

成果発表会開催

- 1) 松山知弘、田口明彦、芳川浩男、相馬俊裕、中山博文、「どうすれば脳・血管は若返るか」、平成17年度研究成果等普及啓発事業発表会（長寿科学総合研究）、平成17年11月19日、兵庫医科大学平成記念会館、西宮

学術講演

- 1) 松山知弘（2005）脳梗塞モデルにおけるシロスタゾール急性期投与の治療効果. Fighting Vascular Events in Osaka-4, 9.10, 大阪.
- 2) 松山知弘（2004）血管新生と神経再生, 金沢大学21世紀COE公開シンポジウム「発達・学習・記憶と障害の革新的脳科学の創生」, 11.25, 金沢
- 3) 松山知弘（2005）脳梗塞に対する血管再生療法, 第3回福井大学21世紀COEプログラムワークショップ「神経科学における多様なイメージング技術の応用と統合」, 3.14, 福井
- 4) 田口明彦（2004）脳血管障害におけるCD34陽性細胞測定の意義, 第1回脳血管障害の病態・治療研究会—急性期から慢性期まで—, 11.9, 豊中
- 5) 松山知弘（2005）脳卒中の治療戦略. 西宮市医師会総合臨床研究会, 2.9, 西宮
- 6) 松山知弘, 田口明彦, 芳川浩男（2004）脳梗塞に対する血管再生療法. 第三回大阪神経治療研究会, 4.15, 大阪

知的財産権の出願・登録状況

- 1) 松山知弘、田口明彦、芳川浩男（2004）「脳梗塞疾患モデルマウス」、特願2004-108500（特開2005-287430）、科学技術振興機構（JST）
- 2) 松山知弘、田口明彦、芳川浩男（2005）「神経幹細胞の調整法」、特願2005-35032（国際出願PCT/JP2006/302350）、財団法人新産業創造研究機構、財団法人ヒューマンサイエンス振興財団

研究成果の刊行に関する一覧表

書籍

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Taguchi, A., Matsuya ma, T., et al.	Circulating CD34-positive cells provide an index of cerebrovascular function.		Circulation			2004	109:297 2-2975
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Circulating CD34-Positive Cells Provide an Index of Cerebrovascular Function

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Background—Increasing evidence points to a role for circulating endothelial progenitor cells, including populations of CD34- and CD133-positive cells present in peripheral blood, in maintenance of the vasculature and neovascularization. Immature populations, including CD34-positive cells, have been shown to contribute to vascular homeostasis, not only as a pool of endothelial progenitor cells but also as a source of growth/angiogenesis factors at ischemic loci. We hypothesized that diminished numbers of circulating immature cells might impair such physiological and reparative processes, potentially contributing to cerebrovascular dysfunction.

Methods and Results—The level of circulating immature cells, CD34-, CD133-, CD117-, and CD135-positive cells, in patients with a history of atherothrombotic cerebral ischemic events was analyzed to assess possible correlations with the degree of carotid atherosclerosis and number of cerebral infarctions. There was a strong inverse correlation between numbers of circulating CD34- and CD133-positive cells and cerebral infarction. In contrast, there was no correlation between the degree of atherosclerosis and populations of circulating immature cells. Analysis of patients with cerebral artery occlusion revealed a significant positive correlation between circulating CD34- and CD133-positive cells and regional blood flow in areas of chronic hypoperfusion.

Conclusions—These results suggest a possible contribution of circulating CD34- and CD133-positive cells in maintenance of the cerebral circulation in settings of ischemic stress. Our data demonstrate the utility of a simple and precise method to quantify circulating CD34-positive cells, the latter providing a marker of cerebrovascular function. (*Circulation*. 2004;109:2972-2975.)

Key Words: cerebral infarction ■ cerebral ischemia ■ antigens, CD34 ■ stem cells

Although it had traditionally been assumed that replacement of damaged endothelium resulted only from outgrowth of preexisting vasculature, recent studies have identified endothelial progenitor cells (EPCs) that appear to contribute to vascular homeostasis and repair.¹ Clinical trials to assess the therapeutic potential of bone marrow-derived mononuclear cells, a rich source of immature cells including EPCs, in hind-limb^{2,3} and cardiac ischemia⁴ have been initiated and have, thus far, provided promising results. Furthermore, immature cells, including CD34-positive (CD34⁺) cells, have been shown to contribute to maintenance of the vasculature, not only as a pool of EPCs but also as the source of growth/angiogenesis factors.⁵ Bone marrow-derived immature cells have also been shown to participate in neovascularization of ischemic brain after experimental stroke.⁶ On the basis of these results, we hypothesized that levels of circulating immature cells might be proportional to

the resilience of the cerebral circulation to ischemic stress; ie, lower numbers of circulating immature cells might be associated with cerebral ischemia and infarction.

Methods

The institutional review board of the National Cardiovascular Center approved this study. All subjects provided informed consent. Circulating CD34⁺ cells in 50 μ L of peripheral blood were quantified according to the manufacturer's protocol (ProCOUNT, Becton Dickinson Biosciences). To minimize intersample variation for measurements of CD34⁺ cells, several methods were used: A nucleic acid dye was added as a threshold reagent; a no-wash technique was performed to eliminate cell loss, and reverse pipetting was used; an internal reference particle was added for determination of absolute cell numbers; and an isotype control, matched for the concentration of anti-CD34 antibody and fluorochrome-to-protein ratio, was included. All measurements were performed in triplicate (Figure 1A, control; Figure 1B, CD34). To quantify other stem cell populations (besides CD34⁺ cells), immature mononuclear cells were enriched

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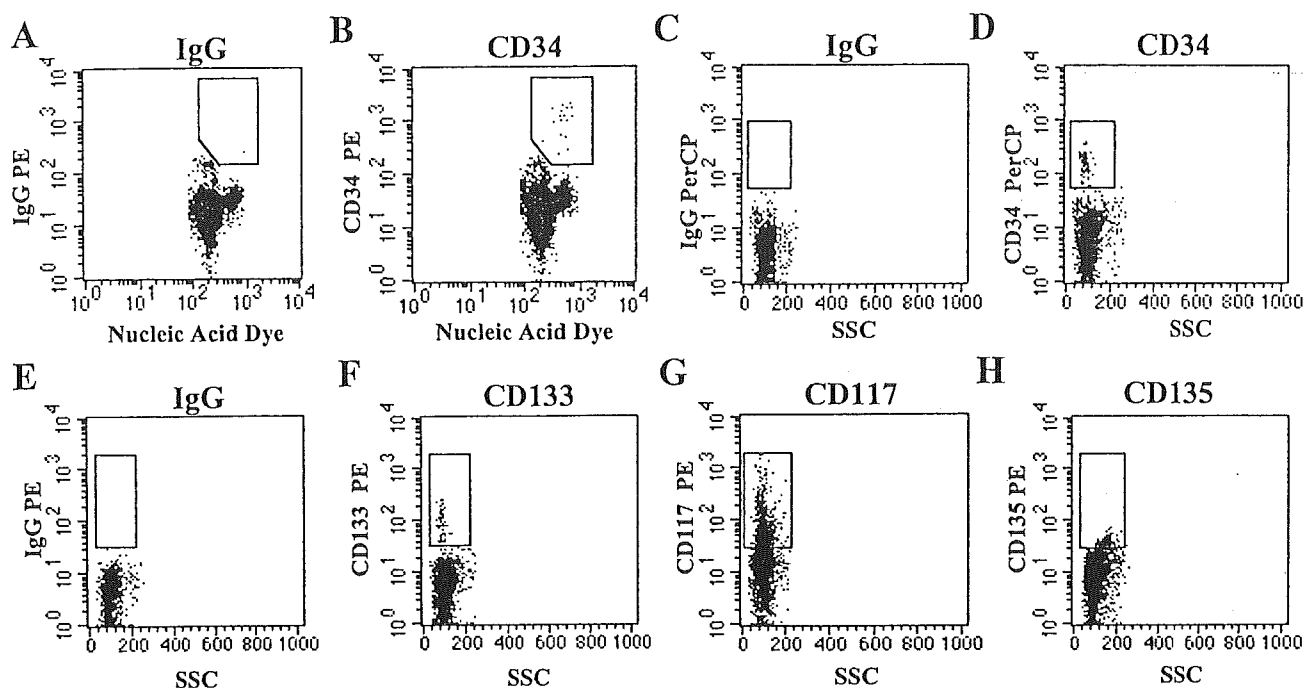


Figure 1. Quantification of circulating immature cells in patients with stroke. Nucleic acid dye versus CD34 PE dot plot was gated on lymphocytes and CD45 dim leukocytes. A, Results with an isotype-matched control antibody. B, Results with anti-CD34 antibody. Enriched immature mononuclear cells were double stained with PerCP-conjugated CD34 antibody (D) and PE-conjugated CD133 (F), CD117 (G), or CD135 (H) antibody. The number of cells in a region including brightly stained cells was counted. C and E, Staining with isotype nonimmune control antibody.

from 2 mL of peripheral blood by antibody-mediated depletion of mature cells according to the manufacturer's protocol (StemCell Technologies) using depletion cocktail, including antibodies to CD2, CD3, CD14, CD16, CD19, CD24, CD56, and CD66b. Enriched immature mononuclear cells were double-stained with peridinin chlorophyll protein (PerCP)-conjugated CD34 antibody (Figure 1D) and phycoerythrin (PE)-conjugated CD133 (Figure 1F), CD117 (Figure 1G), or CD135 (Figure 1H) antibody. The number of cells in a region including brightly stained cells was counted, and immature cells were quantified using CD34⁺ cells as an internal control. The cumulative intra-assay coefficient of variation was 14%, 13%, 14%, and 15%, with CD34⁺, CD133⁺, CD117⁺, and CD135⁺ cell measurements, respectively, from 5 stroke patients.

Atherosclerosis in the common and internal carotid arteries was analyzed by ultrasonography to determine plaque score as described previously.⁷ Cerebral infarcts (diameter >5 mm) were counted independently by a neurologist blinded to other parameters under study (number of circulating CD34⁺, etc) using T1-weighted, T2-weighted, and fluid-attenuated inversion-recovery MRI obtained with a 1.5-Tesla MRI scanner. The diagnosis of hypoperfusion was made angiographically. Regional cerebral blood flow (CBF), cerebral blood volume, oxygen extraction fraction (OEF), and cerebral metabolic rate of oxygen (CMRO₂) were quantified by conventional steady-state ¹⁵O PET using a PET scanner (Shimadzu) as described.⁸ Cerebrovascular function was evaluated in patients with chronic hypoperfusion caused by major cerebral artery (carotid artery or M1 portion of the middle cerebral artery) occlusion or severe stenoses (>90%) without a major stroke. Twelve patients with 15 major arterial occlusions or stenoses had PET examinations.

To investigate the mobilization of immature cells after acute cerebral infarction, peripheral blood was quantified at 6 hours and 3, 7, 14, and 30 days after the onset of stroke. The episodes of acute cerebral infarction were confirmed by the diffusion image of brain MRI. Age-matched volunteers who had no history of cerebrovascular diseases and no neuronal deficiency were enrolled as controls (mean age, 67±4 years). Test-retest intraclass correlations were 0.88, 0.75, 0.86, and 0.86 for CD34, CD133, CD117, and CD135,

respectively, obtained from 5 volunteers tested twice with an interval of at least 10 days between samples.

Univariate correlations were performed using Pearson's correlation coefficient and Spearman's correlation coefficient. Statistical comparisons among groups were determined using analysis of variance. Individual comparisons were performed using Student's *t* test. In all experiments, mean±SE is reported.

Results

First, we investigated mobilization of immature cells after acute cerebral infarction (n=5), focusing on CD34⁺ cells. The level of CD34⁺ cells gradually increased to day 7 and remained significantly above the prestroke baseline on days 7 and 14, returning to baseline levels by day 30 (Figure 2A). On the basis of these data, we enrolled 25 patients with a history of atherothrombotic cerebral ischemic events, excluding those who had suffered cerebrovascular or cardiovascular acute ischemic episodes in the 30 days before study, as well as premenopausal females. In this group (>30 days after stroke), no correlation was observed between the interval after stroke and the level of circulating CD34⁺ cells ($r=0.009$, $P=0.97$). Characteristics of this group included mean age of 68±2 years, 20 men and 5 women, 23 patients receiving antiplatelet therapy, 11 patients receiving antihypertensive therapy, 6 patients receiving therapy for hyperlipidemia, 5 patients receiving therapy for diabetes mellitus (DM), and 16 patients with a current or past history of smoking.

