser Flowmetry) was attached to the skull. Body and skull temperatures were monitored and maintained at 36.5 to 37.5 °C. Both common carotid arteries were occluded for 12 min with micro-aneurysm clips and then reperfused. As described previously, only mice that showed less than 13% of baseline control microperfusion during the first minute of occlusion were used in subsequent experiments [17].

2.3. Bromodeoxyuridine-labeling protocols

Bromodeoxyuridine (BrdU; Roche Diagnostics, Indianapolis, IN), a thymidine analogue, was used to label proliferating cells. In the first experiment, mice with no surgery

(control), mice with sham ischemia, and mice with ischemia were intraperitoneally injected with BrdU (50 mg/kg) four times every 2 h over 6 h to conclude 9 days after ischemia. The next day, mice were killed under deep pentobarbital anesthesia and their brains were immersion-fixed in methanol at 4 °C overnight.

In the second experiment, examining the effect of COX in cell proliferation after ischemia, both right and left common carotid arteries were occluded for 12 min as described above. Ischemic mice were divided randomly into three groups. Indomethacin (Cayman Chemical, Ann Arbor, MI; 10 mg/kg), NS-398, (Cayman Chemical; 20 mg/kg), or vehicle alone was given at 9:00 AM and 6:00 PM on day 8, and at 9:00 AM on day 9 after ischemia. NS-398 inhibits COX-2 1000 times more potently than COX-1 [18,19]. We intraperitoneally administered BrdU (50 mg/kg) four times every 2 h for 6 h, starting 2 h after the last injection of each COX inhibitor.

In the third experiment, to further investigate the role of cyclooxygenase in hippocampal neurogenesis, we subjected COX-2 knockout mice including homozygous (−/−), heterozygous (+/−), and wild-type littermates to the ischemia and BrdU-labeling protocols above.

2.4. Immunohistochemistry

For quantification of BrdU-positive cells, each tissue block was dehydrated after fixation and embedded in paraffins. Tissue sections (4 μm) from the dorsal hippocampus were examined with cresyl violet. The protocol of BrdU immunohistochemistry was described previously [4]. In brief, after DNA denaturing, sections were incubated with a rat monoclonal anti-BrdU antibody, 1:100 (Harlan Sera-Labo, Loughborough, UK) at 4 °C overnight. After washing, the sections were incubated with a biotinylated secondary antibody, and further incubated with a streptavidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA). The sections were finally reacted with 0.05% 3′3-diaminobenzidine in the presence of 0.01% H_2O_2.

2.5. Quantification

To count BrdU-labeled cells in paraffin sections as identified by the immunoperoxidase reaction, five 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 and SGZ, defined as a zone two-cell bodies wide along the border of the GCL and hilus, were considered together for quantification. The mean density of BrdU-labeled cells in each mouse was calculated as the number of labeled nuclei divided by the area.

Table 1
The enhancement of dentate gyrus neurogenesis following ischemia was attenuated by COX inhibitors

	Control	Ischemia
Vehicle	16.3 ± 10.5/mm^2	56.0 ± 30.3/mm^2
Indomethacin	11.8 ± 5.7/mm^2	35.6 ± 22.0/mm^2
NS-398	13.7 ± 8.1/mm^2	34.2 ± 25.8/mm^2

Table 2
The enhancement of dentate gyrus neurogenesis following ischemia was attenuated in COX-2 knockout mice

	Control	Ischemia
Wild type	23.3 ± 8.7/mm^2	79.6 ± 32.5/mm^2
COX-2 (+/ −)	17.7 ± 7.5/mm^2	48.3 ± 24.6/mm^2
COX-2 (− / −)	14.5 ± 6.2/mm^2	39.3 ± 21.2/mm^2

Data in the text and figure are described as mean ± S.D. Multiple comparisons were evaluated statistically by the analysis of variance, followed by Scheffe's post hoc tests.

3. Results

A few BrdU-positive cells were observed in the SGZ of the hippocampal dentate gyrus in the control (21.6 ± 10.7/mm^2). The number of BrdU-positive cells in the SGZ reached a peak at 10 days. Semi-quantitative analysis in the SGZ showed the number of BrdU-positive cells to be significantly increased compared to the control by five times at 10 days after ischemia (98.7 ± 26.6/mm^2). Moreover, all mice subjected to ischemia showed neuronal loss in the hilus of the dentate gyrus.

