表6－16　脳出血の部位と代表的な症状・徴候

| | 被殻出血 | 視床出血 | 橋出血 | 小脳出血 | 皮質下出血 |
|---|---|---|---|---|---|
| 意識障害 | 大血腫であり | しばしばあり（血腫が小さくても） | 大血腫であり | | 大血腫であり |
| けいれん | | | | | しばしばあり |
| 頭痛 | | | | しばしばあり | しばしばあり |
| 麻痺 | 対側 | 内包後脚への圧迫で対側にあり | 対側あるいは四肢 | | 前頭葉の出血であり |
| 眼症状 | | | | | |
| 瞳孔 | | 時にあり（対光反射減弱，縮瞳） | 両側縮瞳 | | |
| 共同偏視 | 病側 | 時にあり（病側，まれに対側） | | | 前頭葉の出血であり（病側） |
| 眼振 | | | | しばしばあり | |
| Ocular bobbing | | | しばしばあり | | |
| Skew deviation | | | 時にあり | まれにあり | |
| その他 | | | 内側縦束症候群 | | |
| 顔面麻痺 | 対側（中枢性） | | 病側（核性） | | |
| 構音障害 | | | しばしばあり | 時にあり | |
| 感覚障害 | | 対側 | 対側あるいは四肢 | | 頭頂葉の出血であり |
| 同名半盲 | 血腫の後方進展であり | | | | 側頭葉，後頭葉であり |
| 運動失調 | | 失立失歩 | 時にあり | 体幹，病側の上下肢 | |
| 失語（優位半球） | 時にあり | 時にあり | | | 前頭葉，頭頂葉，側頭葉であり |
| 半側空間無視，病態失認（劣位半球） | | 時にあり | | | 頭頂葉であり |

注：症状，徴候は出血量，血腫の進展部位によってさまざまであり，表には典型的なものを示した。

② 脳出血：出血の生じる部位，出血量により症状はさまざまであるが，突然の頭痛，嘔吐，意識障害に加え，運動麻痺，感覚障害あるいは半盲，失語などの高次脳機能障害を生じることが多い。痙攣で発症することもある。図6－19に脳出血の好発部位，表6－16に部位と代表的な症状・徴候の関係を示した。診断にはCTが有用である（図6－18右）。

③ 脳梗塞：梗塞に陥る部位，体積により症状はさまざまであるが，種々の程度の意識障害，運動麻痺，感覚障害あるいは半盲，失語などの高次脳機能障害を生じることが多い。診断には，急性期はCTでは病変がはっきりしないことも多く，MRIが有

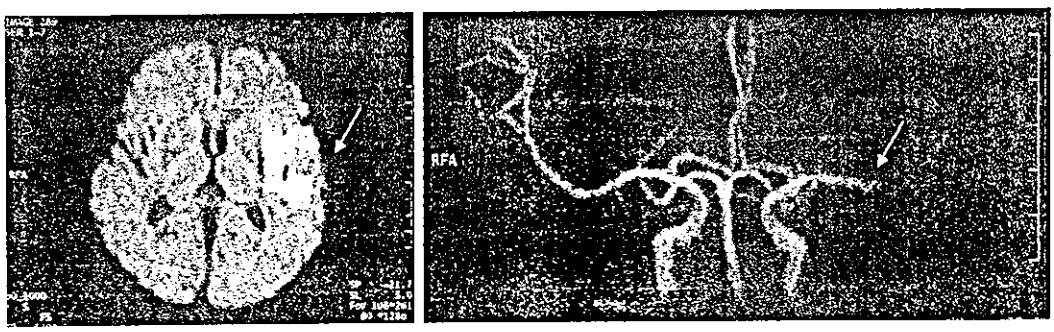


図6－20　脳梗塞のdiffusion MRI（左，矢印が梗塞像）及びMRA（右，矢印が閉塞部位）

用である。とくに，diffusion MRI（図6－20左），magnetic resonance angiography（MRA）（図6－20右）が梗塞巣の早期検出，閉塞血管の特定に有用である。

(6) 治 療

① くも膜下出血：くも膜下出血を生じた原疾患に対する治療が行われる。原因の大部分を占める脳動脈瘤に対しては，再破裂防止のため開頭による動脈瘤頸部のクリッピング術や血管内手術によるコイルを用いた動脈瘤塞栓術が行われる。動脈瘤が適切に処置されれば，再出血の恐れはなくなるが，術後2週間以内に脳血管攣縮による脳梗塞を生じることがあり，これに対する予防と治療が重要となる。原疾患が脳動静脈奇形の場合は，部位，大きさなどを考慮し，手術による摘出，血管内手術，ガンマナイフ治療といった治療法が選択される。

② 脳出血：血圧，呼吸，頭蓋内圧の管理，合併症の予防といった治療が基本となるが，意識障害を早期に回復させることや神経症状の悪化を予防する目的で手術による血腫除去が選択されることがある。重症の小脳出血は最もよい適応である。重症の被殻出血，皮質下出血も適応となることがあるが，視床出血，脳幹出血は基本的に手術適応がない。急性水頭症が起きた場合は脳室ドレナージを行う。血腫除去には，開頭術，CTを用いた定位的血腫吸引除去術，エコーガイド下による血腫吸引除去術などの方法があり，症例に応じて選択される。

③ 脳梗塞：脳梗塞は脳細胞に酸素とブドウ糖を供給している血管が何らかの機序により閉塞し，脳細胞が壊死することなので，発症から6時間以内といった超急性期であれば，閉塞した血管を再開通させる血栓溶解療法を行えれば理想的である。実際，経静脈的あるいは選択的動脈内投与による血栓溶解療法が有効であったとの臨床試験も報告されている。ただし，現実には，血栓溶解療法は出血性梗塞の合併といった危険性を伴うこと，発症6時間以降に病院を受診する患者が少なくないこと等の問題点があり，現時点では実施症例は少数に留まっている。大部分の症例では，梗塞巣の拡大防止や再発予防を目的とした抗血小板・抗凝固療法あるいはフリーラジカルスカベンジャーによる脳細胞保護療法，抗脳浮腫剤の投与，酸素の投与，発熱，血圧（基本的には下げない）のコントロールといった治療が行われている。

(7) 食事療法

脳卒中の安静期，リハビリ訓練中，再発予防の時期別に食事療法について述べる。

① 安静期：脳卒中患者では，嚥下障害を生じることが多いため，急性期には末梢静脈栄養が行われることが多い。さらに，呼吸器感染症を恐れるあまり，経腸栄養が遅れがちである。しかし，静脈栄養が長期化すると全身の栄養状態が悪化し，感染防御能が低下する恐れがある。したがって，呼吸器感染症に注意を払いつつ経腸栄養を

基本原則とすべきと考えられている。経腸栄養法としては意識障害のある場合，あるいは意識障害がなくても嚥下障害が明らかである場合には，経鼻胃管栄養，経内視鏡的胃ろう造設術（percutaneous endoscopic gastrostomy：PEG），経鼻空腸管栄養，間欠的口腔-食道栄養法（intermittent oro-esophageal catheterization：IOE）などの経管栄養が選択される。また，急性期であっても意識障害がなく嚥下機能にも問題がなければ直ちに経口摂取を開始すべきと考えられる。急性期を過ぎても嚥下障害が持続する場合は，嚥下テストにより評価を行った後，可能であれば経口摂取の訓練を行うのが一般的である。

(i) 経管栄養：少量で最大の栄養量を確保するため，高たんぱく，高エネルギーがよい。1 mlで1 kcal以上で調整する。その他，粒子を細かくすること，栄養バランスを保つこと，消化管粘膜に対する刺激が少ないことなどが求められる。一般に基礎代謝量以上を投与したいが，逆流による誤嚥の恐れもあるため，少量より開始し，増量していくのが望ましい。

(ii) 嚥下障害時の経口摂取：脳卒中急性期に誤嚥性肺炎を合併することは時に致命的になりうるので，経口摂取を無理に急ぐべきではない。しかし，嚥下テストにより誤嚥の恐れが低く，経口摂取可能と判断されれば，嚥下の能力にあわせ食物の形態を工夫し，食事を開始する。嚥下障害のあるときは酸味の強いものは避け，消化のよいある程度の硬さを持つ半流動から開始するとよい。また，当初は経口摂取のみで必要な量を摂取することは困難な場合もあり，経管栄養と併用してもよい。経口摂取への移行訓練としては氷片を口内に入れて，咀嚼，嚥下，むせについてチェックするとよい。

② リハビリ訓練中：脳卒中患者では，とくに脳梗塞を中心に早期からリハビリテーションが開始されることが多い。その進行にあわせ，適正な栄養量を提供する必要がある。エネルギーは1500～1600Kcal，たんぱく質は60g，脂質は40g，塩分は8～10g程度を目安に，年齢，体格，合併症の有無を考慮し調整する。

③ 再発予防：前述した危険因子をコントロールすることが再発予防にも非常に重要であるので，食事療法についてもそれに沿う形となる。いずれの生活習慣病に関しても肥満の関与は大きいので，適正な食事，運動が重要であることはいうまでもない。最大のリスクファクターである高血圧の予防のためには，塩分制限（1日10g以下），エネルギー制限，糖質，脂質の制限，良質なたんぱく質の摂取，カリウムの摂取等が挙げられる。また，脳卒中予防には，野菜や果物の十分な摂取が推奨されている。ビタミンEや魚等に含まれるエイコサペンタエン酸の摂取も動脈硬化の予防に有効であるとされ，脳卒中再発予防にも有効である可能性がある。

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# 4 大規模臨床試験（J-STARS）の予備調査への応用

▶J-STARSは，スタチンによる脳卒中再発予防効果を検証する医師主導の臨床試験である．
▶J-STARSの参考とするために，脳卒中データバンクと協同で横断的および縦断的予備調査（J-STARS-CおよびL）が実施されている．
▶予備調査では，脂質代謝異常を有する非心原性脳梗塞患者の臨床像の一端が明らかになった．

## J-STARSとは[1, 2]

J-STARS（Japan Statin Treatment Against Recurrent Stroke）は，厚生労働科学研究費補助金の効果的医療技術の確立推進臨床研究事業の「脳血管疾患の再発に対する高脂血症治療薬HMG-CoA還元酵素阻害薬の予防効果に関する研究（主任研究者：松本昌泰）」として，平成14年度から始まった医師主導の臨床試験である．本試験は，平均的な血清コレステロール値を有する虚血性脳血管障害の既往のある被験者を対象として，HMG-CoA還元酵素阻害薬（以下スタチン）による脳卒中の再発防止，痴呆の発症予防，動脈硬化の進展の抑制に対する有効性と安全性を評価することを，主目的としている．試験デザインはPROBE（Prospective, Randomized, Open, Blinded-Endpoint）を採用し，全国132の施設で3,000例の症例登録を目標としている．試験の概要，進捗状況はホームページで公開されている（http://plaza.umin.ac.jp/~jstars/index.html）．