Several factors were found to influence the number of circulating CD34⁺ cells. Statistical analysis revealed a significant decrease in circulating CD34⁺ cells in patients with DM ($0.5±0.1$; non-DM, $1.2±0.1$ cells/ μ L; $P=0.01$). In contrast,

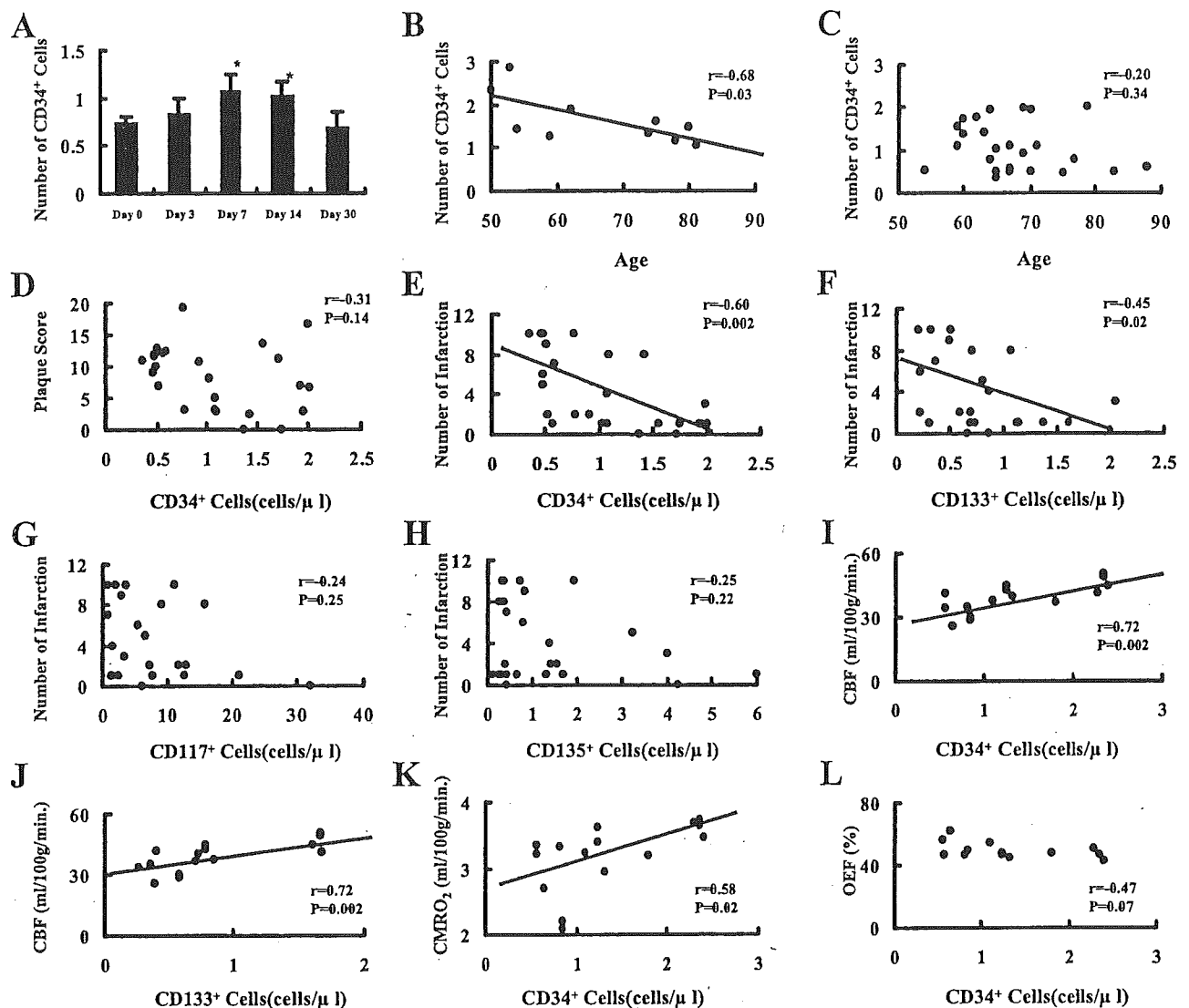


Figure 2. Levels of circulating CD34⁺ cells and stroke. Circulating CD34⁺ cells increased after the onset of stroke and peaked on day 7. A significant increase in circulating CD34⁺ cells was observed on days 7 and 14 (A). A decrease of circulating CD34⁺ cell was observed with aging in the control group (B), but no such correlation was observed in the stroke patient group (C). No correlation was observed between the number of circulating CD34⁺ cells and the degree of arteriosclerosis in major cerebral arteries (D). However, there was a correlation between cerebral infarctions and circulating CD34⁺ (E) and CD133⁺ cells (F). In contrast, there was no correlation between cerebral infarction and CD117⁺ (G) or CD135⁺ cells (H). Correlation between circulating CD34⁺ (I) and CD133⁺ (J) cells and CBF in areas of chronic hypoperfusion was observed. Lower levels of circulating CD34⁺ cells were correlated with a decrease in CMRO₂ (K) but not with a change in OEF (L). **P*<0.05 compared with day 0 (based on 2-way ANOVA).

no change was observed in patients with hypertension (*P*=0.61), with hyperlipidemia (*P*=0.81), with smoking (*P*=0.64), or based on gender (*P*=0.36). In addition, treatment with HMG-CoA reductase inhibitors (*P*=0.81), compared with patients without hyperlipidemia, did not impact the number of CD34⁺ cells. In the control patient group, a decrease of circulating CD34⁺ cells was observed with aging (Figure 2B), although this was not observed in the patient group (Figure 2C). Comparing baseline levels of circulating CD34⁺ cells, there was a significant decrease in the patient group compared with age-matched controls (stroke, 1.1 ± 0.1 ; control, 1.6 ± 0.2 cells/ μ L; *P*=0.02).

We sought a possible correlation between circulating immature cells and the degree of arteriosclerosis of the common and

internal carotid arteries in the patients with atherothrombotic cerebral ischemic events. However, there was no significant correlation between arteriosclerosis and circulating CD34⁺ (Figure 2D). This result was not surprising, because multiple risk factors and cell types contribute to progression of vascular lesions in major arteries. In contrast, because disruption of vascular homeostasis and repair are associated with cerebral infarction, we reasoned that a history of cerebral infarction might correlate with circulating immature cells. A strong correlation was observed between the number of infarcts and the absolute number of circulating CD34⁺ cells (Figure 2E) and CD133⁺ cells (Figure 2F). However, no significant correlation with regard to cerebral infarcts was observed with circulating CD117⁺ cells (Figure 2G) and CD135⁺ cells (Figure 2H).

In view of the critical role of endothelium in maintaining CBF, we evaluated cerebrovascular function in patients with chronic hypoperfusion. Direct correlations were observed between CBF (in the chronically hypoperfused area) and circulating CD34⁺ cells (Figure 2I) and CD133⁺ cells (Figure 2J). In addition, lower numbers of circulating CD34⁺ cells (Figure 2K) correlated with diminished CMRO₂, although there was no significant increase in the OEF (Figure 2L). These observations suggest a contribution of CD34⁺ cells in homeostasis and repair of the cerebral circulation and maintenance of brain metabolism. No correlation was observed with the above parameters of vascular function and circulating CD117⁺ and CD135⁺ cells. Measurement of angiogenic growth factors in patient plasma, vascular endothelial growth factor, basic fibroblast growth factor, hemopoietic growth factor, and insulin-like growth factor-1 also demonstrated no correlation with indices of cerebrovascular function or the number of CD34⁺ cells (not shown).

Discussion

We have found that circulating immature cell populations, especially CD34⁺ and CD133⁺ cells, are associated with maintenance and repair of the cerebral vasculature. In our study, we used a simple and precise method to count the absolute number of circulating CD34⁺ cells in a small sample of peripheral blood. Our results indicate that the level of CD34⁺ cells serves as an index/marker for cerebrovascular function. Analysis of CD133⁺, CD117⁺, and CD135⁺ cells, which identify other populations of immature cells, demonstrated that only CD133⁺ cells correlated with cerebrovascular function in a manner paralleling CD34⁺ cells.

Patients with diabetes displayed a significant reduction in the number of circulating CD34⁺ cells. In view of the microvascular dysfunction that is characteristic of diabetes, this may not be surprising. Similarly, decreased circulating CD34⁺ cells with increasing age in healthy individuals may be associated with limited vascular renewal in older individuals. It was also of interest to note no change between levels of CD34⁺ cells in patients taking HMG-CoA reductase inhibitors. The latter results might reflect the positive effect

of such drugs countering the negative effect of hyperlipidemia on circulating CD34⁺ cells. Such conclusions, of course, are at best tentative, because in this first report we have identified associations rather than proved a cause-effect relationship.

These observations suggest that diminished numbers of CD34⁺ and CD133⁺ cells impact maintenance and repair of cerebral vasculature. Precise measurement of circulating CD34⁺ cells provides a marker for cerebrovascular function in the setting of ischemic stress.

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Administration of CD34⁺ cells after stroke enhances neurogenesis via angiogenesis in a mouse model

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Thrombo-occlusive cerebrovascular disease resulting in stroke and permanent neuronal loss is an important cause of morbidity and mortality. Because of the unique properties of cerebral vasculature and the limited reparative capability of neuronal tissue, it has been difficult to devise effective neuroprotective therapies in cerebral ischemia. Our results demonstrate that systemic administration of human cord blood-derived CD34⁺ cells to immunocompromised mice subjected to stroke 48 hours earlier induces neovascularization in the ischemic zone and provides a favorable environment for neuronal regeneration. Endogenous neurogenesis, suppressed by an antiangiogenic agent, is accelerated as a result of enhanced migration of neuronal progenitor cells to the damaged area, followed by their maturation and functional recovery. Our data suggest an essential role for CD34⁺ cells in promoting directly or indirectly an environment conducive to neovascularization of ischemic brain so that neuronal regeneration can proceed.

Introduction

Thrombo-occlusive atherosclerotic cardiovascular disease is a major cause of death and disability in developed countries. In the acute phase, therapeutic maneuvers include fibrinolytic therapy to restore blood flow to the ischemic site. In the longer term, formation of new blood vessels is necessary to fully supply tissue metabolic and functional requirements. Although it had been assumed that postnatal development of neovessels resulted only from outgrowth of pre-existing vasculature, it has become evident that circulating endothelial progenitor cells (EPCs), contained in a CD34⁺ cell population enriched in cord blood, have the capacity to participate in neovascularization of ischemic tissues (1, 2). Thus, a new strategy proposed for enhancing recovery due to ischemic stress is administration of EPCs to stimulate formation of neovasculature. In this context, recent reports have demonstrated that infusion of EPCs results in their incorporation into neovasculature at the ischemic site and limitation of tissue damage in animal models (3). Furthermore, human CD34⁺ cells were shown to secrete numerous angiogenic factors, including VEGF, HGF, and IGF-1 (4). On the basis of these observations, clinical trials of cell transplantation in hindlimb (5, 6) and cardiac ischemia (7) have been initiated with promising results.

Stroke is another setting of occlusive thromboatherosclerotic disease in which acceleration of angiogenesis might be expected to enhance the outcome. Despite the improvement of poststroke neurological outcome by administration of human cord blood cells (8) or bone marrow-derived cells (9) (both potentially a rich sources of stem cells including CD34⁺ cells) in rodent models, few of the administered cells could be demonstrated in brain parenchyma expressing neuronal markers, raising a question as to the underlying mechanism. The results of our study demonstrate that systemic administration of human CD34⁺ cells to immunocompromised mice subjected to stroke 48 hours earlier accelerates neovascularization of the ischemic zone. Such a rich vascular environment, along with generation of other nurturing neuronal mediators by CD34⁺ cells, such as VEGF, FGF2, and IGF-1 (10–12), enhances subsequent neuronal regeneration; endogenous neurogenesis is accelerated as neuronal progenitors migrate to the damaged area, followed by their maturation and survival when CD34⁺ cells have stimulated the formation of increased vascular channels. In contrast, in the presence of an antiangiogenic agent, the beneficial effect of CD34⁺ cells was lost. Our results provide the first direct link between vasculogenesis and neurogenesis in the repair of ischemic brain lesions.

Results

Induction of stroke and proliferation of endothelial cells in situ. A reproducible model of stroke in the middle cerebral artery (MCA) cortex, sparing the striatum, was developed in SCID mice by permanent ligation of the M1 distal portion of the left MCA. Subsequent infusion of carbon black showed strongly decreased staining in the affected area. Nonviability of affected tissue was confirmed by 2,3,5-triphenyltetrazolium (TTC) staining. Values of cortical width index (see Methods section) were highly reproducible (–0.34–0.36)

Nonstandard abbreviations used: anterior cerebral artery (ACA); cerebral blood flow (CBF); chloromethylbenzamide (CM-Dil); doublecortin (DCX); endothelial progenitor cell (EPC); erythropoietin (EPO); fetal liver kinase-1 (Flk-1); high-power field (HPF); middle cerebral artery (MCA); neuronal progenitor cell (NPC); neuron-specific nuclear protein (NeuN); phycoerythrin (PE); polysialylated neuronal cell adhesion molecule (PSA-NCAM); subventricular zone (SVZ); 2,3,5-triphenyltetrazolium (TTC).