In contrast to control mice, in the ischemic group, mice treated with indomethacin and NS-398 showed a significant decrease in the number of BrdU-positive cells in the SGZ compared with vehicle-treated mice at 10 days after ischemia (Table 1).

In nonischemic controls of COX-2 knockout mice, the difference of numbers of BrdU-positive cells in the SGZ of COX-2 +/+, +/ − , and − / − mice was not significant (Table 2). In the ischemic groups, BrdU-positive cells in the SGZ 10 days after ischemia in COX-2 +/+ mice were significantly more numerous than in COX-2 +/+ non-ischemic control mice. The number of BrdU-positive cells following ischemia was significantly less in COX-2 +/ − and COX-2 − / − mice than in COX-2 +/+ mice (Table 2).

4. Discussion

Our data demonstrated that transient forebrain ischemia increased neurogenesis in the dentate gyrus of the adult mouse hippocampus. The enhancement of dentate gyrus neurogenesis following ischemia was attenuated by COX inhibitors and in COX-2 knockout mice. Kumihashi et al. [20] recently reported that enhanced neurogenesis following ischemia was suppressed by treatment with acetylsalicylic acid. We for the first time demonstrated the involvement of COX-2 in ischemia-induced neurogenesis.

COX-2 has been shown to be involved with cell cycle activity [21,22]. As mitotic proliferation is a critical component in adult neurogenesis, COX-2 may affect the proliferation of neural progenitor cells in the SGZ.

COX-2 may affect neurogenesis in the dentate gyrus at least partly through generation of PGE2. A recent study demonstrated that PGE2 transactivated a receptor

for epidermal growth factor (EGF) [23]. Interestingly, EGF receptor-like immunoreactivity was detected in proliferating cells in the dentate gyrus [24] and involvement of FGF2 in hippocampal neurogenesis after ischemia already has been shown [25].

Alternatively, COX-2 may effects on the neurogenesis via the production of reactive oxygen species or the link to the NO production.

Further studies may be necessary for the elucidation of molecular pathways via COX-2 underlying enhancement of neurogenesis for the regulate application of neurogenesis in the stroke patients.

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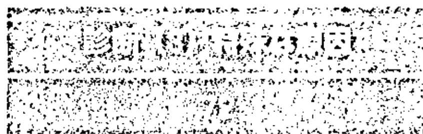


頚部回旋が誘因となる脳卒中

松本 昌泰（広島大）

水泳，ヨガ，車の運転が脳梗塞を誘発することもある

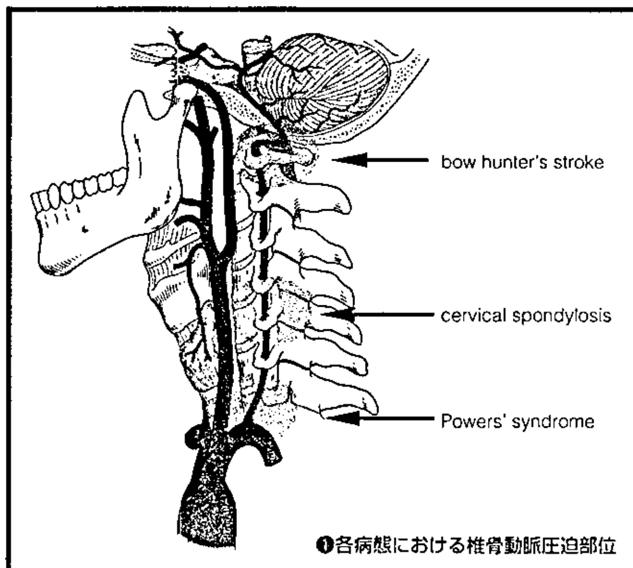
椎骨脳底動脈系の脳梗塞では，まれではあるが天井の塗装中，首の回旋を伴う水泳，椅子での仮眠，車の運転中，カイロプラクティック，ヨガやフィットネススクールでの首の運動などの頚部の回旋を伴う各種の体位や運動により誘発されたと考えられる症例が報告されている[1~6]．

