

断において末梢血中血管系幹細胞の定量的評価は有用な検査法として発展が可能であると考えている。

研究方法

1. 研究計画書の作成および審査

兵庫医科大学倫理委員会において承認を受け、十分なインフォームドコンセントに基づいた本人（本人にその能力のない時は、その代理人）の意志で同意書を得た後に検査を施行した。また、施設外に出すサンプルは採取後に完全に匿名化を行い、個人識別情報は厳重に管理し、プライバシーの保護に努めている。

2. 対象患者群および病態評価

アルツハイマー型痴呆患者、痴呆症状を伴うパーキンソン病患者および脳血管性痴呆症患者を対象とし、NIHSS、modified Rankin scale、Barthel Index、Mini Mental State Examination (MMSE)、長谷川式痴呆スケール(HDS-R)、Global Clinical Dementia Rating (CDR)を行い、末梢血中 CD34 陽性細胞測定用の採血を行った。

3. 血管血球系幹細胞解析方法

解析方法によるばらつきを抑えるため、匿名化後、全ての病院のサンプルを国立病院機構大阪南医療センターに集積し、測定による変動や誤差を最小限とするために、我々が開発した非常に精度の高い測定方法にて解析した（解析の詳細に関しては、臨床研究実施チーム(a)組研究報告書を参照）。

また、採取血液中の CD34 陽性細胞の免疫組織化学的検討を行い、末梢血中 CD34 陽性細胞の形態を観察することにより、脳血管障害患者や痴呆患者の末梢血血管系幹細胞の質的評価を試みる。

4. 脳障害患者の病態評価

MMSE、CDR、DSM-III-R、HDS-R により痴呆の判定を行い、各種画像検査と共に病態の評価を行うとともに、エントリー後の経時的変化に関する追跡調査を行っている。

研究結果

本研究は末梢血中血管血球系幹細胞と患者予後との関連の解明を目的とした、前向き経時的コホート研究であり、各種痴呆症患者を対象にした臨床研究プロトコールを作成し、兵庫医科大学倫理委員会において承認後、患者エントリーを開始している。平成17年3月の時点で60名の患者の病態評価と末梢血中幹細胞の測定を終えており、引き続き患者登録を行っている。患者平均年齢は74.5歳(55~93歳)で、60名のエントリー患者のうち、脳梗塞既往のあるものは45名、痴呆を有する者は30名であった(表1)。痴呆患者のうち13例はアルツハイマー型痴呆、3例はパーキンソン病にともなう痴呆と診断されている(表2)。

本研究では、患者末梢血中幹細胞(CD34陽性細胞)を量的、質的に評価し、12ヶ月後にも再評価することで、機能予後や痴呆の進行に関しても検討する予定である。また、予備研究において末梢CD34陽性細胞は単一の丸い核を有するほぼ円形の細胞であり、形態的にmononuclear cellであることが確認された(図19)。細胞の形態学的変化が病態によって変化するかどうかを評価できれば血管系幹細胞機能を評価できる新たな有用な検査法として発展が可能であると考えている。

考察

我々はすでに末梢血中のCD34陽性細胞やCD133陽性細胞などの血管血球系幹細胞の減少が脳梗塞の発症と強く関連していること、そしてこれらの血管血球系幹細胞の減少が単に血流低下に関与しているだけではなく、血管新生に関わる血管内皮細胞の機能の低下とも関連していることを示している。このことは、脳血管を構成する血管内皮細胞の平均寿命は約3年であることを考えると、末梢血中の血管血球系幹細胞が脳血管のメンテナンスに関与しているだけでなく、神経組織の代謝や機能にまで影響を与え高次神経機能の低下や痴呆症の発症とも関連しており、血管系幹細胞数が多ければ神経機能が改善する可能性を示唆している。

実際、マウス脳梗塞モデルを用いた動物実験では、脳障害後に投与した血管系幹細胞が脳内で血管内皮細胞に分化して神経再生を亢進させることにより神経機能を改善させることが明らかとなっている。しかし、アルツハイマー型痴呆患者では末梢血中幹細胞数の増加が観察されており、これは血管系幹細胞の機能と神経機能との関連が高い可能性がある。これらの結果を基礎に、前向き経時的コホート研究においては、血管系幹細胞の形態学的検討を組み合わせた評価を行っており、痴呆症の病態解明や治療法の開発に直結する知見も得られると考えている。