Conflict of interest: The authors have declared that no conflict of interest exists.

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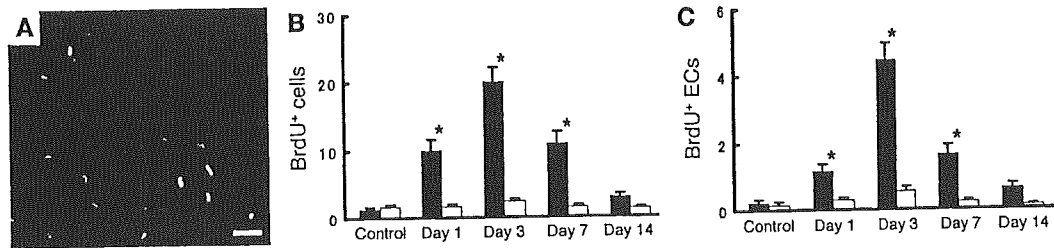


Figure 1

Endothelial proliferation in situ after stroke. On days 1, 3, 7, and 14 after stroke, the number of proliferating cells (BrdU⁺) and proliferating endothelial cells (co-staining for BrdU and CD31) was determined in the left cortical area of 1–1.5 mm distal from the midline. (A) Immunohistological analysis of proliferating cells labeled with BrdU (green), anti-mouse CD31 IgG (red), and both (yellow). The number of cells visualized with BrdU (B) and the subpopulation BrdU⁺ cells also displaying mouse CD31 (i.e., double positives) (C) are shown. Ten HPFs were evaluated for each animal ($n = 6$ per group) by two investigators blinded to the experimental protocol. Note in C, cells displaying mouse CD31 are termed endothelial cells (ECs). Black bars, ipsilateral; white bars, contralateral. * $P < 0.05$ versus control. Scale bar: 30 μm .

over the 12-week experimental period. Survival in this stroke model was greater than 95%, and no seizures were observed.

To estimate the optimal time to administer human CD34⁺ cells, proliferation of endothelial cells in vasculature of the penumbral region (at the leading edge of viable tissue) was assessed by in vivo BrdU labeling. Sections were visualized with antibody to BrdU and mouse-specific antibody to CD31 by confocal microscopy. Cellular profiles co-staining for both markers were considered proliferating endothelial cells (Figure 1A). On days 1 and 3 after stroke, a subpopulation of BrdU⁺ cells also stained with mouse CD31, indicating an endothelial origin of this signal (Figure 1, B and C). By day 7, although endothelial proliferation continued, it had begun to decrease. In contrast, BrdU-labeled cells were present in a constant, small amount on the contralateral (nonstroke) side (Figure 1, B and C). These data indicated that administration of CD34⁺ cells on day 2 after stroke would buttress the endogenous proliferative component of the vascular response to cerebral ischemia.

Administration of CD34⁺ cells after stroke. Human CD34⁺ cells (95% pure CD34⁺ cells) isolated from human cord blood or control cells (CD34⁻ cells with <0.2% CD34⁺ cells, also from human cord blood) were administered intravenously via tail vein 48 hours after stroke. Analysis of cell surface markers revealed that $1.5\% \pm 0.1\%$ and $0.9\% \pm 0.1\%$ of the CD34⁺ cell population expressed the endothelial lineage markers fetal liver kinase-1 (Flk-1) (1) and P1H12 (13), respectively ($n = 4$). The effect of CD34⁺ cells was evident within 24 hours of their transplantation. Labeling vasculature by infusion of carbon black ink demonstrated neovasculature at the border of the MCA and anterior cerebral artery (ACA) cortex (staining with TTC demarcates viable and nonviable tissue) in animals treated with CD34⁺ cells (Figure 2, A and B), compared with those receiving CD34⁻ cells (Figure 2C) or PBS alone (Figure 2D). Determination of the angiographic score confirmed the impression of increased neovasculature in animals transplanted with CD34⁺ cells, compared with other groups (Figure 2E). To evaluate vascular activation in affected cerebral vessels, we used mouse-specific antibody to CD13, an antigen expressed by endothelial cells in angiogenic, but not quiescent, vasculature (14). Visualization of mouse CD13 in brain sections 24 hours after cell transplantation showed that cells bearing this activated endothelial marker were most evident in sections from mice treated with CD34⁺ (Figure 2F), compared with those receiving CD34⁻ cells (Figure 2G) or PBS (Figure 2H). Increased density of vasculature in the ischemic territory of animals treated with CD34⁺ cells translated to significantly enhanced cerebral blood flow (CBF) (Figure 2I).

To analyze the effect of subpopulations within the general CD34⁺ cell population, we compared the effect of poststroke transplantation of the same number of CD34⁺ cells (containing Flk-1⁻ and Flk-1⁺ cells) with CD34⁺/Flk-1⁻ cells on vascular activation and neovasculature. Brain tissue was examined 7 days after cell transplantation, because EPCs are known to incorporate into capillary walls at ischemic sites by this time point after the ischemic episode (2). FACS analysis confirmed that the CD34⁺/Flk-1⁻ population contained less than 0.1% Flk-1⁺ cells ($n = 4$). On the basis of CD13 staining (using the same mouse-specific antibody mentioned earlier), there was similar activation of endogenous endothelium after transplantation of either CD34⁺ cells (including both Flk-1⁻ and Flk-1⁺ subpopulations) and CD34⁺/Flk-1⁻ cells (not shown). Although neovasculature was observed at the border of the MCA and ACA cortex in animals treated with CD34⁺ cells (Figure 2J) and CD34⁺/Flk-1⁻ cells (Figure 2K), mice treated with CD34⁺ cells displayed increased neovasculature based on angiographic score (scores of 22 ± 3 and 13 ± 2 , for CD34⁺ and CD34⁺/Flk-1⁻ cells, respectively; $P < 0.05$, $n = 6$).

Transplantation of CD34⁺ cells and poststroke functional recovery. Stroke causes motor deficits and behavioral abnormalities (15). Dysfunction of the cortex is closely linked to disinhibition of behavior (16). Compared with sham-operated controls, mice that received CD34⁻ cells or PBS displayed significant behavioral abnormalities on day 90 after cell transplantation ($n = 12$, for each group). Rearing counts under lighted conditions were 8.4 ± 0.8 (PBS), 8.7 ± 0.5 (CD34⁻ cells), 4.2 ± 0.5 (CD34⁺ cells), and 3.0 ± 0.5 (sham-operated controls) for each of the groups. Counts of locomotion were 5.1 ± 0.4 (PBS), 5.0 ± 0.4 (CD34⁻ cells), 3.7 ± 0.4 (CD34⁺ cells), and 3.3 ± 0.4 (sham). ANOVA revealed hyperactivity with respect to both rearing ($P < 0.01$) and locomotion ($P < 0.01$) in the CD34⁻ cell- and PBS-treated groups, compared with sham-operated controls. In contrast, mice treated with CD34⁺ cells showed no significant hyperactivity compared with sham-operated controls ($P > 0.05$), and displayed significant improvement in both behavioral tests compared with animals that received PBS or CD34⁻ cells ($P < 0.05$). Mice treated with CD34⁻ cells or PBS after stroke showed loss of this “dark” response, with respect to rearing ($P > 0.05$) and locomotion ($P > 0.05$). In contrast, animals treated with CD34⁺ cells displayed the expected increase in rearing and locomotion in the darkness ($P < 0.01$).

Using another behavioral paradigm, excessive startle consequent to auditory stimulation was observed in poststroke animals treated with CD34⁻ cells and PBS. Startle amplitudes were 0.9 ± 0.1 volts (PBS), 0.8 ± 0.1 (CD34⁻ cells), 0.5 ± 0.1 (CD34⁺

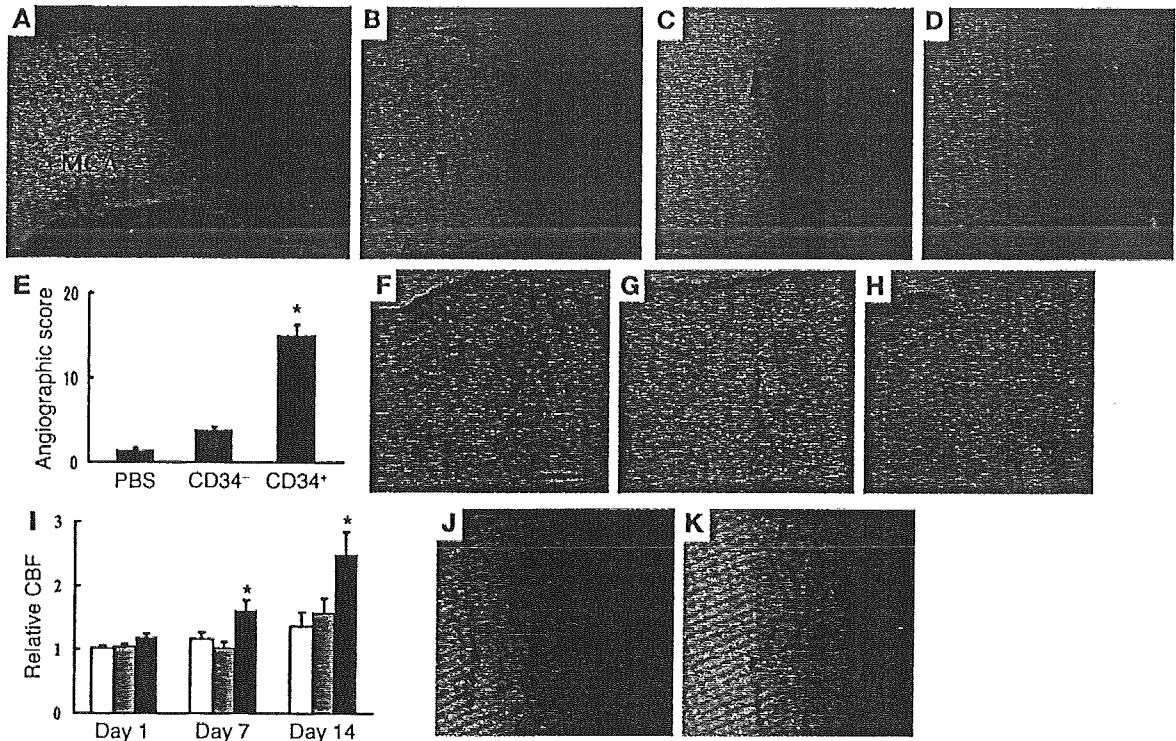


Figure 2 Transplantation of CD34⁺ cells after stroke accelerates neovascularization. (A–D) Mice subjected to stroke received CD34⁺ cells (A and B), CD34⁻ cells (C), or PBS alone (D) on day 2. Animals were infused with carbon black ink and killed at 24 hours after cell transplantations. Sections were stained with TTC. Neovascularization was noted at the border zone between the ACA and MCA areas (arrowheads show microvessels), especially in animals treated with CD34⁺ cells compared with those receiving CD34⁻ cells or PBS alone. (E) An angiographic score for each experimental condition based on analysis of 6 mice per group. (F–H) Activated endothelial cells were observed with antibody specific for mouse CD13 in the ACA area. F: CD34⁺ cells; G: CD34⁻ cells; H: PBS. (I) CBF was measured in the MCA area just outside of the penumbra, and values in animals treated with CD34⁺ cells (black bars), CD34⁻ cells (gray bars), or PBS (white bars) were compared with values before cell transplantation at times corresponding to days 1, 7, and 14 after cell transplantation (*n* = 6 per group). Data shown are relative CBF versus day the measurement was performed. (J and K) Labeling vasculature by infusion of carbon black ink demonstrated neovasculture at the border of the MCA and ACA cortex in animals treated with CD34⁺ cells (J) and CD34⁺/Flk-1⁻ cells (K) on day 7 after cell transplantation. Scale bars: 0.5 mm (A) and 0.1 mm (B, F, and J). **P* < 0.05 versus PBS.

cells), and 0.4 ± 0.1 (sham) on day 90. ANOVA revealed hyperactivity in the CD34⁻ cell- and PBS-treated groups, compared with sham-operated controls (*P* < 0.05). In contrast, mice treated with CD34⁺ cells showed no significant hyperactivity compared with sham-operated controls (*P* > 0.05). Shortening of the latency period with fear conditioning was also observed in poststroke animals treated with CD34⁻ cells and PBS compared with sham-operated controls (*P* < 0.05). In contrast, mice treated with CD34⁺ cells showed no significant hyperactivity (*P* > 0.05). Similar behavioral abnormalities were also observed on day 35 after cell transplantation. Since ischemic injury in our stroke model was limited to the cortex (hippocampus and striatum were intact), it was not surprising that spatial learning (water maze testing) and passive avoidance remained unchanged and comparable in all groups when assessed 5 weeks after stroke (data not shown). Mice showed rapid recovery from focal motor deficits, and by day 16 after stroke no motor deficits were detected using a modified three-point scale (17) (data not shown).