その病態としては，頚部回旋による椎骨動脈の機械的圧迫により椎骨動脈が傷害され，血栓形成や動脈解離などが起こり，血流障害や artery-to-artery の塞栓症などにより脳梗塞を発症する機序が想定されている．なかでも椎骨動脈の C1 ～ C2 レベルの atlas loop（V3）では，頚部の回旋により回旋方向の対側の椎骨動脈が圧迫されることが多く，頚部の回旋による椎骨動脈解離の 70% が本部位の椎骨動脈に発生するとされている[7]．

以下に，このような頚部回旋による椎骨動脈の機械的圧迫による脳血流の低下を観察しえた症例の所見を提示し，本病態への注意を喚起したい．

椎骨動脈の機械的圧迫をきたす病態

頚部の回旋・伸展などの身体の動きにより症状の誘発がみられるものとしては，Powers' syndrome[8]，頚椎症[9]，bow hunter's stroke[10] などが知られており（❶），それぞれに原因に応じた治療が必要となる．例えば bow hunter's stroke では，頚部の回旋により回旋方向とは反対側の椎骨動脈が環椎-軸椎間で圧迫され閉塞するため，環椎-軸椎の後方固定や横突起の unroofing などの外科的治療を行うこともある．



❶各病態における椎骨動脈圧迫部位

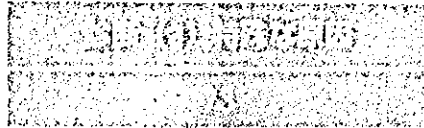
また，Powers' syndrome では頚部回旋時に回旋方向と反対側の椎骨動脈が鎖骨下動脈からの分岐部において前斜角筋・甲状頚動脈により圧迫されるのに対し，頚椎症では回旋と同側の椎骨動脈が骨棘により圧迫され，それぞれ椎骨動脈の血流障害により椎骨脳底動脈循環不全（vertebro-basilar insufficiency）によるめまいなどの症状を呈する．

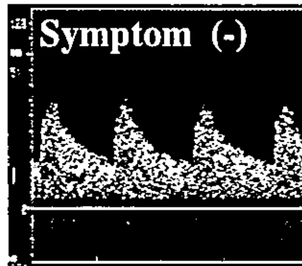
経頭蓋超音波ドプラ法が有用である

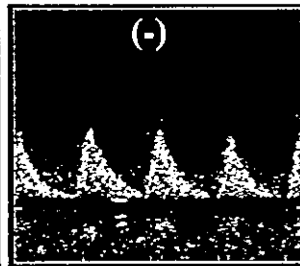
回旋による椎骨動脈の機械的圧迫の証明は，これまで頚部回旋時の血管造影検査により証明されてきたが，経頭蓋超音波ドプラ法（TCD）により頚部回旋時の後大脳動脈血流速の変化をモニターすることにより（❷），失神様めまい発作の症状発現に先立ち脳血流低下を検出することが可能となった．また，本例では脳血流 SPECT により頚部回旋時の脳血流低下も同時に証明することができ（❸），頚部回旋時の TCD によるモニターにより脳循環病態のダイナミックな評価の有用性が示された．

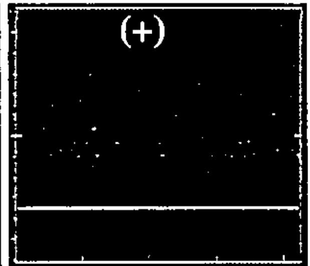
予知・予防には事前の十分な問診や TCD による評価が重要

❷❸に提示した症例は，bow hunter's stroke と同様の機序により左の椎骨動脈が圧迫閉塞され，副血行路の発達が悪いために，失神様のめまいを生じた例である．通常は側副血行路の発達（対側の椎骨動脈や後交通枝による代償）のため，軽度のめまいのみだったり，症状がほとんどみられない場合もありうる．したがって，頚部の回旋が繰り返される職業や，頚部の回旋状態の持続が想定される手術時の体位が必要な場合などには，事前に十分な問診を行うとともに，頚部回旋による椎骨動脈の圧迫の有無や程度を TCD などにより評価しておくことが，椎骨動脈の機械的圧迫に起因する脳梗塞を回避するうえで重要と思われる．