## J-STARSの2つの予備調査[2]

近年行われた多くの大規模臨床試験およびそのメタアナリシスにより，脳卒中の一次予防にスタチンが有用であることはすでに明らかにされている．しかし，二次予防に関する臨床試験を始めるにあたっては，プロトコール作成のために，以下のような疑問に対する知見が得られれば役立つと考えた．

① 虚血性脳血管障害の既往のある患者の年齢分布，高血圧あるいは糖尿病の有病率，虚血性心疾患の既往のある割合，臨床病型の内訳はどのようであるか，そしてそれらは高脂血症の有無により異なるのか？
② 虚血性脳血管障害の既往のある患者の，脳卒中を含む心血管イベント発生率は年間どの程度であるか，そしてそれらは高脂血症の有無あるいは程度により異なるのか？

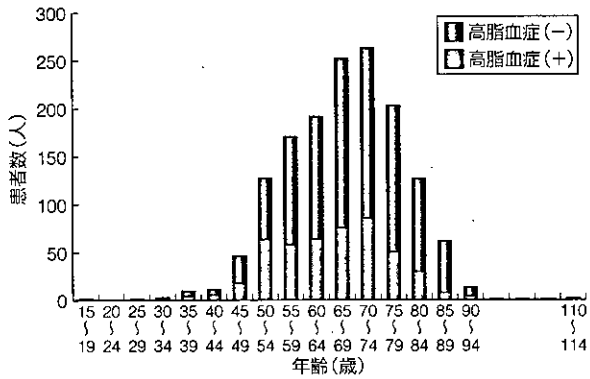
高脂血症の有無あるいはその重症度別に，これらを多数例で検討したデータはほとんどないことから，筆者らはJ-STARSの前に予備調査を計画した．迅速に必要とされるデータを得るため，①に関してはJSSRS groupと協議し，脳卒中データバンクにすでに蓄積されたデータの解析を行った（横断的研究，J-STARS-cross-sectional; J-STARS-C）．データの守秘の観点から，解析はJSSRS groupのメンバーである筆者により行われた．②に関しては，脳卒中データバンクに登録された虚血性脳血管障害の症例を前向きに追跡することで，データを得ることとした（縦断的研究，J-STARS-longitudinal；J-STARS-L）．脳卒中データバンクの参加施設から協力施設を募り（現在34施設），患者本人（あるいはその家族）から同意を得たうえで，専用のソフト（Statin Trial System，図1）に登録した．ソフトには脳卒中台帳の必須項目のデータを取り込む機能に加え，脂質代謝を中心とした生活習慣病のデータの記入欄，心血管イベント発生の有無の記入欄，頸部血管エコーの結果の記入欄などを設けた．データの提出方法は，脳卒中データバンクと同様の方法を採用し，第1回追跡調査では372例のデータが集積された．

## J-STARS-CおよびJ-STARS-Lから得られたもの

J-STARS-Cでは，脳卒中データバンクに2002年度までに登録された初発の脳梗塞のうち，NINDS分類でアテローム血栓性脳梗塞あるいはラクナ梗塞と診断され，退院時のADLがmodified Rankin Scale（mRS）で0〜3であった1,487例を解析の対象とした．これらの，高脂血症の有無別の年齢分布，高血圧，糖尿病，虚血性心疾患の合併率などについて検討した．結果として，高脂血症の割合は加齢とともに低下し（図2），高血圧，糖尿病の合併率が高かった．しかし，虚血性心疾患の合併率，アテローム血栓性脳梗塞の占める割合については，高脂血症を伴わない群と差を認めなかった．また，高脂血症を伴う群の52.7％は，高脂血症に対して加療（食事，運動療法あるいは薬物療法）が行われていなかった．以上の結果も参考に

**図1　J-STARS-Lのために作成された脳卒中台帳と共有可能なソフト**


**図2　退院時転帰が良好であったアテローム血栓性脳梗塞およびラクナ梗塞の年齢分布（J-STARS-C）**




高脂血症の有病率は有意に加齢とともに減少している．

**表1　J-STARSに登録が期待される患者の臨床背景（J-STARS-C）**

| | |
|---|---|
| 年齢（平均±標準偏差） | 64.4±8.9 |
| 男性の割合（%） | 61.2 |
| 高血圧（%） | 71.6 |
| 糖尿病（%） | 44.0 |
| 高血圧かつ糖尿病（%） | 32.9 |
| 心房細動（%） | 3.3 |
| 虚血性心疾患（%） | 10.9 |
| アテローム血栓性脳梗塞（%） | 42.1 |
| 毎日飲酒（%） | 33.8 |
| 毎日喫煙（%） | 40.0 |

**表2　高脂血症の有無による臨床背景および心血管イベント発症の比較（J-STARS-L）**

| | 高脂血症なし | 高脂血症あり | p値 | 総コレステロール 180〜240 mg/dL |
|---|---|---|---|---|
| 症例数 | 136 | 127 | | 130 |
| 年齢（平均±標準偏差） | 68.9±9.6 | 67.4±10.9 | N.S. | 67.9±10.6 |
| 男性の割合（%） | 67.6 | 62.2 | N.S. | 62.3 |
| BMI | 23.2±3.0 | 24.3±3.2 | 0.0062 | 23.8±3.1 |
| 入院時の総コレステロール（mg/dL） | 186.6±33.3 | 226.3±44.0 | ＜0.0001 | 208.0±16.5 |
| 高血圧（%） | 57.4 | 75.4 | 0.0032 | 69.0 |
| 糖尿病（%） | 24.3 | 36.2 | 0.048 | 27.7 |
| 虚血性心疾患の既往（%） | 10.3 | 10.2 | N.S. | 10.0 |
| アテローム血栓性脳梗塞の割合（%） | 41.2 | 40.9 | N.S. | 40.0 |
| 全心血管イベントの発症数（発症率/1年間） | 2（0.015） | 11（0.183） | 0.0162 | 9（0.116） |
| 脳卒中の再発数（発症率/1年間） | 2（0.015） | 10（0.093） | 0.0284 | 8（0.067） |

N.S.：有意差なし．BMI：body mass index.

して，J-STARSでは臨床試験への参加可能年齢を45歳以上80歳以下とし，高血圧，糖尿病の有無で層別して登録割付を行うこととした．45歳以上80歳以下の高脂血症を伴う群の臨床背景を表1に示す．

J-STARS-Lでは，中間追跡調査で集積された症例のうち，心原性脳塞栓を除く263例を検討すると，追跡期間中に発症した全心血管イベントのうち92%が虚血性脳血管障害であった．高脂血症の有無により2群に分類して検討すると，高脂血症ありの群の心血管イベントの発症率が有意に高かった（表2）．また，脳卒中台帳登録時の総コレステロールがJ-STARSの選択基準である180〜240 mg/dLの群の，臨床背景およびイベント発生率を表2にまとめた．

以上の予備調査の結果から，臨床的に高脂血症と診断された虚血性脳血管障害患者は，発症年齢が若く，他の生活習慣病の合併率が高く，その後再び虚血性脳血管障害を発症する危険性が高いことが明らかにされた．J-STARSでこれらの患者にスタチンを投与することが脳卒中再発予防につながるか，非常に興味がもたれるところである．

●文献　1）松本昌泰．脳血管疾患の再発に対する高脂血症治療薬のHMG-CoA阻害剤の予防効果に関する研究．厚生科学研究費補助金，効果的医療技術の確立推進臨床研究事業．平成14年度総括・分担研究報告書．2003.
2）松本昌泰．脳血管疾患の再発に対する高脂血症治療薬のHMG-CoA阻害剤の予防効果に関する研究．厚生科学研究費補助金，効果的医療技術の確立推進臨床研究事業．平成15年度総括・分担研究報告書．2004.

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循環器疾患等総合研究事業

# 脳血管疾患の再発に対する高脂血症治療薬のHMGCoA阻害剤の予防効果に関する研究

（H14－効　果（生活）－023）
（H15－効　果（生活）－020）
（H16－循環器（生習）－003）

平成14年度～16年度　総合研究報告書

3/7

**雑誌（I）**

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# 雑　誌（I）

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**Diabetologia**


*Articles*

# A phosphodiesterase inhibitor, cilostazol, prevents the onset of silent brain infarction in Japanese subjects with Type II diabetes

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**Abstract**

*Aims/hypothesis.* This study aimed to evaluate the effect of a phosphodiesterase inhibitor, cilostazol, on the prevention of silent brain infarction in diabetic patients without symptoms of vascular events.
*Methods.* A total of 89 subjects were allocated at random to the cilostazol group ($n = 43$) or the control group ($n = 46$).
*Results.* After the study period ($3.2 \pm 0.5$ years), carotid intima-media thickness (IMT) (means ± SD) had increased ($p < 0.01$) by $0.18 \pm 0.19$ mm in the control group. In the cilostazol group, intima-media thickness showed almost no change ($-0.00 \pm 0.16$ mm). In the control group, 2 out of 46 subjects showed symptomatic brain infarctions and 10 out of 34 subjects without infarct-like region assessed by standard brain MRI examination showed silent brain infarctions after the observation period. On the other hand, no subjects in the cilostazol group showed silent brain infarction or strokes during the study period. Both at the beginning and end of the study period, the number of infarct-like regions positively correlated with IMT ($r = 0.335$, $p < 0.001$ or $r = 0.347$, $p < 0.001$ respectively). The progression of infarct-like regions was directly related to the increase in IMT during the study period ($r = 0.299$, $p = 0.004$).
*Conclusion/interpretation.* These data demonstrated that cilostazol could prevent the onset of silent brain infarction in Japanese subjects with Type II (non-insulin-dependent) diabetes mellitus. Also, an increase in intima-media thickness of the carotid artery wall could be able to predict the onset of silent brain infarction. [Diabetologia (2002) 45: 188–194]

**Keywords** Silent brain infarction, intima-media thickness, Type II diabetes, antithrombotic drug.

Silent brain lesions detected by magnetic resonance imaging (MRI) are fairly common not only in first-ever stroke but also in normal elderly subjects without any symptoms [1–5]. It has been shown that a considerably higher incidence of white matter lesions exist in neurologically normal subjects with several risk factors for stroke than in those without risk factors. Recently, subclinical silent brain infarction has been identified as a risk factor for clinical stroke [6]. Thus, the detection of silent brain lesion is a useful clinical predictor of stroke. Antithrombotic drugs, such as aspirin and ticlopidin, can effectively reduce the recurrence of brain infarction in subjects with brain infarction or coronary heart disease. Diabetic patients without previous myocardial infarction have as high a risk of myocardial infarction as nondiabetic subjects with previous myocardial infarction [7]. The Stroke Council of the American Heart Association recently recommended antithrombotic drugs to prevent stroke in subjects with a high risk of atheroscle-

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*Abbreviations:* API, ankle pressure index; APTT, anti-prothrombin time; dBP, diastolic blood pressure; HDL-Chol, HDL cholesterol; IMT, intima-media thickness of the carotid artery; sBP, systolic blood pressure; T-Chol, total cholesterol; TG, triglycerides

rosis [8]. However, there have been no data on the effect of antithrombotic drugs on the primary prevention of stroke.