Effect of poststroke CD34⁺ cell transplantation on endogenous neurogenesis and cortical expansion. These results suggested that cortical function and neoangiogenesis, potentially supporting integrity of the MCA cortex, might be better maintained in animals treated with CD34⁺

cells after stroke. Such maintenance of cortical integrity might reflect CD34⁺ cell-mediated enhancement of the survival of ischemic neurons or acceleration of endogenous neurogenesis. Representative brains from poststroke animals in each group (treated with PBS alone, CD34⁻ cells, or CD34⁺ cells) are shown in Figure 3, A–F. There was a prominent increase in cortical width index in animals receiving CD34⁺ cells at 14, 35, and 90 days after cell transplantation (Figure 3G). Thus, cortical expansion in animals treated with CD34⁺ cells was most likely to reflect the result of ongoing neurogenesis, rather than maintenance of the integrity of ischemic neurons from the time of induction of stroke.

Cell trace analysis with a fluorescent dye revealed that labeled transplanted CD34⁺ cells were not co-stained with antibody to a neuronal (neuron-specific nuclear protein; NeuN) or an astrocyte (glial fibrillary acidic protein) marker (not shown). However, activation of endogenous neurogenesis was observed after stroke, and this was accentuated in mice receiving CD34⁺ cells. On day 7 after administration of CD34⁺ cells, cells expressing the polysialylated neuronal cell adhesion molecule (PSA-NCAM), a marker of migrating neuronal progenitor cells (NPCs) (18), were observed in the subventricular zone (SVZ), and migration of these NPCs toward the ischemic zone was visualized on day 14 using markers

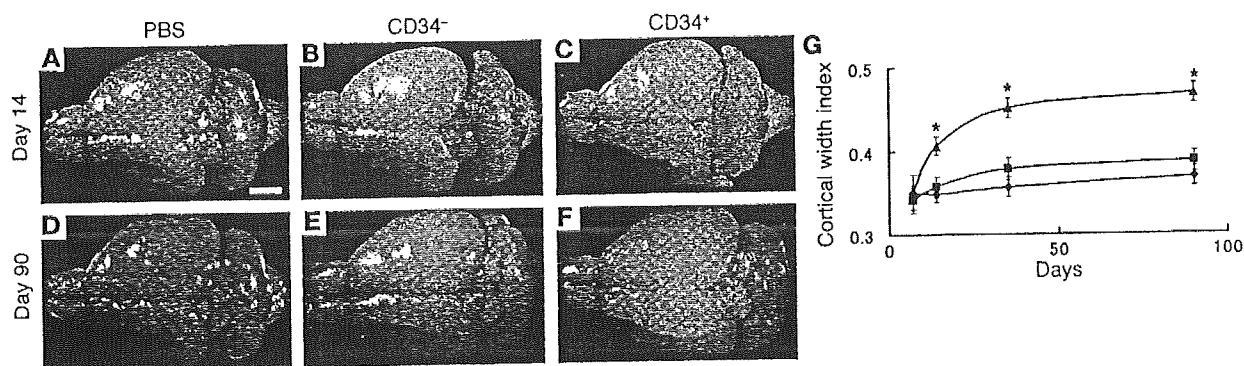


Figure 3 CD34⁺ cell transplantation induces cortical expansion after stroke. (A–F) On day 14 (A–C), and day 90 (D–F) after cell transplantation, the brains of mice were evaluated grossly. Compared with poststroke mice treated with PBS (A and D) or infused with CD34⁻ cells (B and E), animals transplanted with CD34⁺ cells (C and F) showed an increase in area occupied by the left cortex. (G) Cortical regeneration was induced by CD34⁺ cells transplantation: triangles, CD34⁺ cells; squares, CD34⁻ cells; diamonds, PBS. In each case, there were 6 animals per group. Scale bar: 2 mm (A). **P* < 0.05 versus PBS.

for PSA-NCAM (Figure 4, A and B, corresponding to lower and higher magnifications, respectively), as well as the neuronal stem cell marker, Musashi-1 (Figure 4C) (19). Expression of doublecortin (DCX), a marker of immature, or early, neurons (20), was principally restricted to NPCs close to the SVZ (Figure 4D). In contrast, expression of a neuronal cell marker, NeuN, was observed after cell migration from the SVZ and appeared as a signal of lower intensity compared with nearby mature neurons (Figure 4E). The situation was quite different on the contralateral side in poststroke animals treated with CD34⁺ cells; no such movement of precursors (PSA-NCAM⁺ cells) was observed (Figure 4F). Although migrating NPCs were also seen in poststroke animals treated with PBS (Figure 4G) and CD34⁻ cells, this finding was considerably magnified in mice receiving CD34⁺ cells (Figure 4H).

To investigate mechanisms underlying cortical expansion, immunohistological analysis was conducted to visualize PSA-NCAM, NeuN, and MAP-2. On day 14 after transplantation of CD34⁻ cells after stroke, mature cortical neurons displaying neuronal markers NeuN (Figure 5A) and MAP-2 (Figure 5B) were observed up to the periphery of the ischemic area, whereas only a thin layer of migrating PSA-NCAM⁺ NPCs was observed at the ischemic edge (Figure 5C). In contrast, transplantation of CD34⁺ cells after stroke expanded cortical areas displaying a low density of NeuN⁺ (Figure 5D) and MAP-2⁺ cells (Figure 5E) beyond the boundary demarcating mature neurons. Migration of NPCs into this expanded area was also observed by PSA-NCAM staining (Figure 5F).

Next, we investigated the fate of NPCs migrating from the SVZ to the ischemic area. Poststroke animals treated with CD34⁻ cells displayed many cells with TUNEL⁺ nuclei around the lower part of cortical ischemic edge (Figure 5G). In contrast, there were a minimal number of TUNEL⁺ nuclei observed in the same region in animals subject to transplantation of CD34⁺ cells after stroke (Figure

5, H and I). Thus, animals treated with CD34⁺ cells after stroke display migration and survival of NPCs, which eventually contribute to a population of more mature neurons.

To confirm this evidence of neurogenesis, animals transplanted with CD34⁺ cells, CD34⁻ cells, or PBS after stroke were infused with

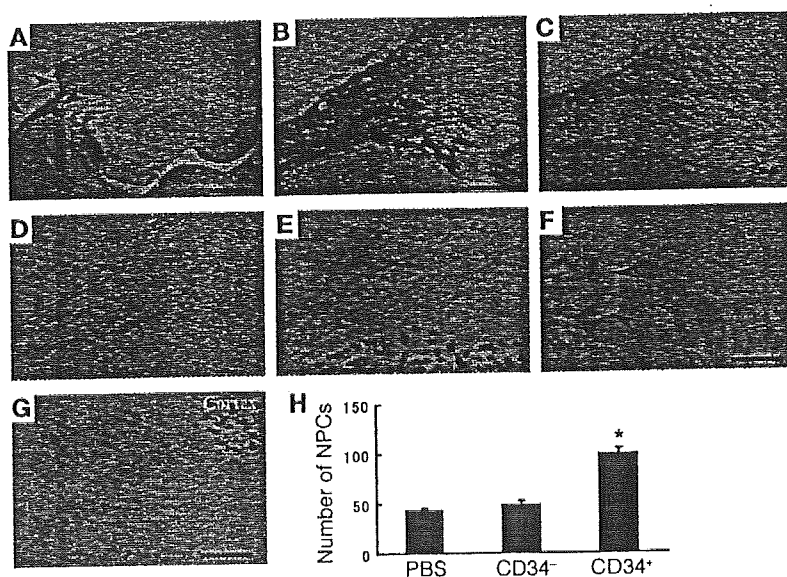


Figure 4 CD34⁺ cell transplantation accelerates neuronal regeneration after stroke. (A and B) On day 14, animals receiving CD34⁺ cells after stroke displayed migration of NPCs toward the ischemic area by PSA-NCAM immunostaining. (C–E) Analysis of serial sections displayed expression of neuronal stem cell markers, Musashi-1 (C) and DCX (D). Note that expression of DCX was limited to the area proximal to the SVZ. PSA-NCAM⁺ NPCs also expressed NeuN (E). Small NeuN⁺ nuclei were observed in PSA-NCAM⁺ NPCs, whereas more intensely staining and larger nuclei represent mature neurons. (F) On day 14 on the contralateral side, PSA-NCAM⁺ NPCs were limited to the SVZ; that is, no migration of NPCs was observed. (G) Migration of NPCs with small NeuN⁺ nuclei toward cortex was observed in poststroke mice treated with PBS on day 14 after cell transplantation. (H) The average number of NPCs in the white matter at the lower left of the left cortex per HPF from 5 animals under each condition. Three sections were evaluated in each animal, and *n* = 5 per group. Arrowheads delineate individual NPCs or demarcate areas rich in NPCs. Scale bars: 1 mm (A), 0.2 mm (B and F), and 0.4 mm (G). **P* < 0.05 versus PBS.

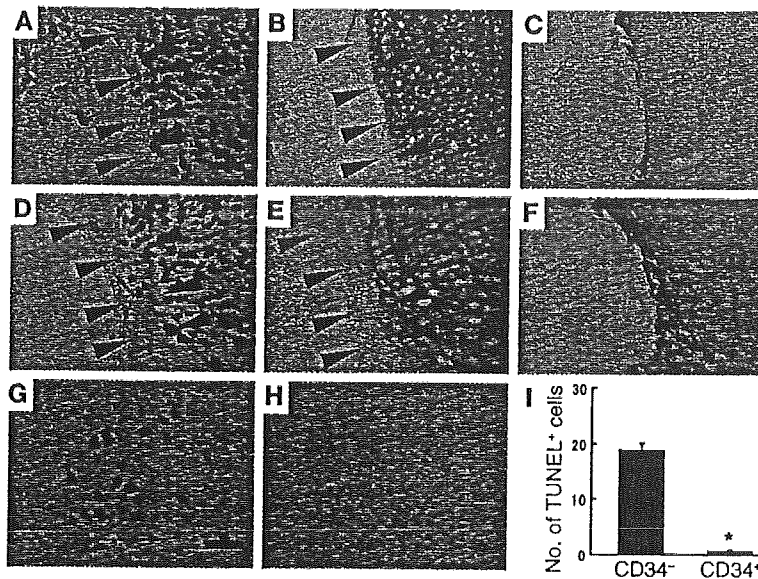


Figure 5

Therapeutic neovascularization, due to CD34⁺ cell transplantation after stroke, enhances neurogenesis. (A–F) On day 14 after CD34⁻ cell transplantation, mature cortical neurons were observed up to the edge of the ischemic region displaying neuronal markers, NeuN (A) and MAP-2 (B), whereas only a thin layer of migrating PSA-NCAM⁺ NPCs was observed at the ischemic edge (C). In contrast, after transplantation of CD34⁺ cells, expanded cortical areas displaying a low density of NeuN⁺ (D) and MAP-2⁺ cells (E) were observed beyond the boundary demarcating mature neurons. Migration of NPCs into this expanded area was also observed by PSA-NCAM staining (F). (G–I) On day 14, TUNEL⁺ cells were visualized around the lower part of the expanded cortical area. Whereas massive cell death was observed in animals receiving CD34⁻ cell transplantation (G), the number of TUNEL⁺ profiles was strongly reduced in mice transplanted with CD34⁺ cells (H). (I) The average number of TUNEL⁺ cells per HPF. Three sections were evaluated in each animal; $n = 5$ per group. Arrowheads indicate the expanded cortical areas displaying a low density of indicated marker. Scale bars: 100 μm (A) and 50 μm (G). * $P < 0.05$ for CD34⁺ versus CD34⁻ cell transplantation.

BrdU every second day for a total of 14 days and then killed. BrdU- and NeuN-bearing cell populations were visualized to determine if a group of neurons might be undergoing cell division. In animals treated with PBS alone (Figure 6A) or CD34⁻ cells (Figure 6B), double staining for BrdU and NeuN displayed little evidence of cells (i.e., nuclei) expressing both markers. Only in animals infused with CD34⁺ cells after stroke did we consistently observe double-labeled cell profiles with BrdU and NeuN to produce a merged image with cells displaying both markers (Figure 6C). Quantification of these results showed a significant increase in double-positive profiles (i.e., cells co-staining with antibody to BrdU and NeuN) in poststroke animals infused with CD34⁺ cells (Figure 6D). Consistent with these data, total neuronal counts (Figure 6E) from sections stained with NeuN in the left cortex 90 days after cell transplantation demonstrated a significant increase in the number of cells visualized in animals transplanted with CD34⁺ cells compared with those receiving CD34⁻ cells or PBS. In addition, vasculature was visualized in the ischemic zone of the forebrain of animals treated with CD34⁺ cells 90 days later using anti-mouse CD31 as a marker. A distinct vascular network was seen in the expanded cortex associated with neurogenesis after stroke (Figure 6F). Such vessels displayed a pattern quite distinct from that observed on the contralateral (nonischemic) side (Figure 6G). In these vessels on the ischemic side, some endothelial cells were

observed to express human CD31 antigen (Figure 6H). On day 90, mature MAP-2⁺ dendrites were observed in the expanded cortex of poststroke animals treated with CD34⁺ cells (data not shown). These data are consistent with the formation of a stable neovasculature to support regeneration of cortical neurons.