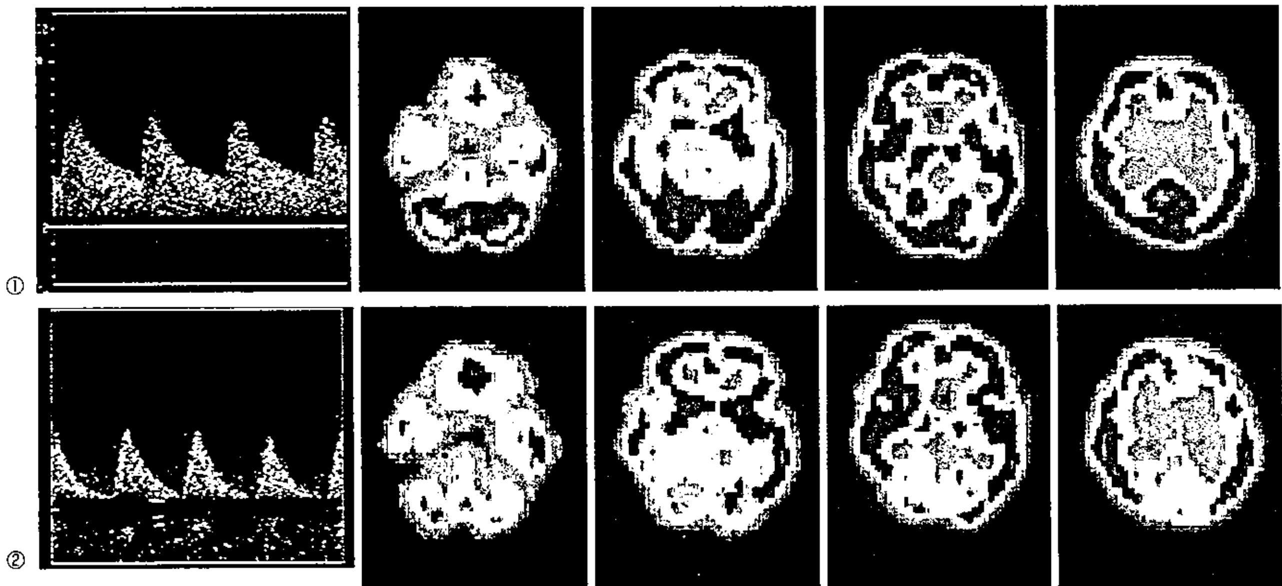






①　②　③

❷左椎骨動脈造影像と経頭蓋超音波ドプラ検査（TCD）所見．
①安静時，②頭部右後上方への回旋時．症状の出現はみていないが左後大脳動脈血流のTCDモニターでは，安静時に比し拡張期血流速の顕著な低下が観察される．③頭部右後上方への最大回旋時（約100～120°），頭部浮遊感などの症状が出現した．TCDでは血流の途絶の所見があり，椎骨動脈造影ではV3での機械的圧迫による椎骨動脈の閉塞が確認された．矢印は圧迫による閉塞部位を示す．



❸頭部の回旋と脳血流SPECT像
①安静時，両側小脳の血流が最も多い．
②頭部回旋時，経頭蓋超音波ドプラ検査を併用しながら症状が出現する直前まで頭部を右後方へ回旋（約90°）して，脳血流SPECT用トレーサ（^{99m}Tc-HMPAO）を静注し約2分間この頭位を保持した．この頭部回旋負荷の間，左後大脳動脈（L.P1）の拡張末期血流速度が0 cm/秒であったが，症状が出現することはなかった．両側小脳および後頭葉の血流低下が明瞭にとらえられた．

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9 病型別にみた脳血管狭窄性病変と重症度・予後

- TIAやラクナ梗塞では脳血管狭窄は軽度の例が多いが，アテローム血栓性梗塞では中等から高度の狭窄性病変の存在頻度が高く，心原性脳塞栓では栓子によると思われる頭蓋内主幹動脈の閉塞が高頻度に観察された．
- 脳血管の狭窄性病変の有無，程度が入院時JSS・退院時JSSに影響を与えていることが示唆された．
- ラクナ梗塞では，JSSで評価した症状の経過に脳血管の狭窄性病変の有無，程度が関与すると考えられた．
- 脳血管の狭窄性病変の存在が，入院後症状進行，再発，出血性脳梗塞発症の増悪因子である可能性が示唆された．