The intima-media thickness (IMT) of the carotid artery is used as a surrogate of definite atherosclerosis in subjects with a high risk of vascular events [9–12]. The risk of brain infarction increases continuously with increasing IMT of the common carotid artery [13]. Also, the Cardiovascular Health Study pointed out that the infarct-like lesions detected by MRI show strong and consistent relationships with increasing carotid IMT and stenosis degree [14]. We have recently shown that long-term antithrombotic therapy with aspirin or ticlopidine can reduce progression of the IMT of subjects with Type II diabetes [15]. Thus, antithrombotic therapy could lessen the progression of early atherosclerosis and thus lessen the progression of brain infarction especially in patients with Type II diabetes.

The new antithrombotic drug, cilostazol, shows beneficial effects such as increasing peripheral blood flow [16] and the inhibition of the proliferation of vascular smooth muscle cells [17] as a type 3 phosphodiesterase inhibitor as well as ameliorating insulin resistance [18, 19]. In this study, therefore, we aimed to clarify the effect of cilostazol on the primary prevention of brain infarction of subjects with Type II diabetes. We conducted a randomized intensive study of antithrombotic drug on patients with Type II diabetes and found that cilostazol can reduce the appearance and progression of infarction-like lesions as well as IMT of the carotid artery in subjects with Type II diabetes.

## Materials and methods

Ultrasonographic scanning of the carotid arteries was performed using an echotomographic system (EUB-450, Hitachi Medico, Tokyo, Japan) with an electrical linear transducer (midfrequency of 7.5 MHz). The axial resolution of this system was at least 0.3 mm. Scanning of the extracranial common carotid artery, carotid bulb, and internal carotid artery in the neck was performed bilaterally from three different longitudinal projections (ie. anterior-oblique, lateral and posterior-oblique) as well as the transverse projection, as reported in our previous studies [20–22]. All the images were photographed. The scanning session lasted for an average of 30 min. The detection limit of this echo system using 7.5 MHz is 0.1 mm.

The intima-media thickness (IMT) defined by Pignoli et al. [23–25] was measured as the distance from the leading edge of the first echogenic line to the leading edge of the second echogenic line. The first line represents the lumen-intimal interface, and the second line is produced by the collagen-containing upper layer of the tunia adventitia. For each longitudinal projection, the site of the greatest thickness including a plaque lesion was sought along the near and far arterial walls from the common carotid artery to the internal carotid artery. Three measurements of intima-media thickness were conducted at the site of the greatest thickness and at two points, 1 cm upstream and 1 cm downstream from this site. These three measurements were averaged. The greatest value among the six averaged intima-media thicknesses (three from the left and three from the right) was used as the representative value (IMT) for each individual. All scans were conducted by physicians who were unaware of the clinical characteristics of the subjects. Determination of IMT on the photograph was performed by a physician. The reproducibility of the IMT measurement was examined by conducting another scan on eight participants 1 week later. The mean difference in IMT between these two determinations was 0.01 mm, and the standard deviation was 0.04 mm, demonstrating good reproducibility for repeated measurements, as described previously [20]. The threshold of IMT for normal subjects were less than 1.1 mm [20, 21].

MRI was done using 1.5-T MRI. We used the T2-weighted image (TR, 4000 ms; TE, 102 ms) and T1-weighted image (TR, 500 ms; TE 15 ms; flip angle 82) of coronal slices (6 mm thick). We considered a focal and sharply demarcated high intensity on a T2-weighted image of larger than 3 mm to be brain infarction when it coincided with low density area of the a T1-weighted image. Hyperintense images visible only on T2 images were not counted as infarctions so as to exclude perivascular space. The number of infarct-like lesions in each patient was counted in a blinded fashion. The physicians evaluating MRI findings were unaware of patients' characteristics and IMT evaluation.

A total of 91 subjects with Type II diabetes between 41 and 75 years of age were recruited from among outpatients of Belland Hospital and Osaka University Hospital. The determination of Type II diabetes was based on World Organization criteria. Each patient in this study fulfilled the following criteria: no episodes of ketoacidosis and absence of ketonuria; diagnosis of diabetes after 30 years of age; insulin therapy (if any) started after duration of diabetes for at least 5 years; absence of overt diabetic nephropathy or other renal tract disease; and absence of active diabetic proliferative retinopathy. The subjects were allocated at random into two groups with and without cilostazol. The subjects in the cilostazol group received cilostazol at a dose of 100–200 mg/day, while those in the control group did not receive any antithrombotic drugs. Informed consent was obtained from the subjects studied. Patient characteristics are shown in Table 1. This study is approved by the Osaka University Ethics Committee.

**Table 1.** Baseline patient characteristics

| | Control (46) | Cilostazol (43) | *p* |
|---|---|---|---|
| Age (years) | 61.0 ± 7.2 | 60.3 ± 7.9 | |
| Gender (female/male) | 18/28 | 26/17 | |
| Duration (years) | 12.7 ± 12.6 | 10.4 ± 9.6 | |
| BMI (kg/m2) | 23.1 ± 4.66 | 22.9 ± 4.10 | |
| $HbA_{1c}$ (%) | 7.63 ± 1.73 | 7.33 ± 1.87 | |
| T-Chol (mmol/l) | 5.34 ± 1.09 | 5.36 ± 0.70 | |
| TG (mmol/l) | 1.57 ± 1.21 | 1.51 ± 1.23 | |
| HDL-Chol (mmol/l) | 1.46 ± 0.59 | 1.39 ± 0.47 | |
| sBP (mmHg) | 134 ± 14 | 138 ± 13 | |
| dBP (mmHg) | 77.1 ± 7.1 | 80.3 ± 8.0 | 0.0462 |
| Fibrinogen (mg/l) | 283 ± 66 | 296 ± 52 | |
| Treatment | 16/8 | 15/7 | |
| Hypertension (–/ + ) | 26/20 | 25/18 | |
| Hyperlipidaemia (–/ + ) | 12/34 | 15/28 | |
| Nephropathy (–/ + ) | 37/9 | 34/9 | |
| MRI[a] | 34/8/2/2/0 | 27/8/7/1/0 | |
| API | 0.97 ± 0.18 | 0.98 ± 0.10 | |
| IMT (mm) | 1.09 ± 0.29 | 1.10 ± 0.34 | |

[a] MRI was shown as number of patients who had brain lesions or showed an increase in number of brain lesions as follows (no lesion/1 lesion /2 lesions /3 lesions /4 lesions or more)

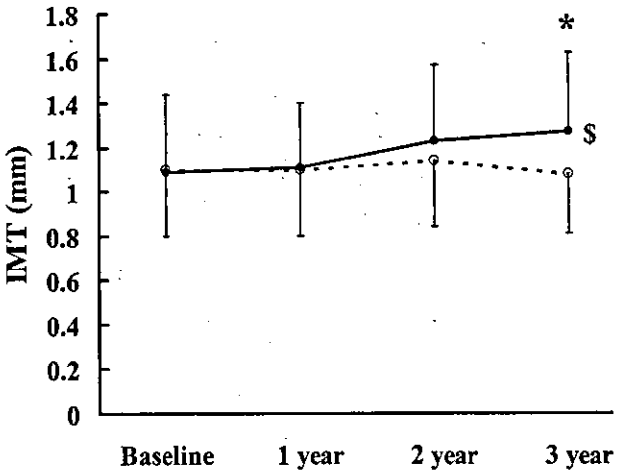


**Fig. 1.** Annual change in IMT of subjects with Type II diabetes with (○) and without (●) cilostazol. Data are shown as means ± SD. * Indicates a significant ($p < 0.01$) difference between with and without cilostazol. $ indicates a significant difference between before and after the observation period

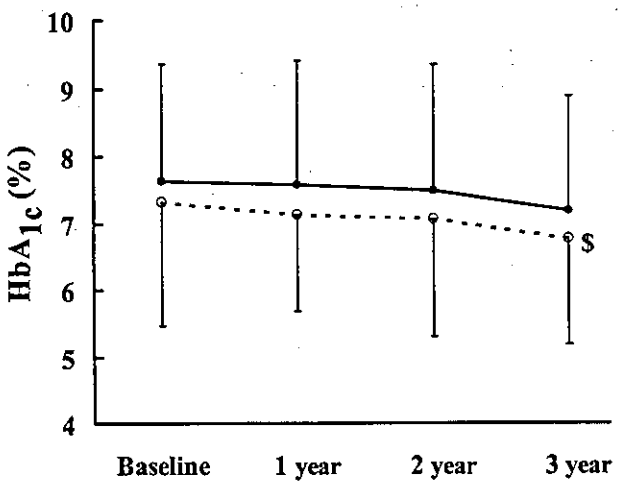


**Fig. 2.** Annual change in $HbA_{1c}$ of diabetic subjects with (○) and without (●) cilostazol. Data are shown as means ± SD. $ indicates a significant difference between before and after the observation period

Exposure to smoking was estimated as the mean number of cigarettes smoked daily. Blood pressure was measured with a mercury sphygmomanometer. After a supine rest of 5 min, three measurements in the sitting position were conducted, and the mean value was used. Ankle pressure index (API) was calculated as systolic blood pressure measured on ankle divided by systolic blood pressure measured on brachial artery. At the baseline determination, blood was withdrawn for analyses of serum total cholesterol and HDL cholesterol, serum triglycerides, plasma glucose, haemoglobin $A_{1c}$ ($HbA_{1c}$), fibrinogen, activated partial thromboplastin time, prothrombin time, and antithrombin III by standard laboratory techniques. Urinary albumin of a fasting urine specimen and a specimen collected at least 4 weeks later was measured by radioimmunoassay. The concentration of albumin in urine was divided by the urinary creatinine concentration and expressed as milligrams per gram of creatinine. The existence of nephropathy was determined if urinary albumin excretion rate was more than 30 mg / g creatinine. During the observation period of 3.2 ± 0.5 years, the lipid profile, blood pressure, IMT and API were determined every year. Brain MRI was taken at the beginning and end of the study period.