Inhibition and acceleration of angiogenesis by antiangiogenic and angiogenic agents. To investigate the hypothesis that neovascularization is essential to support endogenous neuronal regeneration, we used an antiangiogenic agent, Endostatin. The latter is known to inhibit proliferation of endothelial cells (21) and to have a direct inhibitory effect on EPCs (22). Mice were subjected to stroke, CD34⁺ or CD34⁻ cells were administered, and animals were divided into two groups; one group received Endostatin and the other PBS ($n = 5$, for each group). On day 7 after cell transplantation, treatment with Endostatin suppressed endothelial proliferation to $18\% \pm 3\%$ and $22\% \pm 3\%$ in mice transplanted with CD34⁻ cells and CD34⁺ cells, respectively, compared with proliferation observed in PBS-treated controls. Administration of Endostatin also impaired cortical expansion due to transplantation of CD34⁺ cells. On day 14, the cortical width index was 0.33 ± 0.01 and 0.41 ± 0.01 with and without Endostatin, respectively ($P < 0.01$). Animals treated with CD34⁺ cells and Endostatin after stroke displayed a reduction in migrating NPCs; 101 ± 7 cells per high-power field (HPF), for animals treated with CD34⁺ cells + PBS, and 21 ± 2 cells/HPF for animals treated with CD34⁺ cells + Endostatin ($P < 0.01$). Similarly, on day 14, the number of neurons in the poststroke cortex was less in mice treated with CD34⁺ cells + Endostatin ($1,469 \pm 53$), compared with animals treated with CD34⁺ cells + PBS ($2,213 \pm 36$; $P < 0.01$).

To gain further support for our hypothesis, we used the proangiogenic agent erythropoietin (EPO).

EPO, well known for its essential role in regulating proliferation and differentiation of erythroid cells, has recently been found to promote mobilization of EPCs (23) and to have angiogenic potential (24). On day 4 after stroke, a significant increase in circulating CD34⁺ cells was observed with EPO injection ($5,244 \pm 1,267$ and $1,333 \pm 389$ cells/ml in the EPO and PBS groups, respectively; $n = 6$ per group, $P < 0.05$). In addition, mice treated with EPO displayed enhanced neovascularization based on angiographic score (5.4 ± 0.8 and 1.2 ± 0.4 , in EPO and PBS groups, respectively; $n = 5$ per group, $P < 0.05$). On day 28 after stroke, treatment with EPO increased the number of cells co-staining for BrdU and NeuN (3.4 ± 0.3 and 0.4 ± 0.1 /HPF in the EPO and PBS groups, respectively; $n = 5$ per group, $P < 0.05$). It should be noted that the magnitude of the stimulatory effect of EPO treatment on angiogenesis and neurogenesis in these experiments was less than that observed in studies with CD34⁺ cell transplantation, probably because of the greater number of cells transferred in the latter case.

Neurogenesis in 24-week-old mice. To analyze the effect of increased age on accelerated neurogenesis associated with CD34⁺ cell transplantation, 24-week-old SCID mice were used. Compared with poststroke mice treated with CD34⁻ cells (Figure 7A), transplantation of CD34⁺ cells (Figure 7B) showed a significant increase in area occupied by the left cortex on day 35 (Figure 7C). In addi-

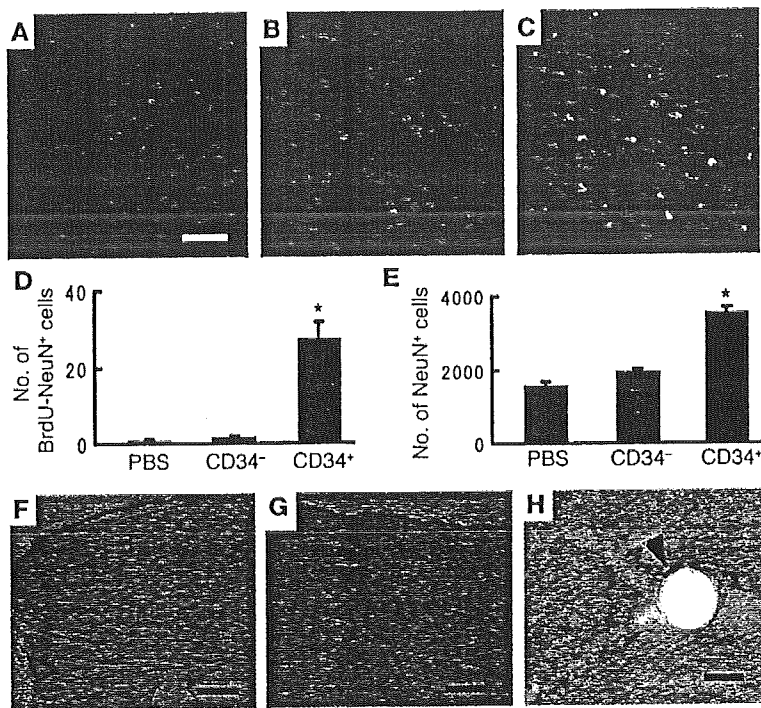


Figure 6

Therapeutic neovascularization supports survival of regenerating neurons. (A–C) Animals treated with PBS alone (A), CD34⁻ cells (B), or CD34⁺ cells (C) were infused with BrdU, killed, and studied immunohistochemically with antibody to BrdU (red), NeuN (green), or both (yellow). (D) The average number of double-positive cells (stained with antibody to BrdU and NeuN) per HPF on day 90 after cell transplantation. Ten fields were evaluated in each animal, and *n* = 6 per group. **P* < 0.05 versus PBS. (E) On day 90 after cell transplantation, brain sections from animals treated with PBS alone, CD34⁻ cells, or CD34⁺ cells were stained with antibody to NeuN, and the number of total neurons in the left cortex was counted. (E) The average number of total NeuN⁺ cells in the left cortex. *n* = 6 per group; **P* < 0.05 versus PBS. (F and G) On day 90 after cell transplantation, mouse CD31 was visualized immunohistologically in forebrain sections from poststroke animals. Newly formed vascular networks were observed in the expanded cortex. The vascular pattern displayed by these neovessels on the ipsilateral (F, ischemic side) was different from that observed on the contralateral side (G). (H) On day 90, human CD31 was visualized immunohistologically. Human endothelial cells were observed in the regenerating cortex of animals treated with CD34⁺ cells after stroke. The arrowhead shows a human CD31⁺ endothelial cell. Scale bars: 50 μm (A), 100 μm (F and G), and 20 μm (H).

tion, evidence of vascular activation, as judged by reactivity with anti-CD13 antibody, was observed in mice treated with CD34⁺ cells (Figure 7D, CD34⁺ cells; Figure 7E, CD34⁺ cells). Migration of NPCs was observed in mice treated with CD34⁻ cells (Figure 7F) and CD34⁺ cells (Figure 7G), but a significant enhancement was observed in animals receiving CD34⁺ cells (Figure 7H). Only a thin layer of migrating PSA-NCAM⁺ NPCs was observed at the ischemic edge in animals treated with CD34⁻ cells (Figure 7I). In contrast, CD34⁺ cell transplantation resulted in a much thicker layer of PSA-NCAM⁺ cells (Figure 7J). Comparing these data in 24-week-old animals with our previous results using 5-week-old mice, the

older animals displayed a similar cortical width index (5 weeks old, 0.45 ± 0.01; 24 weeks old, 0.43 ± 0.02; *P* > 0.05), although older age was associated with a decrease in NPCs migrating into the cortex (5 weeks old, 71 ± 3 cells/HPF; 24 weeks old, 43 ± 4 cells/HPF; *P* < 0.05) on day 35 after CD34⁺ cell transplantation.

Discussion

Our findings show that administration of human CD34⁺ cells to immunocompromised mice 48 hours after stroke enhances neovascularization at the border of the ischemic zone followed by endogenous neurogenesis. Furthermore, suppression of the neo-

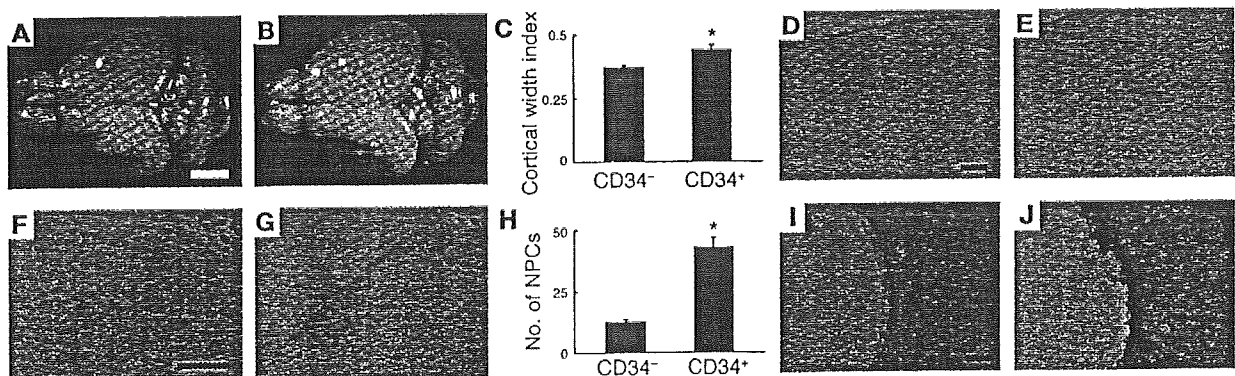


Figure 7

CD34⁺ cell transplantation in 24-week-old animals. (A–C) On day 35 after cell transplantation, the brains were evaluated. Compared with poststroke mice treated with CD34⁻ cells (A), animals transplanted with CD34⁺ cells (B) showed an increase in area occupied by the left cortex. Significant cortical regeneration was induced by CD34⁺ cells transplantation (C). (D and E) Compared with CD34⁻ cell transplantation (D), increased evidence of activated vasculature was observed in animals receiving CD34⁺ cells (E), as detected with mouse specific anti-CD13 antibody. (F–H) Migration of NPCs (small NeuN⁺ nuclei migrating toward the cortex) was observed in poststroke mice treated with CD34⁻ cells (F) and with CD34⁺ cells (G). However, a significant increase in migrating NPCs was induced by CD34⁺ cell transplantation (H). (I and J) A thin layer of migrating PSA-NCAM⁺ NPCs was observed at the ischemic edge of the cortex in animals treated with CD34⁻ cells (I), compared with a much thicker layer in those receiving CD34⁺ cells (J). Scale bars: 2 mm (A), 0.1 mm (D and I), and 0.4 mm (F). *n* = 4 in each group; **P* < 0.05 versus CD34⁻ cells.



vascularization by an antiangiogenic agent impaired neurogenesis. On the basis of these data, accelerated neovessel formation seems to be essential for enhancing endogenous neurogenesis and improving functional recovery.

The generation of new neurons in the adult is largely restricted to two regions: the SVZ lining the lateral ventricles and the subgranular zone of the dentate gyrus (25). In the transient cerebral ischemia model, evidence has been provided that neuronal regeneration occurs (10, 20). However, even in a transient ischemia model in which integrity of the microcirculation is maintained, it was shown that greater than 80% of newly formed neurons died, most likely because of unfavorable environmental conditions including lack of trophic support and exposure to products of damaged tissue. These considerations may underlie the observation that only 0.2% of nonviable ischemic neurons were replaced through neurogenesis (20). Such previous observations are consistent with our current results in animals treated with CD34⁺ cells or PBS, in which there was no enhancement of neovascularization, no neurogenesis, and no functional recovery.

Consistent with these data, Endostatin-mediated suppression of endothelial proliferation and direct effects on EPCs abrogated the beneficial effect CD34⁺ cells on neurogenesis and cortical expansion, and at the same time inhibited formation of neovasculature after stroke. In contrast, when EPO was used as a proangiogenic agent (recently, EPO has also been found to promote mobilization of EPCs) (23, 24), accelerated formation of neovasculature was accompanied by enhanced neurogenesis after stroke in our murine model. Our data provide the first direct link between therapeutic neovascularization after stroke and enhanced neurogenesis; formation of neovasculature after stroke supported neurogenesis. Consistent with a previous report (26), activation of NPCs after stroke was induced in adult, as well as young, murine brain. The number of activated NPCs was less in the adult animals, but a significant increase was still observed consequent to CD34⁺ cells transplantation, with evidence of activated vasculature in brains of the older animals. These findings support our hypothesis that administration of CD34⁺ cells provides a milieu favorable for neovascularization and endogenous neurogenesis, even in the mature brain.