虚血性脳血管障害の分類にはNINDSの臨床病型分類が用いられることが多い．一方，MRIや超音波検査の普及により，簡便に頭蓋内外の血管の評価が可能となり，心原性脳塞栓やラクナ梗塞に分類される症例においても脳血管の狭窄病変の合併が少なからず発見されるが，臨床病型ごとにその意義について詳細に検討した報告は少ない．本稿では，脳血管の狭窄性変化と病型，重症度，入院後の症状進行，再発，出血性脳梗塞の発症についての検討を行った．

対象は脳卒中データバンク（JSSRS）で2001年度までに集積した虚血性脳血管障害の症例から，t-PA（組織プラスミノーゲンアクチベーター）などの血栓溶解療法施行例を除外し，検討項目のデータがそろっている2,254例とした．病型別の患者背景は表1に示す通りであるが，病型間で年齢，性別，高血圧・糖尿病・高脂血症有病率に有意な差があった（one-factor ANOVAおよびχ^2独立性の検定）．

臨床病型別分類と脳血管検査所見

臨床病型分類別の脳血管検査所見の頻度を示す（図1）．TIA（一過性脳虚血発作）やアテローム血栓性梗塞では頭蓋外動脈の高度狭窄や閉塞所見が比較的高頻度にみられるのに対し，ラクナ梗塞では正常～軽度硬化を高頻度に認めた．また心原性脳塞栓では他病型に比し正常～高度狭窄の頻度が低いが，頭蓋内主幹動脈閉塞の頻度が高い．それぞれの病型の成因の違いを反映した結果であると推察される．今回は，脳血管検査所見のうち正常～軽度硬化を除いた症例を狭窄率50％未満と狭窄率50％以上に分類し，以下の検討を行った．この2群間に高血圧，糖尿病，高脂血症の有病率に統計学的な有意差を認めなかった．

脳血管狭窄性病変とJSS

対象症例全体の検討では，狭窄率50％未満群よりも狭窄率50％以上群のほうが，入院時JSS・退院時JSSがともに高値であった（Student's t-test）（図2）．JSSの値は入院後2群とも改善していたが，JSSの変動に2群間で有意差を認めなかった（反復測定分散分析）．病型別の検討では，TIAでは，入院時・退院時JSSとも2群間で有意差を認めず，JSSの変動にも2群間で有意差を認めなかった．アテローム血栓性梗塞および心原性脳塞栓では，入院時・退院時JSSともに狭窄率50％未満群のほうが低値であったが，JSSの変動に差はなかった（図2）．その一方，ラクナ梗塞では，入院時JSSに差はなかったが，退院時JSSは狭窄率50％未満群のほうが低値であり，変動パターンに明らかな有意差を認めた（各病型はいずれも反復測定分散分析）（図2）．これは，症状の経過に脳血管の狭窄性病変が関与している可能性が推察され，興味深い結果であった．

脳血管狭窄性病変と入院後症状進行，再発，出血性脳梗塞発症

入院後に出血性梗塞をきたした症例，入院後症状の進行を認めた症例，入院後再発をきたした症例は，いずれも狭窄率50％未満群よりも狭窄率50％以上群において有意に高頻度であり（表2），脳血管の狭窄性病変が発症後の増悪因子となっていると思われる．これを臨床病型別に検討すると，TIA（表3）では，症例数は少ないものの入院後再発は，狭窄率50％未満群よりも狭窄率50％以上群において有意に高頻度に認めた．ラクナ梗塞（表4）では，出血性梗塞，入院後再発は少なく，入院後の症状進行は11.7％と比較的高頻度であったが，その頻度は2群間で差がなかった．アテローム血栓性梗塞（表5）では，入院後再発は狭窄率50％未満群よりも狭窄率50％以上群において有意に高頻度であり，出血性梗塞や入院後の症状進行もその傾向があった．心原性脳塞栓（表6）では，入院後再発には2群間に有意差がなかったが，出血性梗塞，入院後の症状進行は狭窄率50％未満群よりも狭窄率50％以上群において有意に高頻度であった（表2～6はいずれもχ^2独立性の検定）．これらの臨床病型別の検討からは，ラクナ梗塞を除く脳卒中病型において，脳血管の狭窄性病変が脳梗塞発症後の増悪因子となっている可能性が示唆された．