Forty-five randomly selected patients with diabetes were given cilostazol. After oral administration of cilostazol, two subjects who showed side effects (headache) were advised to terminate drug administration and were excluded from this study. Forty-three diabetics were controlled with diet only, 31 with oral hypoglycaemic agents, 15 with insulin injection once or more daily. Thirty-eight patients showed hypertension (systolic blood pressure greater than 160 mmHg or diastolic blood pressure above 145 mmHg) or were given anti-hypertensive drugs (diuretics, beta-blockers, alpha-blockers, Ca-channel blockers, and angiotensin converting enzyme inhibitors). Sixty-two patients showed dyslipidaemia (total cholesterol greater than 220 mg/dl or HDL cholesterol less than 40 mg/dl) or were given anti-hyperlipidaemic drugs (clofibrates, probucol, and 3-hydroxy-3-methylglutaryl coenzyme reductase inhibitors). The same doses of drugs are administered during the observation period.

Data are presented as means ± SD. The laboratory data were compared by Student's and paired $t$ test or one-way ANOVA. The difference in number of brain lesions was evaluated by Wilcoxon's rank-sum test. Stepwise multivariate regression analyses were performed to account for the effects and interactions of different variables on foci of silent brain infarction in diabetic patients treated with and without cilostazol. In this analysis, F values for inclusion and exclusion of variables were set at 2.0. These statistical analyses were carried out using the HALBAU (Gendai Sugaku-sha, Kyoto, Japan) statistical package on a personal computer. A $p$ value of less than 0.05 or as an $F$ value greater than 2.0 for stepwise multivariate regression analyses were considered to be statistically significant.

## Results

Diabetic patients not given an antithrombotic drug (control group) showed a significant progression of intima-media thickness during the observation period (0.17 ± 0.19 mm). However, diabetic patients given cilostazol (cilostazol group) showed almost no progression of IMT (0.00 ± 0.20 mm), which was significantly ($p < 0.001$) smaller than that in the control group. Thus, after the observation period, the IMT of the subjects given cilostazol were significantly lower than those of the subjects not given it (1.08 ± 0.27 vs 1.27 ± 0.36 mm, $p < 0.001$, Fig. 1). During the study period, $HbA_{1c}$ and diastolic blood pressure decreased significantly in cilostazol group (Fig. 2). However, the differences in change of $HbA_{1c}$ (–0.43 ± 1.56 vs –0.54 ± 1.15 %, respectively) and diastolic blood pressure (1.1 ± 12.1 vs –2.9 ± 12.4 mmHg, respectively) between the control group and the cilostazol group showed no statistical significances. Also, both groups showed a significant increase in total cholesterol level. API increased but not significantly in both groups (Table 2).

**Table 2.** Patients' characteristics before and after follow-up period

| | Control ($n = 46$) | | | Cilostazol ($n = 43$) | | |
|---|---|---|---|---|---|---|
| | before | after | $p$* | before | after | $p$* |
| BMI (kg/m2) | 23.1 ± 4.66 | 22.7 ± 4.38 | | 22.9 ± 4.10 | 22.8 ± 4.06 | |
| $HbA_{1c}$ (%) | 7.63 ± 1.73 | 7.20 ± 1.71 | | 7.33 ± 1.87 | 6.78 ± 1.60 | 0.0045 |
| T-Chol (mmol/l) | 5.34 ± 1.09 | 5.65 ± 0.91 | 0.0032 | 5.36 ± 0.70 | 5.67 ± 0.70 | 0.0121 |
| TG (mmol/l) | 1.57 ± 1.21 | 1.41 ± 0.69 | | 1.51 ± 1.23 | 1.40 ± 1.30 | |
| HDL-Chol (mmol/l) | 1.43 ± 1.21 | 1.60 ± 0.59 | 0.0036 | 1.39 ± 0.47 | 1.51 ± 0.35 | |
| sBP (mmHg) | 134 ± 14 | 135 ± 10 | | 138 ± 13 | 134 ± 10 | |
| dBP (mmHg) | 77.1 ± 7.1 | 76.0 ± 6. 3 | | 80.3 ± 8.0 | 77.4 ± 6.9 | 0.0283 |
| Bleeding time (sec) | 150 ± 65 | 158 ± 72 | | 140 ± 57 | 143 ± 47 | |
| Prothrombin time (sec) | 20.9 ± 43.0 | 10.9 ± 3.7 | | 10.8 ± 0.5 | 10.2 ± 0.4 | |
| APTT (%) | 37.0 ± 45.1 | 35.8 ± 3.3 | | 29.2 ± 2.2 | 39.2 ± 35.8 | |
| Antithrombin III | 95.5 ± 21.8 | 98.2 ± 32.2 | | 103 ± 11 | 97.8 ± 12.3 | |
| Fibrinogen (mg/l) | 283 ± 66 | 290 ± 41 | | 296 ± 52 | 279 ± 77 | |
| IMT (mm) | 1.09 ± 0.29 | 1.27 ± 0.36 | < 0.001 | 1.10 ± 0.34 | 1.08 ± 0.27 | |
| MRI[a] | 34/8/2/2/0 | 24/10/5/4/3 | < 0.001 | 27/8/7/1/0 | 27/8/6/1/1 | |
| Change in MRI[a] | 30/8/6/2/0 | 41/2/0/0/0 | | | | |
| API | 0.97 ± 0.18 | 1.05 ± 0.13 | 0.0418 | 0.98 ± 0.10 | 1.00 ± 0.14 | |

Data are shown as means ± SD
[a]MRI and change in MRI were shown as number of patients who had brain lesions or showed the increase in number of brain lesions as follows (no lesion/1 lesion /2 lesions /3 lesions / 4 lesions or more).
*$p$ before vs after

**Table 3.** Multivariate regression analysis to evaluate efficacy of cilostazol, IMT, blood pressures in affecting progression of infarct-like lesions by MRI

| | Initial MRI findings | | | Final MRI findings | | | Progression of MRI findings | | |
|---|---|---|---|---|---|---|---|---|---|
| Parameter | Partial regression coefficient (mm/years) | $p$ value | Partial correlation coefficient | Partial regression coefficient | $p$ value (mm/year) | Partial correlation coefficient | Partial regression coefficient (mm/year) | $p$ value | Partial correlation coefficient |
| Initial IMT (per 1 mm) | 0.767 | 0.003 | 0.312 | 0.945 | 0.006 | 0.294 | 0.320 | 0.142 | 0.161 |
| Change in IMT (per 1 mm) | | | | | | | 0.781 | 0.034 | 0.230 |
| Initial sBP (per 1 mmHg) | 0.022 | 0.006 | 0.292 | 0.046 | < 0.001 | 0.436 | 0.024 | < 0.001 | 0.382 |
| Initial dBP (per 1 mmHg) | − 0.033 | 0.024 | − 0.242 | − 0.063 | 0.001 | − 0.337 | − 0.032 | 0.011 | − 0.276 |
| Cilostazol (per administration) | | | | − 0.341 | 0.114 | − 0.172 | − 0.399 | 0.009 | − 0.281 |
| $R^{2-}$ | 0.191 | | | 0.291 | | | 0.316 | | |

Stepwise multivariate regression analysis was done on 89 diabetic subjects

In the control group, 16 out of 46 subjects had an increased number of infarct-like lesions detected by MRI at the end of the study. In the cilostazol group, 2 out of 43 subjects showed increases in the number of infarct-like lesions. The difference was statistically significant ($p < 0.001$). At the baseline examination, IMT was significantly related to the number of infarct-like lesions ($r = 0.335$, $p = 0.001$). After the observation period, IMT was significantly related to the number of infarct-like lesions ($r = 0.347$, $p = 0.001$) in both groups. During the observation period, the progression of IMT correlated significantly and positively with the increase in the foci of infarct-like lesions ($r = 0.299$, $p = 0.004$, Fig. 3). During the observation period, the change in systolic blood pressure did not significantly correlate with the increase in the foci of infarct-like lesions (Fig. 4). In the control group, 10 out of 34 subjects without infarct-like lesions at the baseline MRI examination showed such lesions after the observation period. On the other hand, in the cilostazol group, none of the 28 subjects without infarct-like lesions at the baseline MRI examination showed such lesions after the observation period ($p = 0.001$).

Stepwise multivariate regression analysis was done to evaluate the risk factors for the infarct-like lesions detected by MRI. The initial IMT, baseline systolic blood pressure and baseline diastolic blood pressure were significant risk factors for the infarct-like lesions at the baseline examination. For the infarct-like lesions of the final MRI examination, the initial IMT, baseline systolic blood pressure, baseline diastolic blood pressure and administration of cilostazol were deduced as b[illegible]g significant factors. Baseline systolic and diastolic [illegible]od pressures, the change in IMT, and administrati[illegible]f cilostazol affected ($p < 0.05$) the increase in the [illegible]nber of infarct-like lesions (Table 3).

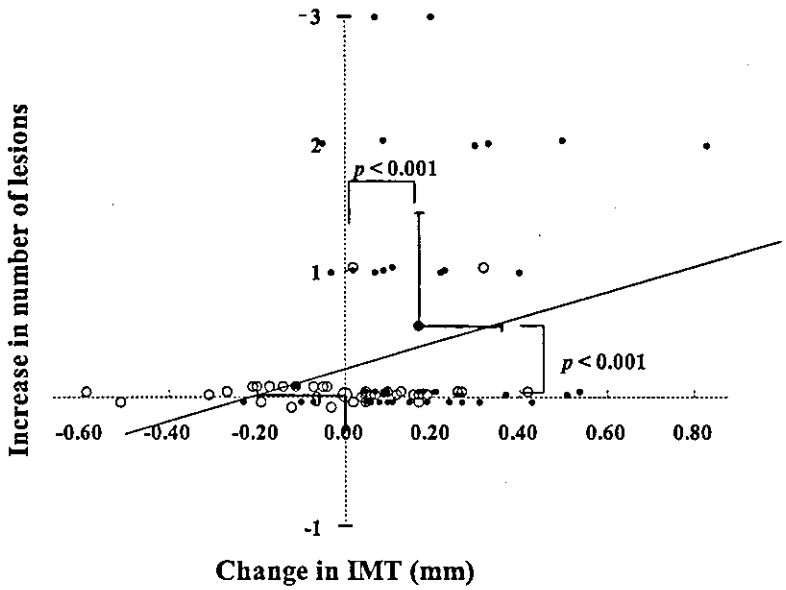


**Fig. 3.** Relationship between the change in IMT and the increase in number of infarct-like lesions in subjects diabetic subjects with (○) and without (●) cilostazol after the observation period. Average data were given with SD of IMT and SD of the number of lesions

## Discussion

This is the first study showing the effectiveness of antithrombotic agents on arresting the appearance or progression of silent brain infraction as well as arresting progression of the carotid intima-media thickness of subjects with Type II diabetes without symptomatic coronary vascular diseases.