A relationship between angiogenesis and neurogenesis would be consistent with regeneration of parenchymal cells in other organs subject to therapeutic angiogenesis (27, 28). Mechanisms underlying this observation might include more optimal preparation of the ischemic tissue bed for neuronal regeneration by accelerated removal of debris and toxic products, and/or the enhanced production of chemokines and trophic agents by neovasculature. Factors involved might be FGF2 (11), PDGF (29), brain-derived neurotrophic factor (30), and IL-8 (31), which have the capacity to induce mitogenesis, differentiation, recruitment, and survival of NPCs and newly generated neurons. In addition, mediators produced by CD34⁺ cells (4), such as VEGF, FGF2, and IGF-1, have been shown to accelerate endogenous neurogenesis (10-12). The effects of these and other factors derived from CD34⁺ cells (32, 33) acting on the vasculature are also likely to have an important role in providing an environment conducive to neurogenesis. Newly formed neurons in the setting of neovascularization have been shown to integrate into neuronal networks in adult animals (34); in fact, lack of participation of neurons in such neuronal circuits is probably associated with cell death (20). Our results provide strong support for the hypothesis that neovascularization consequent to administration of CD34⁺ cells induces neurogenesis by providing the necessary supportive environment. Whereas perturbation of the neurovascular unit has been proposed to contribute to tissue dam-

age in stroke, neoangiogenesis and accompanying neurogenesis could be considered to be rebuilding crucial elements of the neurovascular unit (35). Previous reports have demonstrated that endothelial cells in newly formed vessels after stroke mainly originate from pre-existing endothelial cells (due to proliferation) with a contribution of circulating EPCs (36, 37). Consistent with these findings, we observed proliferating mouse endothelial cells around the ischemic area soon after stroke. The latter endothelial cells could be visualized, at least in part, with antibody specific for human CD31, after transplantation of human CD34⁺ cells. Furthermore, diminished neovascularization was observed after stroke following transplantation of CD34⁺/Flk-1⁻ cells, compared with CD34⁺ cells (containing both Flk-1⁻ and Flk-1⁺ cells) despite similar activation of murine endothelial cells in the ischemic territory in each case. These results support potential contribution of CD34⁺/Flk-1⁺ cells (a population known to be rich in EPCs) in expansion of the vascular network after stroke.

The actual pattern of the newly formed vascular network was quite different on the ipsilateral (ischemic) from the contralateral side in poststroke mice treated with CD34⁺ cells. This observation suggests that formation of vasculature consequent to transplantation of CD34⁺ cells after stroke does not simply reconstitute the original vascular network. Rather, a new vascular pattern arises (a true neovasculature) that is capable of supporting neurogenesis, followed by functional recovery, even though it displays a relatively "aberrant" pattern, at least anatomically, compared with the contralateral vasculature.

Our observations provide evidence of a crucial role for neovessel formation, achieved through the administration of CD34⁺ cells after stroke, in processes that underlie neurogenesis. These data strongly suggest that neovascularization is essential for neuronal regeneration after stroke and that therapeutic neovascularization is a potentially effective means of enhancing functional recovery. Our observations might explain the mild therapeutic effect achieved by neuronal cell transplantation after stroke reported in humans (38). This leads to the hypothesis that therapeutic neovascularization may be required to achieve optimal "take" of transplanted neuronal precursors in the setting of ischemia. Although the current data bear most directly on the endogenous neuronal response to cerebrovascular ischemia, it is possible that enhanced formation of neovasculature may also be important for survival of embryonic (39) and neural (40) stem cell transplants in other circumstances, such as neurodegenerative disorders.

Methods

All procedures were done in accordance with the National Cardiovascular Center Animal Care and Use, and Human Assurance Committees. Quantitative measurements and behavioral tests were conducted by investigators blinded to the experimental protocol and identity of the sections and animals under study.

Induction of focal cerebral ischemia. Permanent focal cerebral infarction was produced by ligation and disconnection of the distal portion of the left MCA. Male SCID mice (5 weeks old; Oriental Yeast Co. Ltd., Tokyo, Japan) were used for experiments involving human cell transplantation. Male C57BL/6 mice (24 weeks old; Clea Japan Inc., Tokyo, Japan) were used for experiments with human recombinant EPO (Kirin, Tokyo, Japan). Under halothane inhalation (3%), the left MCA was isolated, electrocauterized, and disconnected just distal to its crossing of the olfactory tract (distal M1 portion). CBF in the MCA area was monitored as described (41). Mice that showed decreased CBF by approximately 75% immediately after and 24 hours after ligation were used for our experiments (success rate of >95%). Body temperature was maintained at 36.5-37°C using a heat lamp during



the operation and for 2 hours after MCA occlusion. The ratio of CBF after cell transplantation to CBF before cell transplantation was calculated to evaluate the change of CBF (termed relative CBF).

Analysis of perfusion and brain infarction after MCA occlusion. Carbon black ink (Fuekinori Kogyo Co. Ltd., Osaka, Japan) was used to delineate the perfused area after MCA ligation as described (42). Viability of brain tissue was evaluated using TTC (Sigma-Aldrich, St. Louis, Missouri, USA). Coronal sections (1 mm thick) of the forebrain were stained with 1% TTC, and the images of sections from the exact center of the forebrain were captured using a microscopic digital camera system (Olympus, Tokyo, Japan). The width of viable cortex was determined by NIH image.

Administration of CD34⁺ cells after stroke. CD34⁺ cells (containing Flk-1⁻ and Flk-1⁺ cell populations) and CD34⁺/Flk-1⁻ cells were isolated from cord blood using a Direct CD34 Progenitor Cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), CD34 Multisort kit (Miltenyi Biotec), and anti-Flk-1 antibody (Sigma-Aldrich), according to the manufacturers' protocols. CD34⁺ mononuclear cells were also collected for control experiments. In each case, cell populations were analyzed by FACS (BD Biosciences, San Jose, California, USA) using PerCP-conjugated anti-CD34 antibody (BD Biosciences), phycoerythrin-conjugated (PE-conjugated) anti-P1H12 antibody (BD Biosciences), and anti-Flk-1 antibody (Sigma-Aldrich) conjugated with FITC according to the manufacturer's protocol (Zenon Alexa Fluor; Molecular Probes Inc., Eugene, Oregon, USA). For labeling studies, cells were incubated with the fluorescent dye chloromethylbenzamide (CM-DiI; Molecular Probes Inc.). Forty-eight hours after stroke, 5×10^5 CD34⁺, CD34⁺/FLK-1⁻, CD34⁻ cells, or the same volume (100 μ l) of PBS were infused intravenously via the tail vein.

For the analysis of the effect of aging on neurogenesis, 24-week-old male SCID mice were used. Because of the known increased evidence of tumors, infection (for example, excess mucus in the eye), and elevated plasma immunoglobulins in older SCID mice, the presence of each of these conditions was also investigated. Mice with evidence of weight loss, tumors, infection, or elevated plasma immunoglobulins ($>10 \mu\text{g/ml}$) were excluded from further analysis.

Immunohistochemistry. Mice brains were removed and fixed in paraformaldehyde. Coronal sections (20 μm) were prepared using a vibratome (Leica Microsystems Inc., Wetzlar, Germany). To assess cell proliferation and angiogenesis, fresh frozen sections (20 μm) were prepared and fixed in acetone. Sections were subjected to immunohistochemistry with antibody to NeuN (Chemicon International, Temecula, California, USA), mouse-specific antibody to CD31 (BD Biosciences), human-specific antibody to CD31 (DAKO A/S, Glostrup, Denmark), mouse-specific antibody to CD13 (Santa Cruz Biotechnology, Santa Cruz, California, USA), and antibodies to MAP-2 (Sigma-Aldrich), human von Willebrand Factor (DAKO A/S), PSA-NCAM (Chemicon International), DCX (Chemicon International), Musashi-1 (19), TUNEL (Molecular Probes Inc.), or BrdU (Boehringer Ingelheim, Ingelheim, Germany).

Cell proliferation analysis. For assessment of cell proliferation in vivo, BrdU (Sigma-Aldrich) was administered intraperitoneally. In the case of endothelial cell proliferation, BrdU (200 mg/kg) was administered 24 hours before killing. For endogenous neurogenesis, 50 mg/kg of BrdU was injected every second day for a total of 14 days. Cells co-staining for BrdU and mouse-specific antibody to CD31 using confocal microscopy (Olympus) in HPFs ($\times 40$ of objective lens) were counted as proliferating endothelial cells. The cell population co-staining for BrdU and NeuN was counted as regenerating-proliferating neurons. For quantitative analysis, brain sections at the exact center of the forebrain were stained, and the left cortical area of 1 mm (or >1 mm) distal from the midline was examined by two investigators blinded to the experimental protocol. In each case, 10 representative fields were evaluated before quantitative results were analyzed.

Assessment of angiogenesis. For assessment of angiogenesis at the border of MCA and ACA areas, carbon black perfusion was used to visualize the

vasculature and TTC staining to demarcate the border of viable and non-viable tissue. Semi-quantitative analysis of angiogenesis made use of the angiographic score and was calculated by a previously described method (43). Microscopic digital images were scanned into a computer (Olympus), and an overlay, composed of 50- μm -diameter circles arranged in rows spaced 100 μm apart, was superimposed on the image. The total number of grids intersecting the border zone containing a microvessel was counted. Mouse-specific antibody to CD13 was used to visualize 'activated' vasculature.

Assessment of ischemic brain damage and neurogenesis. For determination of the cortical width index, whole-brain images were captured using a microscopic digital camera system (Olympus). The width at the midpoint of the forebrain was measured, and the ratio of left width to right width was defined as the cortical width index. To quantify accelerated neurogenesis, three brain sections around the center of the forebrain on day 14 after cell transplantation were stained with NeuN or TUNEL. The number of small NeuN nuclei-positive cells in the white matter at lower left of the left cortex and the number of TUNEL⁺ cells around the lower part of ischemic cortical edge was counted in HPFs. For determining the number of neurons in the left cortex, brain sections of the exact center of the forebrain on day 90 were stained with NeuN, and the total number of NeuN⁺ cells was quantitated.

Behavioral analysis. To assess cortical function, mice were subjected to behavioral testing in the open-field task (44) on day 35 or 90 after cell transplantation. Auditory startling behavior was tested as described (45). To estimate learning activity, mice were tested by water maze test (46) and passive avoidance test (47). To exclude the contribution of physical deficits directly related to the operative procedure and induction of stroke, motor deficiencies were examined on days 9 and 16 after stroke. Neurological deficits were scored on a three-point modified scale as described (17). Body weight, monitored in each experimental group, displayed no significant differences (data not shown).

Inhibition and promotion of neovascularization. Inhibition of neovascularization was achieved by administering Endostatin (10 μg ; subcutaneous; Calbiochem-Novabiochem, Darmstadt, Germany) once daily for 14 days. To stimulate mobilization of EPCs originating in the host, EPO (1,000 $\mu\text{g/kg}$) was injected subcutaneously 24, 48, and 72 hours after ischemia. Peripheral blood cells were analyzed using FITC-conjugated anti-mouse CD45 (BD Biosciences), PE-conjugated anti-mouse CD34 antibodies (BD Biosciences), and 7-amino-actinomycin D viability Dye (Beckman Coulter Inc., Marseille Cedex, France), as described (48), using counting beads (BD Biosciences) as an internal control.

Data analysis. In all experiments, mean \pm SE is reported. Statistical comparisons among groups were determined using one-way ANOVA. Where indicated, individual comparisons were performed using Student's *t* test.