In this study, cilostazol, a type 3 phosphodiesterase inhibitor, could prevent the progression of carotid IMT of subjects with Type II diabetes (from $0.17 \pm 0.19$ to $-0.0 \pm 0.2$ mm/3 years). Recently, we showed that aspirin at a small dose or ticlopidin could reduce the progression of IMT by almost 50% [15]. Cilostazol also shows an antiatherogenic effect on endothelial cells and vascular smooth muscle cells. Because type 3 phosphodiesterase is present not only in platelets but also in endothelial cells and smooth muscle cells, the inhibition of this enzyme by cilostazol administration results in increased blood flow [16] and attenuation of smooth muscle cell proliferation [17]. Together with these antiatherogenic effects on endothelial cells and vascular smooth muscle cells, cilsotazol could prevent the progression of carotid IMT.

Gene disruption of endothelial nitric oxide synthase causes insulin resistance in mice [26]. Cilostazol increases NO synthesis in smooth muscle cells [27].Thus, cilostazol might improve insulin sensitivity [18, 19]. In this study, $HbA_{1c}$ decreased significantly after administration of cilostazol. It also decreased in subjects not given cilostazol but not significantly. However, according to a previous multiple regression analysis on the effectiveness of the reduction of $HbA_{1c}$ on the inhibition of IMT progression, the decrease of the $HbA_{1c}$ might be too small to arrest the IMT progression shown in this study [15].

Cross-sectional study before and after the observation period showed a positive relationship ($p < 0.001$) between the number of infarct-like lesions and the carotid IMT ($r = 0.335$ or $r = 0.347$, respectively). These results were comparable with the observation that carotid artery IMT is a risk factor for myocardial infarction and stroke in older adults [12]. This study is the first to show that the change in infarct-like lesion after the observation period was significantly and positively related with the change in carotid IMT ($r = 0.299$, $p = 0.004$). This points to the possibility that the increase in carotid IMT could predict the appearance of silent brain infarction.

The diabetic patients without cilostazol had an increase of infarct-like lesions ($p < 0.001$). However, the diabetic patients given cilostazol showed a negligible appearance of infarct-like lesions in the observation period of up to 3 years. These results offer support for the secondary prevention study using cilostazol at 200 mg/day showing a reduction in the appearance of stroke by 43.4% compared with the placebo group [28].

Multivariate regression analysis showed that the initial systolic blood pressure is the primary risk factor (its partial regression coefficient was 0.024) for the progression of MRI finding of subjects with Type II diabetes and also the initial diastolic blood pressure is negatively (its partial regression coefficient was $-0.032$) responsible for the progression of MRI.

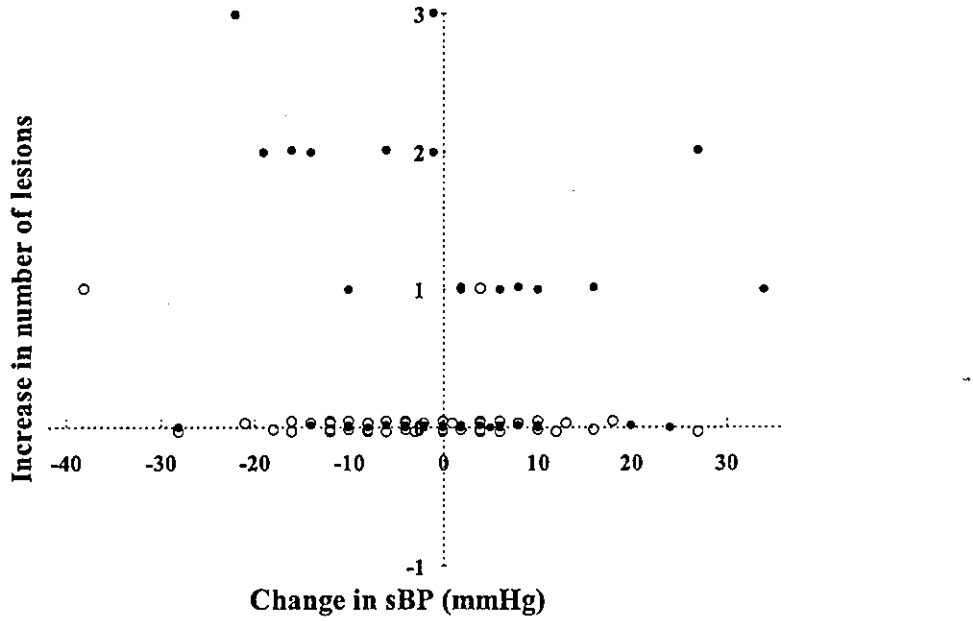


**Fig. 4.** Relationship between the change in systolic blood pressure and the increase in number of infarct-like lesions in diabetic subjects with (○) and without (●) cilostazol after the observation period

This observation could be comparable to the report showing that the thickening of the media of arteries in hypertension is a response to the raised tension in their walls [29]. The patients in the cilostazol group did not show a significant reduction of systolic blood pressure and the change in systolic blood pressure did not correlate with the change in brain lesions. Thus, the reduced appearance of brain lesions in the cilostazol group could not be accounted for by changes in systolic blood pressure.

This study is the first primary prevention study showing that cilostazol is effective for arresting the appearance of silent brain infarction of diabetic patients without symptomatic coronary vascular events. As already described in detail, cilostazol could have favourable antiatherogenic effects, such as improving impaired endothelial function, increasing blood flow, and inhibiting vascular smooth muscle proliferation. It could also improve insulin resistance in Type II diabetic subjects. However, to establish the usefulness of cilostazol for primary prevention in Type II diabetic patients, a large-scale intervention study is needed.

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# Increased Proliferation of Neural Progenitor Cells but Reduced Survival of Newborn Cells in the Contralateral Hippocampus After Focal Cerebral Ischemia in Rats

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**Summary:** Recent studies demonstrated that neurogenesis in the adult hippocampus increased after transient global ischemia; however, the molecular mechanism underlying increased neurogenesis after ischemia remains unclear. The finding that proliferation of progenitor cells occurred at least a week after ischemic insult suggests that the stimulus was not an ischemic insult to progenitor cells. To clarify whether focal ischemia increases the rate of neurogenesis in the remote area, the authors examined the contralateral hemisphere in rats subjected to permanent occlusion of the middle cerebral artery. In the subgranular zone of the hippocampal dentate gyrus, the numbers of bromodeoxyuridine (BrdU)-positive cells increased approximately sixfold 7 days after ischemia. In double immunofluorescence staining, more than 80% of newborn cells expressed Musashi1, a marker of neural stem/progenitor cells, but only approximately 10% of BrdU-positive cells expressed glial fibrillary acidic protein (GFAP), a marker of astrocytes. The number of BrdU-positive cells markedly decreased 28 days after BrdU administration after ischemia, but it was still elevated compared with that of sham-operated rats. In double immunofluorescence staining, 80% of newborn cells expressed NeuN, a marker of differentiated neurons, and 10% of BrdU-positive cells expressed GFAP. However, in the other areas of the contralateral hemisphere including the rostral subventricular zone, the number of BrdU-positive cells remained unchanged. These results showed that focal ischemia stimulated the proliferation of neuronal progenitor cells, but did not support survival of newborn cells in the contralateral hippocampus.
**Key Words:** Hippocampus—Ischemia—Neurogenesis—Neuronal progenitor cells—Musashi-1 (Msi1).

Growing evidence indicates that neurogenesis still occurs in the subgranular zone (SGZ) in the hippocampus (Altman and Das, 1967; Gould et al., 1998; Eriksson et al., 1998; Roy et al., 2000) and the rostral migratory stream forms from the subventricular zone (SVZ) to the olfactory bulb (Pincus et al., 1998; Johansson et al., 1999; Doetsch et al., 1999) in the adult mammalian brain. Recent studies demonstrated increased neurogenesis in the dentate gyrus of the hippocampus after transient global ischemia in gerbils, rats, and mice (Liu et al., 1998; Takagi et al., 1999; Kee et al., 2001; Yagita et al., 2001). Although it was shown recently that increased neurogenesis was suppressed by treatment with glutamate receptor antagonists (Bernabeu and Sharp, 2000; Arvidsson et al., 2001) or with acetylsalicylic acid (Kumihashi et al., 2001), the molecular mechanism underlying increased neurogenesis still remains unclear. The proliferating activity in the SGZ reached a peak 7 to 10 days after ischemic insult. Because CA4 neurons in the hilus are the most vulnerable to ischemic insult (Schmidt-Kastner and Freund, 1991; Matsuyama et al., 1993), neuronal progenitor cells in the SGZ resided close to injured CA4 neurons in a transient global ischemia model. Late stimulation and proliferation of neuronal progenitor cells suggest that the stimulus was not an ischemic insult to progenitor cells themselves, but subsequent production of growth factors in brain tissue. Yoshimura et al. (2001) recently demonstrated involvement of basic fibroblast growth factor on increased neurogenesis after kainate seizures and ischemia, using

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basic fibroblast growth factor knockout mice. In terms of cell proliferation in a remote area, Jin et al. (2001) recently demonstrated increased incorporation of BrdU into cells in bilateral SGZ after focal ischemia. Although increased incorporation of BrdU in the contralateral hippocampus suggests the role of a diffusible or humoral factor, there are still unresolved points such as the characteristics of proliferating cells, cell survival, and neuronal differentiation in the contralateral SGZ. Therefore, we examined the proliferation of neuronal progenitor cells using a neural stem cell marker, Musashi1, and determined cell survival after proliferation and neuronal differentiation of the nonischemic, contralateral hemisphere including the hippocampus after permanent occlusion of the middle cerebral artery.

## MATERIALS AND METHODS

### Animal model

Adult male Wistar rats (2 months old) weighing 250 to 300 g (Charles River Laboratory, Yokohama, Japan) 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. They were fed standard laboratory chow and had free access to water before and after all procedures. Animal care was given according to the guidelines of the Animal Center of Osaka University Graduate School of Medicine.

Each animal was anesthetized with halothane, and occlusion of the middle cerebral artery (MCA) was done according to Koizumi et al. (1986) and Longa et al. (1989). Briefly, the left common carotid artery was exposed through a midline incision, and the internal carotid artery was isolated and carefully separated. A 4–0 nylon monofilament, with its tip rounded by heating, was introduced from a bifurcation of the internal carotid artery and advanced until resistance was felt. Operated rats were used if they showed spastic paralysis of the right forelimb and circling to the right during the ischemic period. Rectal temperatures were monitored routinely during surgery to maintain animals at 37.0 ± 0.5°C. For the sham operation, a nylon filament was introduced from the carotid bifurcation and the tip was kept in the internal carotid artery.

### Bromodeoxyuridine labeling and experimental design

We used bromodeoxyuridine (BrdU) (Boehringer Mannheim, Tokyo, Japan), a thymidine analog, to label proliferating cells. Bromodeoxyuridine was incorporated into newly synthesized DNA. In the first experiment (Fig. 1), rats of the control, sham, and ischemia groups (each time point 1, 4, 7, or 14 days after ischemia: n = 4 in each group) received BrdU (50 mg/kg, i.p.) administration three times every 4 hours during a period of 8 hours. The next day (each time point 2, 5, 8, or 15 days after ischemia, respectively), they were decapitated while they were under deep pentobarbital anesthesia, and their brains were removed and divided into coronal sections (approximately 5-mm thickness) in ice-cold methanol, and immersion fixed in methanol at 4°C overnight. After fixation, each tissue block was dehydrated and embedded in paraffin. The 5-μm-thick sections corresponding to the stereotaxic sections 1.5 mm rostral and 3.0 mm caudal to bregma, containing the caudoputamen and the dorsal hippocampus, respectively, were stained with the immunohistochemical reaction for BrdU. In the second experiment (Fig. 1), control and ischemic rats (n = 4 for each) were perfusion-fixed under deep pentobarbital anesthesia 1 day after BrdU administration. After perfusion with 4% paraformaldehyde in a 50-mmol/L phosphate buffer, the brains were removed and cut into coronal blocks containing the hippocampus, and immediately immersion fixed in 4% paraformaldehyde. Coronal sections (30 μm thick) were cut on a vibratome and processed for the double immunofluorescence. In the third experiment (Fig. 1), rats received BrdU (50 mg/kg i.p.) administration three times 7 days after MCA occlusion, and then control and ischemic rats were killed 1 day (n = 4 for each) or 28 days (n = 4 for each) after BrdU injection. The 5-μm-thick paraffin sections containing the dorsal hippocampus were stained with the immunohistochemical reaction for BrdU. Additional control rats and ischemic rats (n = 4 for each) were perfusion fixed 28 days with 4% paraformaldehyde after BrdU administration, and the vibratome sections were processed for the double immunofluorescence.

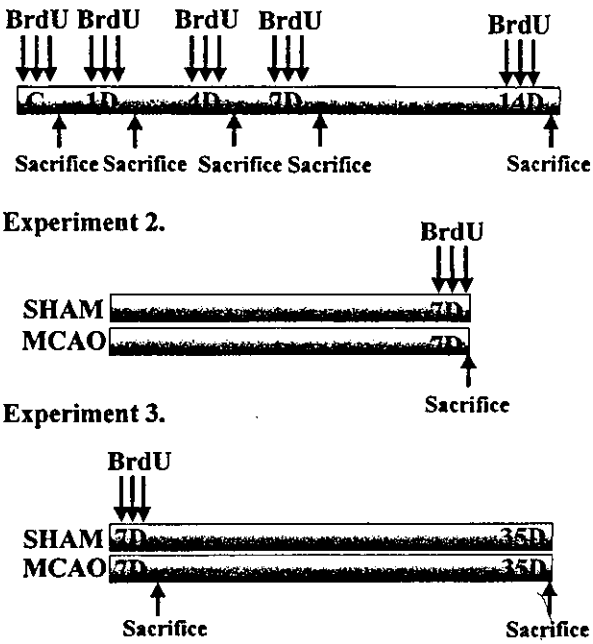


FIG. 1. Bromodeoxyuridine (BrdU) labeling of ischemic brain. Bromodeoxyuridine (50 mg/kg, i.p.) was given three times every 4 hours during a period of 8 hours. In the first experiment (experiment 1), rats received BrdU 1, 4, 7, and 14 days after ischemia (n = 4 in each group), and the next day they were killed and the brains were processed for paraffin sections and used for counting BrdU-positive cells. In the second experiment (experiment 2), rats received BrdU on day 7 after ischemia, and the next day they were killed and the vibratome sections were prepared for double immunofluorescence. In the third experiment (experiment 3), rats received BrdU on day 7 after ischemia, and they were killed 1 day or 28 days after BrdU injection. The paraffin and vibratome sections were prepared for counting BrdU-positive cells and for double immunofluorescence, respectively.

### Immunohistochemistry

For BrdU immunohistochemistry, DNA denaturing was required. Each deparaffinized section was treated in 50% formamide and a 2× saline–sodium citrate buffer at 65°C for 2

hours. After washing in the 2× saline–sodium citrate buffer, sections were incubated in 2N HCl at 37°C for 30 minutes. Sections were rinsed in Tris-buffered saline/0.1% Triton X-100 for 20 minutes, and incubated with a monoclonal anti-BrdU antibody (1:100; Amersham, Arlington Heights, IL, U.S.A.) at 4°C overnight. After washing in Tris-buffered saline/0.1% Triton X-100, the sections were incubated with a biotinylated secondary antibody for 1 hour at room temperature. They were washed and further incubated with a streptavidin–biotin–peroxidase complex (Vector, Burlingame, CA, U.S.A.). The sections were finally reacted with 0.05% 3'3-diaminobenzidine in the presence of 0.01% $H_2O_2$. The control sections were incubated with the nonimmune serum or ascites.

For the double-immunofluorescence technique, vibratome brain sections were incubated with a primary antibody diluted with Tris-buffered saline/0.1% Triton X-100 at 4°C overnight. We used the following antibodies as primary antibodies; a mouse monoclonal anti-BrdU antibody (Amersham 1:100), a rat monoclonal anti-BrdU antibody (1:200; Harlan Sera-labo, Loughborough, U.K.), a mouse monoclonal anti-NeuN antibody (1:200; Chemicon, Temecyla, CA, U.S.A.), a mouse monoclonal anti-microtubule-associated protein 2 (MAP2) antibody (1:200; Sigma-Aldrich, Tokyo, Japan), a rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:200 Sigma), a goat polyclonal anti-doublecortin (DCX) antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), and a rat monoclonal anti-Musashi1 antibody. Double immunostaining was performed using immunofluorescence and confocal microscopy (Zeiss). For double labeling of BrdU and cell markers (NeuN and MAP2: neuron, GFAP: astrocytes, DCX: immature neuron, Musashi1: neuronal progenitor cells and astrocytes), sections were incubated with an anti-BrdU antibody and antibodies for each cell marker at 4°C overnight after DNA denaturation. DCX is a protein required for neuronal migration and it is expressed in both migrating neuroblasts and differentiated neurons (Francis et al., 1999; Nacher et al., 2001). Fluorescein isothiocyanate- or rhodamine-labeled goat or donkey anti-immunoglobulin G (IgG) antibodies were used as the secondary antibodies. The combination of antibodies used in each double-immunostaining experiment was as follows: BrdU-NeuN and BrdU-MAP2; the rat anti-BrdU antibody and mouse anti-NeuN or anti-MAP2 antibody as primary antibodies, and the rhodamine-labeled anti-rat IgG antibody and fluorescein isothiocyanate–labeled anti-mouse IgG as secondary antibodies. BrdU-DCX; the rat anti-BrdU antibody and goat anti-DCX antibody as primary antibodies, and the rhodamine-labeled anti-rat IgG antibody and fluorescein isothiocyanate-labeled anti-goat IgG as secondary antibodies. BrdU-GFAP; the rat anti-BrdU antibody and rabbit anti-GFAP antibody as primary antibodies, and the rhodamine-labeled anti-rat IgG antibody and fluorescein isothiocyanate–labeled anti-rabbit immunoglobulin G antibody as secondary antibodies. BrdU-Musashi1; the mouse anti-BrdU antibody and rat anti-Musashi1 antibody as primary antibodies, and the rhodamine-labeled anti-mouse IgG antibody and fluorescein isothiocyanate-labeled anti-rat IgG antibody as secondary antibodies.

### Quantification

To count BrdU-positive cells in paraffin sections colored by the peroxidase reaction, five sections from the caudoputamen and the hippocampus were obtained every 150 μm beginning at a section 1.5 mm rostral and 3.0 mm caudal to the bregma, respectively. The area for quantification was as follows (Fig. 2): the frontal, cingulate, and parietal cortex, caudoputamen, and thalamus in the contralateral hemisphere, and the rostral subventricular zone and the dentate gyrus of the hippocampus in both hemispheres. In the hippocampus, the granular cell layer (GCL) (approximately 60 μm) and SGZ, defined as a two-cell-body–wide zone (approximately 10 μm) along the border of the GCL and the hilus, were always combined for quantification. The mean density of BrdU-labeled cells in each rat was calculated as the number of labeled nuclei/area. Statistical analysis was performed using analysis of variance followed by Scheffé *post hoc* tests.

To assess the phenotype of BrdU-positive cells after ischemia, we used a double-immunostaining technique. We detected BrdU-positive cells in the SGZ and GCL, and determined whether they expressed Musashi1, DCX, NeuN, MAP2, or GFAP with confocal microscopy. A double positive percentage was calculated as BrdU+/Musashi1+, BrdU+/NeuN+, or BrdU+/GFAP+ cells for total BrdU-positive cells. The mean value of each data was obtained in eight sections from four rats.