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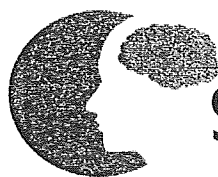
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research article

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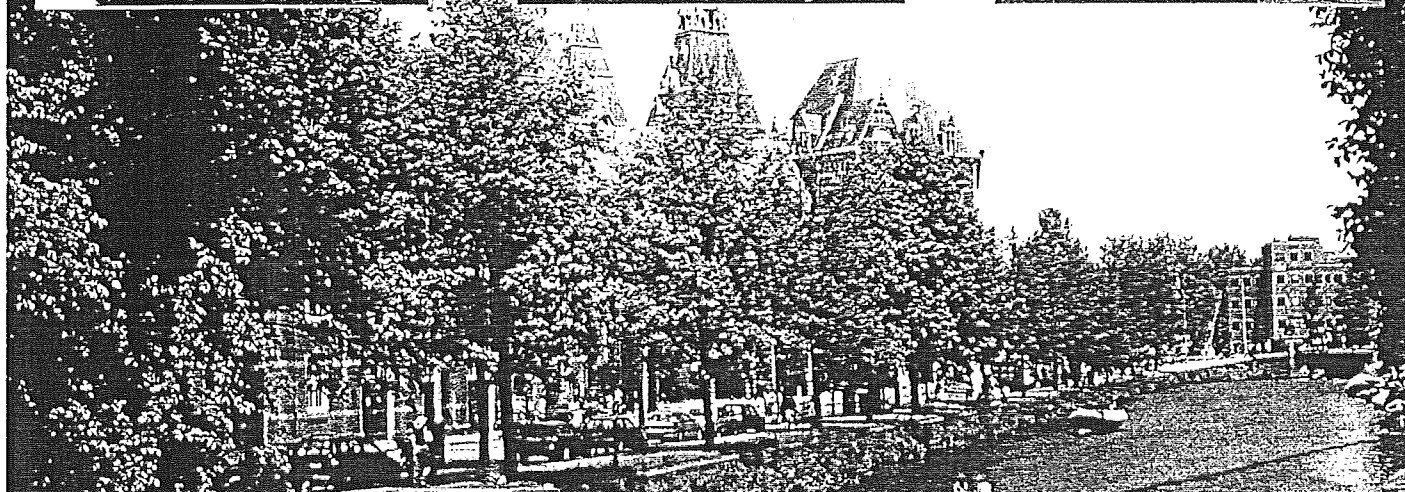
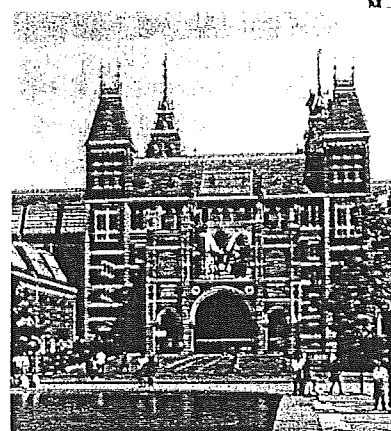
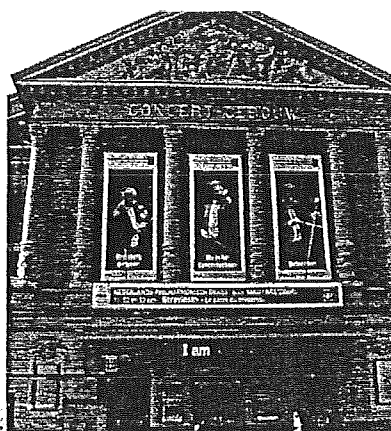
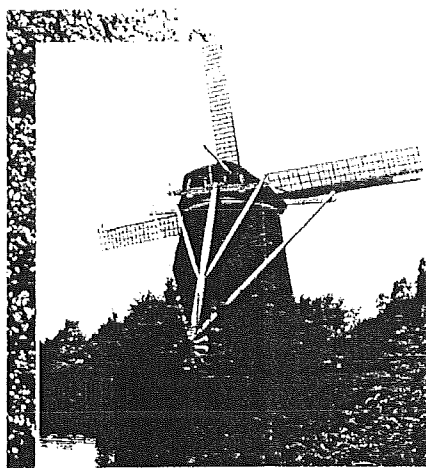
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Series
第7回

脳卒中とその予後 (Post Stroke)

—— 脳保護を目指した治療戦略 ——

今回のKey Word：脳梗塞、認知症、脳保護



座談会

(発言順)

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Stroke Prevention Program in Amsterdam

脳卒中とその予後 (Post Stroke)

——脳保護を目指した治療戦略——

今回のKey Word：脳梗塞、認知症、脳保護

高 齢化社会に伴う認知症(痴呆)や寝たきりの増加が大きな社会問題となっているが、その原因として頻度の高い疾患が脳卒中である。そこで、脳卒中とりわけ脳梗塞と認知症の関連を踏まえながら、「脳卒中とその予後(Post Stroke)——脳保護を目指した治療戦略——」というテーマで、先頃アムステルダムにおいて第22回国際脳循環代謝学会が開催されたのを機に、同地で岡山大学大学院脳神経制御学講座神経病態内科学教授の阿部康二氏(司会)、順天堂大学医学部脳神経内科講師の卜部貴夫氏、兵庫医科大学内科学神経・SCU科講師の松山知弘氏に話し合っていた。

座談会
(発言順)



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社会的にも重要な課題となっている 脳梗塞による認知症や寝たきり

阿部 まず私から脳卒中の動向などについてお話ししたいと思います。地球規模での死亡率ということでは脳卒中が28%とトップを占め、次いで感染症19%、冠疾患12%と続いています(WHO, 2002)。一方、我が国のデータから医療費・受診患者総数をみると医療費は高い順に癌・脳卒中・高血圧・糖尿病となっていますが、受診患者総数は高血圧が圧倒的に多く、次いで糖尿病・脳卒中となっています(国民生活動向2002)。高血圧や糖尿病は、心疾患や脳卒中の予備軍とも言える存在なので、その対策は重要です。2002年山陽脳卒中協議会入院患者統計のデータ(提供:阿部

康二氏)をみても、脳梗塞は脳出血よりも頻度が高く(脳梗塞66.0%、脳出血12.9%)、脳梗塞の内訳はラクナ梗塞21.8%、アテローム血栓・塞栓性23.4%(血栓性19.2%・塞栓性4.2%)、心原性脳塞栓20.8%となっており、ラクナ梗塞よりアテローム血栓・塞栓性梗塞が多くなっていました。また、高齢化による非弁膜症性心房細動も増加しており、それを反映して心原性脳塞栓も増加傾向にあります。今後とも高齢化社会が進行し、こうした脳梗塞の病型が増加していくことは、団塊世代が多くを占める日本人の人口構成からみても明らかです。脳梗塞は環境因子と遺伝因子を基盤に発症するわけですが、環境因子のうちでも高血圧・高脂血症・糖尿病・肥満などの危険因子が一次・二次予防の上で重要です。脳梗

塞のうち約7割が初発、約3割が再発とされていますので、この視点からは特に一次予防が重要であるということになります。先程、データでお示したように、脳卒中は医療費がかかり患者数も多い疾患にランクされていますが、その最大の理由は後遺症にあります。後遺症をかかえて寝たきりになる方もおられますが、脳卒中は我が国の寝たきりの原疾患の約3~4割を占める最大のものであります。また後遺症のうちでも特に認知症は、社会的にもますます重要な課題になっているのは周知の通りです。ですから脳卒中の一次・二次予防、そして慢性期治療における誤嚥性肺炎やうつ病の予防に加えて、認知症予防のための脳保護にも力を注いでいく必要があります。そこで続いて卜部先生から、脳梗塞に対す

る脳保護療法という視点からお話したいきたいと思います。

Poststroke depression (PSD)をめぐって

ト部 我が国における脳卒中の死亡率は第3位なのですが、ただ今、阿部先生からもお話があったように患者数は増加しており、寝たきりの原因としては36.7%とトップを占めています(厚生労働省・平成10年度国民生活基礎調査)。また同調査では高齢による衰弱13.6%、痴呆8.9%という数字も示されていますが、これらの中には脳卒中に関連しているケースがかなり含まれているのではないかと推測されます。そう考えますと、寝たきりの約50%に脳卒中が関連しているということになります。本日のテーマに関連しますが、Post Strokeにおける認知機能という視点からまず重要なのは、うつ病の存在です。このようなPoststroke depression (PSD)は、慢性期脳梗塞患者の25~50%に存在すると考えられています。その責任病巣として左大脳半球病変、頭頂葉+前頭葉の深部白質の虚血病変、あるいはこれらの部位+大脳基底核病変などが報告されていますが¹⁾、まだ明確な結論は得られていません。PSDの臨床的意義としては、①認知機能に有害な影響を与える可能性、②機能的回復の妨げとなる、③長期的予後として死亡率を高める、などが考えられます。我が国の「脳卒中治療ガイドライン2004」の中では、脳梗塞慢性期における選択的セロトニン再取り込み阻害薬(SSRI)を含む抗うつ薬のPSDに対する投与がグレードB(行うように推奨される)に位置づけられています。

国家的課題として取り組みが進行中の認知症

阿部 続いて松山先生、お願いします。

松山 脳卒中診療の最近の動向ということでは、画像診断・治療とともに著しく進歩しました。画像診断のうちMRIでは脳梗塞が出来上がる前に診断が可能、MRAでは血管の状態が針を刺さずに分かる、

超音波検査ではベッドサイドで診断可能、という特徴があります。また治療法としては、血流を回復させるための血栓溶解療法、脳を保護する(死にかけた細胞を助ける)脳保護療法、頭や首に傷をつけないステントやコイルを用いた血管内治療などが広く行われるようになりました。しかし平均余命が延長し続けており、65歳以上の身体障害者数も増加を続けています。しかも要介護者・寝たきり者の大半を脳卒中・認知症が占めており、さらなる新しい治療法が必要とされる状況にあります。一方、厚生労働省は認知症発症2年の抑制効果を医療経済的にまとめていますが(下方ら、2002)、患者数減少16万人、医療費用削減1,600億円、介護費用削減5,600億円となっています。認知症の発症抑制は国家的課題となっており、厚生労働省の外郭団体である財団法人長寿科学振興財団なども、寝たきり老人対策、老人性認知症の解明などに積極的に取り組むことの重要性をうたっています。こうした中、平成17年度厚生労働科学研究推進事業の一環として長寿科学総合研究推進事業(認知症・骨折等総合研究分野)が活動中で、我々も参加させていただいています。

阿部 最近、降圧薬が認知症の発症を抑制したという大規模臨床試験の報告がありますね。

松山 いくつかの大規模臨床試験およびそのサブ解析において、降圧薬が認知症の発症を抑制した、あるいは認知機能の低下を抑制したことが報告されています。その事実、血管性の認知症と変性疾患であるアルツハイマー病による認知症には、共通した危険因子(血管病変)があることを示唆するものとして注目されています。

阿部 脳梗塞慢性期の治療を考える場合には、血管のケアが認知症の発症抑制や治療の上で非常

に大事だということですね。

松山 そのように考えられます。

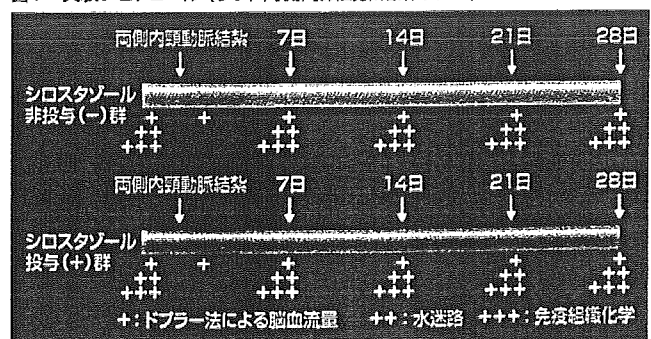
ラット両側内頸動脈結紮モデルで慢性脳虚血に対するシロスタゾールの作用を検討(ト部氏)

阿部 脳保護療法に関して、急性期ではエダラボンなどの新しい薬剤が出てきましたが、慢性期についてはまだ研究が重ねられている最中です。ト部先生、松山先生ともに、そうした慢性期における脳保護のお仕事をしておられますので、その内容をご紹介させていただきたいと思います。まずト部先生からお願いします。

ト部 我々はラットの両側内頸動脈結紮モデルを用いて、慢性脳虚血に対する抗血小板薬シロスタゾール(プレタール)の脳保護作用について検討しました。その実験プロトコルは図1のごとくですが、シロスタゾール非投与(-)群と投与(+)群で脳血流量(ドブラー法による)・water maze task(水迷路試験)・免疫組織化学の3つの検討を行いました。まず行動学的な記憶学習の成果を解析するモリスの水迷路試験の結果ですが、シロスタゾール(-)群ではescape latency(ラットが水中に隠れた踏み台をみつけてそれに至るまでの時間)が次第に延長していくのに対し、シロスタゾール(+)群では偽手術群にはほぼ近いescape latencyが得られており、シロスタゾール投与による学習成果が示唆されました(図2)。