## RESULTS

A few BrdU-positive cells were observed in the SGZ of the hippocampal dentate gyrus in the control (Figs. 3 and 4). In the hippocampus that was ipsilateral to ischemia, the number of BrdU-positive cells increased 1 day after MCA occlusion, reached a peak 4 days later, and

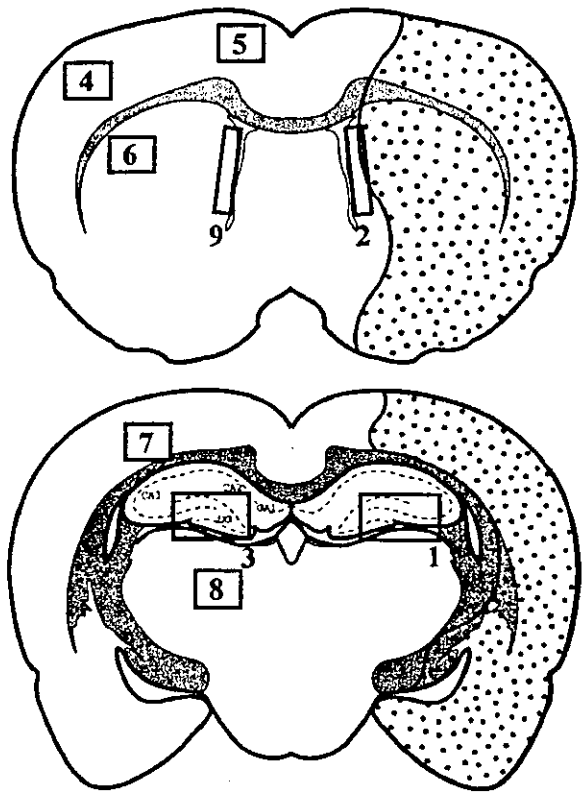


FIG. 2. Diagram of the brain sections in the rat brain after middle cerebral artery occlusion. Infarct area was shown as stippled area. Number of BrdU-positive cells was quantified in nine boxed areas as follows: 1,2: ipsilateral hemisphere; 1: hippocampus; 2: rostral subventricular zone; 3–9: contralateral hemisphere; 3: hippocampus; 4: frontal cortex; 5: cingulate cortex; 6: caudoputamen; 7: frontoparietal cortex; 8: thalamus; 9: rostral subventricular zone.

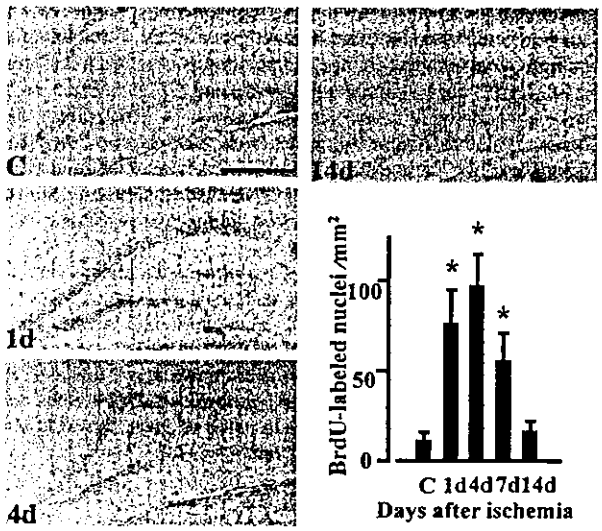


FIG. 3. Bromodeoxyuridine (BrdU)-positive cells in the ipsilateral hippocampus after middle cerebral artery occlusion. All rats were administered BrdU (50 mg/kg, i.p.) three times a day before they were killed. Note the increase in BrdU-positive cells in the subgranular zone 1 day (1d) to 7 days (7d) after ischemia. Bar = 0.5 mm. The significance of differences was determined using analysis of variance followed by Scheffé *post hoc* test. Each column and bar denotes mean ± SD. $^*P < 0.05$ compared with the control group. C: control animal; 1d, 4d, 7d and 14d: 1 day, 4 days, 7 days, and 14 days after middle cerebral artery occlusion.

remained elevated until 7 days after ischemia (Fig. 3). In contrast, in the contralateral hippocampus, the number of BrdU-positive cells in the SGZ remained unchanged until 4 days after MCA occlusion, increased markedly 7 days after ischemia, and then declined to the control level 14 days after ischemia (Fig. 4). Semiquantitative analysis showed that the number of BrdU-positive cells increased approximately sixfold 7 days after ischemia compared with the control (Fig. 4). Only a few BrdU-positive cells were observed in the dentate hilus from 1 to 14 days after ischemia. In other areas of the contralateral hemisphere including the rostral SVZ, the number of BrdU-positive cells remained unchanged from 1 day to 14 days after ischemia (Table 1). In the ipsilateral SVZ, the number of BrdU-positive cells significantly increased 7 days after ischemia.

To examine the phenotype of BrdU-positive cells in the contralateral hippocampus 1 day after BrdU administration, double immunofluorescence for BrdU/Musashi1, BrdU/DCX, or BrdU/GFAP was examined (Fig. 5). In both sham-operated rats and ischemic rats 7 days after MCA occlusion, the majority of BrdU-positive cells expressed both Musashi1 and DCX. In the semiquantitative analysis, approximately 80% of BrdU-positive cells in the SGZ colocalized with Musashi1 (79.8 ± 9.7% in control rats and 80.8 ± 9.8% in ischemic rats), but only 20% of BrdU-positive cells colocalized with GFAP (18.3 ± 4.5% in control rats and 17.8 ± 5.4% in ischemic rats). However, in the ipsilateral hippocampus of ischemic rats, BrdU-positive but Musashi1-negative cells were often observed (Fig. 6). Only 50.5 ± 10.4% of BrdU-positive cells in the SGZ colocalized with Musashi1, and 42.7 ± 4.7% of BrdU-positive cells colocalized with GFAP (Fig. 6). The percentage of both BrdU- and DCX-positive cells per total BrdU-positive cells in the SGZ was 76.7 ± 7.2% and 31.7 ± 9.7% in the contralateral and in the ispsilateral hippocampus, respectively.

To examine the survival or reduction of BrdU-positive cells, the number of BrdU-positive cells in the SGZ and GCL of the contralateral hippocampus 28 days after BrdU administration was counted in both control and ischemic rats. The survival rate in control rats was 92%, whereas that in ischemic rats was only approximately 20% (Table 2), although there were still a significantly larger number of BrdU-positive cells in the ischemic rat hippocampus than those of sham-operated rats (Table 2). In the ipsilateral hippocampus, the number of BrdU-positive cells also decreased from 98.0 ± 22.5/mm$^2$ (1 day after BrdU injection) to 20.3 ± 6.6/mm$^2$ (28 days after BrdU injection); however, it was not possible to calculate the residual rate of BrdU-positive cells because many BrdU-positive cells were Musashi1-negative and would likely be inflammatory cells. We further observed that BrdU-positive postmitotic cells expressed the neuronal antigenic phenotypes, NeuN and MAP2, 28 days after BrdU injection with double immunofluorescence (Fig. 7). In the contralateral hippocampus, the majority of BrdU-positive cells expressed both NeuN and MAP2.

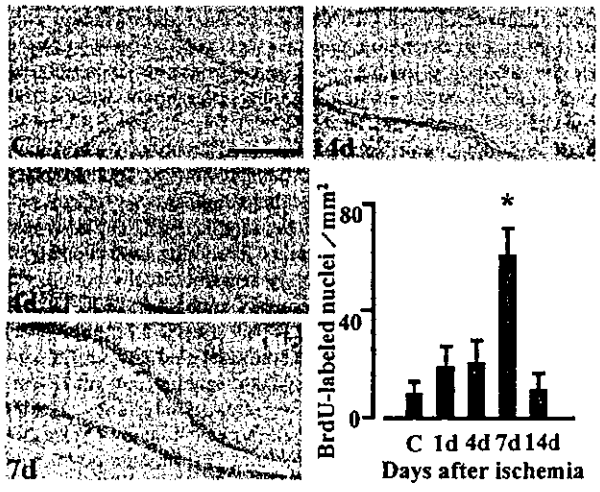


FIG. 4. Bromodeoxyuridine (BrdU)-positive cells in the contralateral hippocampus after middle cerebral artery occlusion. All rats were administered BrdU (50 mg/kg, i.p.) three times a day before they were killed. Note the marked increase in BrdU-positive cells in the subgranular zone 7 days after ischemia (7d), compared with the control (C). Bar = 0.5 mm. The significance of differences was determined using analysis of variance followed by Scheffé *post hoc* test. Each column and bar denotes mean ± SD. $^*P < 0.05$ compared with the control group. C: control animal; 1d, 4d, 7d and 14d: 1 day, 4 days, 7 days, and 14 days after middle cerebral artery occlusion.

**TABLE 1.** *BrdU-positive cells in the bilateral hemisphere after middle cerebral artery occlusion*

| | Control | After MCA occlusion | | | |
|---|---|---|---|---|---|
| | | 1 day | 4 days | 7 days | 14 days |
| Contralateral | | | | | |
| Frontal cortex | 0.33 ± 0.51 | 0.33 ± 0.51 | 0.33 ± 0.51 | 0.83 ± 0.75 | 0.50 ± 0.55 |
| Cingulate cortex | 0.50 ± 0.55 | 0.33 ± 0.51 | 0.67 ± 0.81 | 0.50 ± 0.33 | 0.33 ± 0.51 |
| Caudoputamen | 0.50 ± 0.54 | 0.33 ± 0.51 | 0.67 ± 0.51 | 0.67 ± 0.81 | 0.67 ± 0.81 |
| Parietal cortex | 0.83 ± 0.75 | 0.67 ± 0.81 | 0.33 ± 0.51 | 0.83 ± 0.75 | 0.33 ± 0.51 |
| Thalamus | 0.67 ± 0.51 | 0.50 ± 0.54 | 0.33 ± 0.52 | 1.00 ± 1.09 | 0.33 ± 0.52 |
| Subventricular zone | 1,658 ± 95 | 2,022 ± 209 | 1,770 ± 320 | 1,870 ± 155 | 1,705 ± 475 |
| Ipsilateral | | | | | |
| Subventricular zone | 1,646 ± 40 | 2,152 ± 455 | 2,009 ± 401 | 3,811 ± 350* | 2,640 ± 401 |

Cell number/mm$^2$, *$P < 0.05$ versus control.
BrdU, bromodeoxyuridine; MCA, middle cerebral artery.

In the semiquantitative analysis, approximately 80% of all BrdU-positive cells represented NeuN both in the control and ischemia groups (Table 2). In contrast, only 10% of BrdU-positive cells in the SGZ and GCL colocalized with GFAP (Fig. 7; Table 2).