続いて免疫組織化学検査の結果をご紹介します。ヒトの脳では皮質よりも白質の容量が多く、脳梗塞で白質が障害される割合も大きく関与しているため、各種免

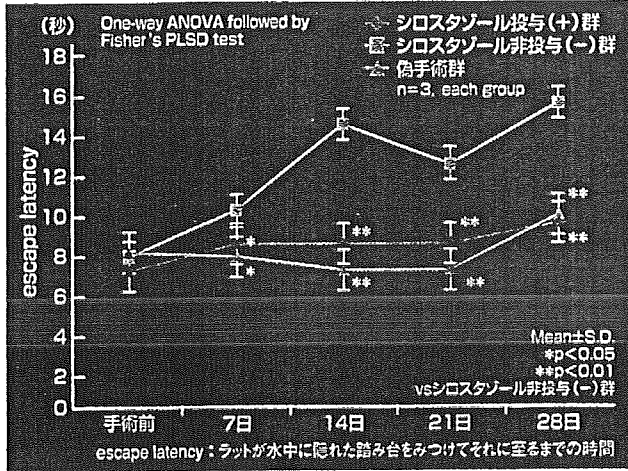
図1 実験プロトコル(ラット両側内頸動脈結紮モデル)



ト部 貞夫ほか: 第46回日本神経学会総会, 2005年5月



図2 モリスの水迷路試験(ラット両側内頸動脈結紮モデル)



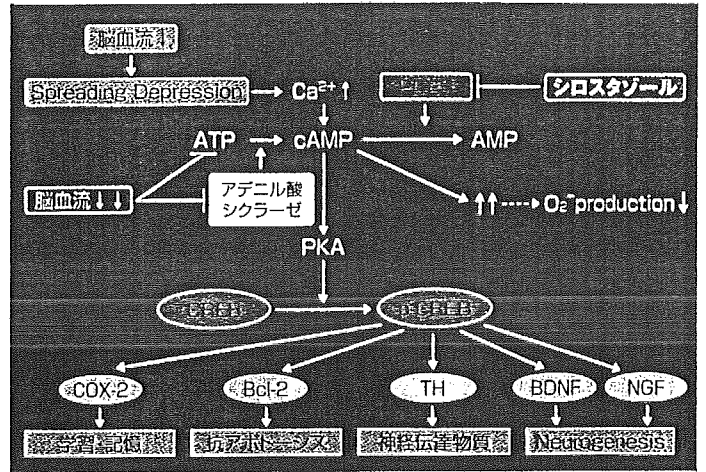
ト部眞夫ほか: 第46回日本神経学会総会, 2005年5月

疫組織染色では白質の観察を行いました。まずCV (cresil violet) 染色での検討ですが、シロスタゾール(-)群では14、21日目あたりから白質組織の粗像化が認められ、28日にはそれが顕著です。しかしシロスタゾール(+群)では既に14日目でこれが改善されており、組織変性が防止されたことが伺われます。アストロサイト(星状膠細胞)のマーカーであるGFAP (glial fibrillary acidic protein) 染色では、シロスタゾール(-)群では7日目からGFAP染色陽性の星状膠細胞が増生活性化されています。その後、徐々に活性化は落ちてきますが28日目でも細胞体をはっきりみえており、活性化が持続していることが伺われます。これに対してシロスタゾール(+群)では、既に7日目の時点で星状膠細胞の増生・活性化が抑制され、それが経時的に持続していました。オリゴデンドロサイト(乏突起神経膠細胞)のマーカーであるGST-pi (glutathione S-transferase-pi) 染色では、シロスタゾール(-)群では14日目で少し染色性が上がっているようにみえましたがその後徐々に低下しており、白質でオリゴデンドロサイトが障害を受けて数が減少している状況を示しているものと思われます。これに対してシロスタゾール(+群)では、最終的には染色性が落ちてしまうのですが、21日目の時点では染色性が強く残存しているとの印象を受けました。シロスタゾールがオリゴデンドロサイトの細胞死予防に何らか

の作用を及ぼしている可能性も考えられます。次にミクログリア(小膠細胞)をIba-1染色しますと、シロスタゾール(+群)で明らかに細胞死が抑制されていました。ですからグリア系に関しては、慢性脳虚血化におけるアストロサイトとミクログリアの活性化がともにシロスタゾールで抑制されるという結果でした。また脂質過酸化の代謝産物をHNE (hydroxynonenal) 染色で検討しました。その結果、シロスタゾール(-)群では7、14日目において白質の細胞群にHNEが蓄積していることが分かりました。これがどの細胞かは、現在、二重染色でセルタイピングを行っています。そのHNE陽性の細胞群は、シロスタゾール(+群)では明らかに抑制されていました。これは脂質過酸化という酸化ストレスに関する細胞毒性を持つ障害因子が、シロスタゾールで抑制されていることを示していると考えられます。遺伝子発現の制御にかかわる転写因子であるCREB (cAMP response element binding protein) のリン酸化と白質障害の関連をp-CREB染色で検討いたしました。興味深いことにシロスタゾール(-)群では染色性は徐々に低下していき、慢性虚血によりCREBのリン酸化が明らかに障害を受けている状況が示されました。しかしシロスタゾール(+群)ではそれが改善されていました。

阿部 ただ今、ト部先生から大変興味深いデータをご紹介いただきました。シロスタ

図3 脳血流障害におけるCREBリン酸化とシロスタゾールの関連



ト部眞夫ほか: 第46回日本神経学会総会, 2005年5月

ゾールには抗血小板作用以外にも内皮細胞保護作用や脳血流増加作用などの多面的作用 (pleiotropic effects) があることが知られていますが、さらに脳代謝にも好影響を与える作用もあることを示唆するデータだと思えます。

ト部 我々はこの慢性脳虚血の実験から得られたデータをシグナル伝達のカスケードで考えてみました(図3)。シロスタゾールはPDE (ホスホジエステラーゼ) 3の阻害薬ですから、cAMPからAMPに至るカスケードを阻害します。この阻害によりcAMP濃度が上昇し、1つの流れとしてPKAを介してCREBがリン酸化されCOX-2、Bcl-2、TH、BDNF、NGFなどの各々の標的分子のアップレギュレーションが起こると考えられます。今回、シロスタゾール投与によりラットの学習能力が向上したことに関しては、COX-2やノルアドレナリンもしくはドーパミン系の神経伝達物質が関与している可能性が推測されます。各種免疫組織染色でみられたシロスタゾール投与による細胞死の防止に関しては、抗アポトーシスに関与するとされるBcl-2のアップレギュレーションが、細胞保護という点で働いている可能性が考えられると思います。白質においてもシロスタゾール投与が神経再生という機序に働きかけている可能性も考えられ、今後、検討したいと思っています。cAMP濃度の上昇によるもう1つの流れとして、活性酸素産生の低下が考え

られます。今回、HNE染色においてみられたシロスタゾール投与による陽性細胞の抑制は、このカスケードが関与することでハイドロキシラジカル産生が抑制され、さらに脂質過酸化が抑制されたためと考えられます。

阿部 ありがとうございます。松山先生、何かコメントがございませうか？

松山 これから我々のデータを紹介させていただきますが、我々のデータはト部先生のデータとは非常に整合性があると思えました。

阿部 シロスタゾール投与により、グリア系の活性化を抑制したり、オリゴデンドロサイトの脱落を抑制したりしているのをどう考えると良いのでしょうか？

ト部 やはりシロスタゾールの直接的効果が発揮されたためと考えています。

阿部 神経細胞に限らずグリア細胞系にもシロスタゾールの直接的な保護作用があるのではないかとどう思われますか？

ト部 はい、そう考えられます。

阿部 大変重要なご指摘だと思います。

では続いて松山先生、ご研究のご紹介をお願いします。

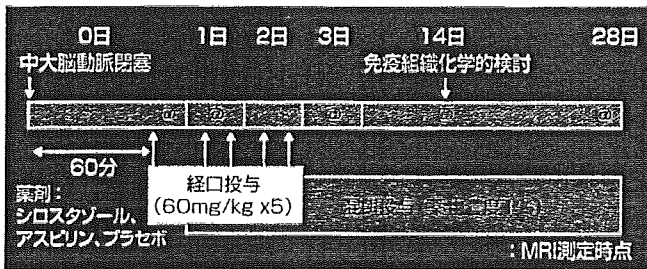
永久閉塞モデルマウスにおいてシロスタゾールのneurogenesisに対する作用を検討(松山氏)

松山 これまで動物実験の問題点として、脳梗塞の治療効果、特に長期予後进行评估する系がないことが問題でした。そこでまず我々は、再現性が良く長期間観察可能な脳梗塞モデルを開発しました。それがSCID(severe compromised immunodeficiency)マウスの中大脳動脈閉塞による脳梗塞モデルです。用いたマウスは5週齢の雄で、左側中大脳動脈起始部を直達法にて永久閉塞しました。脳血流はレーザードブラー法により経頭蓋的に測定し、脳容積算出にはCarbon Black灌流法、TTC染色、MRI(T7)による経時的測定(ADC、T2)、MAP2やNeuNに対する免疫組織化学などを施行しました。脳梗塞容積比率(%I)は脳浮腫の影響を除いた計算式を用いて算出しています。その結果ですが、中大脳動脈閉塞後には均一で安定した血流低下が認められ、生存率は半年間で約95%と高率でした。Carbon Black灌流法やTTC染色での検討では、梗塞は中大脳動脈領域の大脳皮質に限局しており、線条体は

障害されていません。またMRI所見においても、どのマウスでもほとんど同じ大脳皮質領域に梗塞が生じ、再現性の良い脳梗塞モデルであることが分かりました。

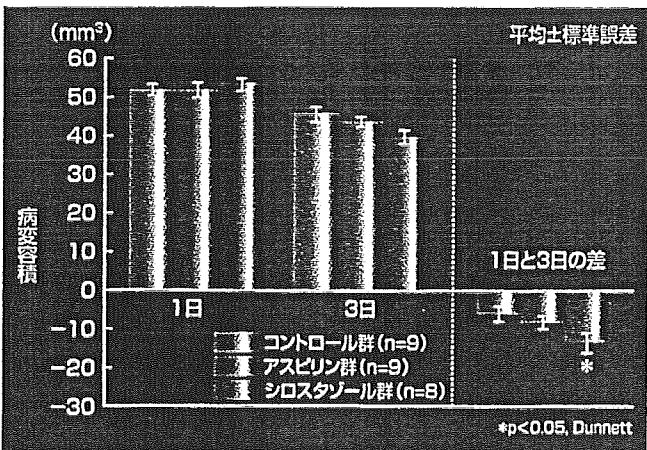
そこでこの永久閉塞モデルマウスを用いてシロスタゾールの脳梗塞に対する作用を検討しました。実験プロトコルは図4のごとくですが、中大脳動脈閉塞1時間後から薬剤(シロスタゾール、アスピリン、プラセボ)の投与を開始します。5回ほどゾンデで強制的に経口投与しますが、その後は自分で食べられるようになるので混餌投与としています。MRI撮像時点は図4にある通りです。その成績をご紹介します。まずMRI所見ですが、ADC画像(拡散係数画像)では既に1.5時間で低信号域が認められ脳梗塞が出来上がっていました。T2強調画像では、高信号域の消失がプラセボ群よりもシロスタゾール群でやや早く認められました。また1日目と3日目に認められたLesion volume(病変容積)を比較すると、シロスタゾール群では有意に大きな減少率が示されました(図5)。T2強調画像で3、14、28日目をコントロール群・アスピリン群・シロスタゾール群で比較すると、3日目では3群ともに高信号域が認められましたが、14日目になると3群ともかなり高信号域が消失し、28日目では梗塞域が分からない状態になっていました。このT2強調画像で重要な現象に気づきました。それはシロスタゾール群では、線条体が大脳の頭蓋容量一杯に膨張しているという事実です。そこで

図4 実験プロトコル(永久閉塞モデルマウス)



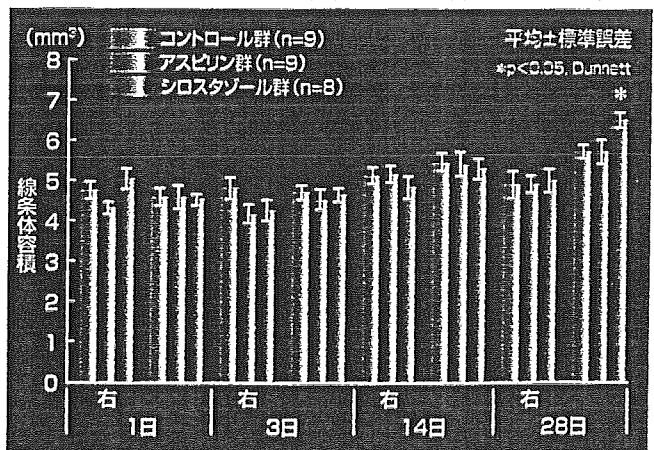
Matsuyama, T. et al. : International Society for Cerebral Blood Flow & Metabolism, JUNE, 2005

図5 脳梗塞後のT2強調画像による病変容積(永久閉塞モデルマウス)



Matsuyama, T. et al. : International Society for Cerebral Blood Flow & Metabolism, JUNE, 2005

図6 脳梗塞後のT2強調画像による線条体容積(永久閉塞モデルマウス)



Matsuyama, T. et al. : International Society for Cerebral Blood Flow & Metabolism, JUNE, 2005