## DISCUSSION

In the present study, we demonstrated that proliferation of neuronal progenitor cells in the SGZ of nonischemic hippocampus increased after focal ischemia. However, survival of those proliferating cells markedly reduced, so focal ischemia resulted in only a slight neurogenesis in the contralateral SGZ. As control, we also examined ipsilateral hippocampus and found a rapid increase in the number of BrdU-positive cells (Table 1). However, the majority of BrdU-positive cells could be not progenitor cells but inflammatory cells or astrocytes residing close to injured neurons, because about half of BrdU-positive cells were Musashi1-negative in the ipsi-

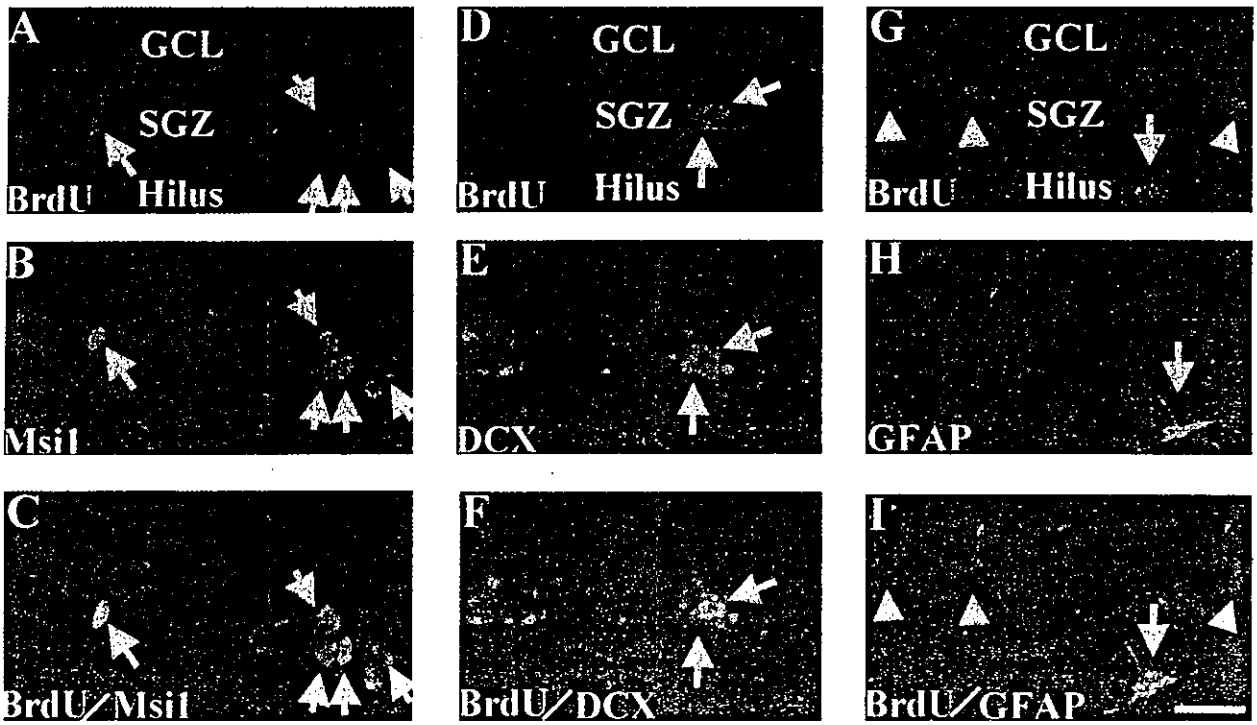


FIG. 5. Proliferation of Musashi1 (Msi1) and doublecortin (DCX)-positive and glial fibrillary acidic protein (GFAP)-negative cells in the contralateral subgranular zone (SGZ). To label newborn cells, rats received bromodeoxyuridine (BrdU; 50 mg/kg, i.p.) administration three times, 7 days after ischemia, and were killed a day after BrdU injection. In the SGZ and granular cell layer (GCL) (A–C; **A:** BrdU, **B:** Msi1, **C:** BrdU/Msi1: D–F, **D:** BrdU, **E:** DCX, **F:** BrdU/DCX), most BrdU-positive cells (red in **A** and **D**), which formed clusters, colocalized with Msi1 (green in **B**) and DCX (green in **E**) as indicated with arrows. In contrast, only a BrdU-positive cell colocalized with GFAP in the hilus, as indicated with an arrow, but few BrdU cells colocalized with GFAP in the SGZ, as indicated with arrowheads (G–I; **G:** BrdU, **H:** GFAP, **I:** BrdU/GFAP). Bar = 30 μm.

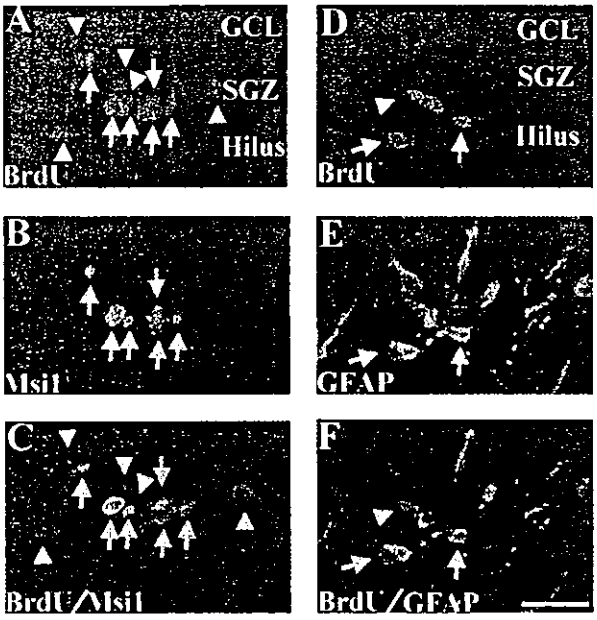


FIG. 6. Proliferation of Musashi1 (Msi1)-negative cells in the ipsilateral subgranular zone (SGZ). The same brain sections in Figure 5 were used to examine proliferating cells in the ipsilateral hippocampus after middle cerebral artery occlusion. In the SGZ and granular cell layer (GCL) of the ipsilateral hippocampus (**A–C**; **A:** bromodeoxyuridine (BrdU), **B:** Msi1, **C:** BrdU/Msi1), more than half of BrdU-positive cells were Msi1-negative. Part of BrdU-positive cells colocalized with glial fibrillary acidic protein (GFAP) (**D–F**; **D:** BrdU, **E:** GFAP, **F:** BrdU/GFAP). Bar = 30 μm.

lateral hippocampus, which is often injured in the nylon suture model (States et al., 1996). The dissociation of the peaks of cell proliferation in the ipsilateral (4 days) and contralateral (7 days) hippocampi is likely ascribed to the different population of proliferative cells. Liu et al. (1998) first demonstrated increased neurogenesis in the hippocampus after transient global ischemia in gerbils. Later, similar findings were observed after transient global ischemia in the hippocampus of mice (Takagi et al., 1999) and rats (Kee et al., 2001). Recently, we demonstrated proliferation of neural stem/progenitor cells using Musashi1 as a marker of neural stem or progenitor cells after transient global ischemia in rats (Yagita et al., 2001). In a focal-ischemia model, Gu et al. (2000) demonstrated neurogenesis in the peripheral area of cerebral infarction. All previous studies demonstrated that the number of BrdU-positive cells in the SGZ reached a peak 7 to 10 days after ischemia, suggesting the involvement of lesion-induced factors, rather than ischemic insult itself, in the increased neurogenesis after ischemia. If this hypothesis is true, focal ischemia and cerebral infarction result in increased neurogenesis in the contralateral SGZ. There are only two reports (Jin et al., 2001; Zhang et al., 2001) about neurogenesis in the contralateral hippocampus after cerebral infarction. Strictly speaking, Jin et al. demonstrated only an increased incorporation of BrdU in the contralateral SGZ. Zhang et al. found no significant difference in number of BrdU-positive cells between the ipsilateral and the contralateral dentate gyrus.

The first novel finding in the present study was the increased proliferation of neural stem/progenitor cells in the contralateral, nonischemic SGZ. We used Musashi1 as a marker of neural stem/progenitor cells as we did in a transient global ischemia model (Yagita et al., 2001). Musashi1 is a neural RNA-binding protein, and is expressed in neural stem/progenitor cells and astrocytes in the adult brain (Sakakibara et al., 1996; Sakakibara and Okano, 1997; Kaneko et al., 2000). Because the majority of astrocytes express GFAP, neural stem/progenitor cells could be recognized as Musashi1-positive and GFAP-negative cells. However, recently, it has been shown that GFAP-positive astrocytes (designated as "type B" cells) in the SGZ of the adult hippocampal dentate gyrus are able to give rise to new neurons via GFAP-negative intermediate neuronal progenitor cells (designated as "type D" cells) (Seri et al., 2001). In the present study, we observed that more than 80% of BrdU-positive cells expressed Musashi1 after the survival time of 1 day in the control SGZ and the contralateral SGZ after MCA occlusion. However, only 20% of BrdU-positive cells expressed GFAP in the same condition (Table 1). This finding could be explained as follows. Although the primary neuronal progenitor cells are GFAP-positive astrocytes (type B cells), GFAP, an intermediate filament, may be degraded soon after the cell division. It would be interesting if cells at this stage become Musashi1 positive. It should be also noted that BrdU may label neurons undergoing cell death as well as newly divided cells (Bjorklund and Lindvall, 2000). However, there were no cells in the contralateral SGZ positive for fragmented DNA with *in situ* nick-end labeling (data not shown); thus, most BrdU-positive cells in that area are likely to be newly divided cells. Proliferation of neural stem/progenitor cells reached a peak 7 days after ischemia, similar to a temporal profile observed in the SGZ after transient global ischemia (Liu et al., 1998; Yagita et al., 2001). Increased proliferation of neural stem/progenitor cells in the contralateral SGZ supported the involvement of lesion- or infarction-induced diffusible or humoral factors such as neurotrophic factors. It was already demonstrated that several growth factors such as epidermal growth factors (Reynolds and Weiss, 1992), basic fibroblast growth factor (Kuhn et al., 1997), brain-derived neurotrophic factor (Ahmed et al., 1995), and insulin-like growth factor-1 (Arsenijevic and Weiss, 1998) are important in the proliferation and differentiation of neural stem cells. However, the loss of a synaptic input from the infarcted hemisphere may be also involved in the proliferation of neural stem/progenitor cells in the contralateral SGZ. In contrast to recently reported