結論

我々は慢性脳低灌流動患者における血管新生による側副血行維持に血管血球系幹細胞が関与すること¹⁾、脳障害動物モデルにおいては、血管血球系幹細胞の移植が、脳機能の改善をもたらすことを明らかにしてきた²⁾。これらの研究は、脳障害患者に対する血管血球系幹細胞移植による治療法の可能性を示すものである。さらに現在、血管再生と神経再生との関連についての検討、および霊長類を用いた血管系幹細胞移植実験を開始している。今後は本研究で得られた知見と総合し発展させることにより脳障害患者に対する新しい治療法が確立されると考えている。

文献

- 1) Taguchi A, Matsuyama T, Soma T, et al. (2004) Circulating CD34-positive cells provide an index of cerebrovascular function. *Circulation* 109:2972-2975.
- 2) Taguchi A, Soma T, Nishimura H, Yoshikawa H, Matsuyama T, et al. (2004) Administration of CD34⁺ cells post-stroke enhances angiogenesis and neurogenesis in a murine model. *J Clin Invest* 114: 330-389.

研究成果の刊行に関する一覧表

雑誌

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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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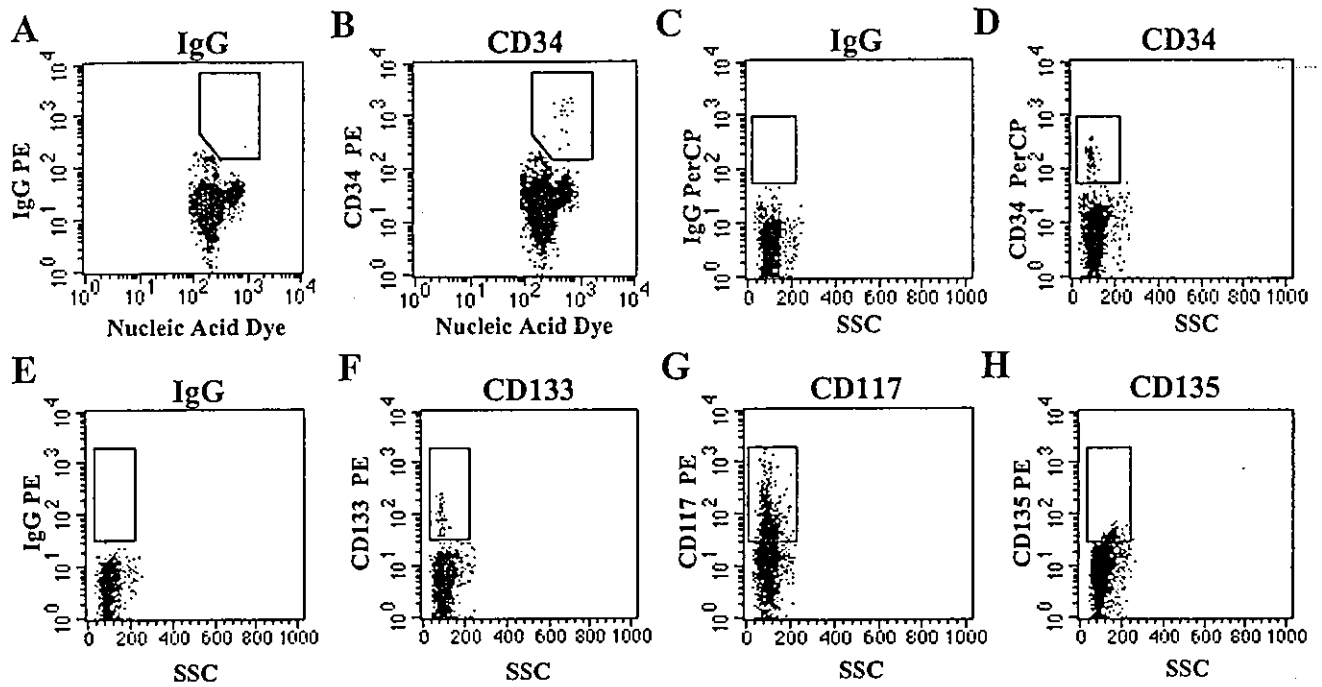


Figure 1. Quantification of circulating immature cells in patients with stroke. Nucleic acid dye versus CD34 PE dot plot was gated on lymphocytes and CD45 dim leukocytes. A, Results with an isotype-matched control antibody. B, Results with anti-CD34 antibody. Enriched immature mononuclear cells were 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/μL; *P*=0.01). In contrast,

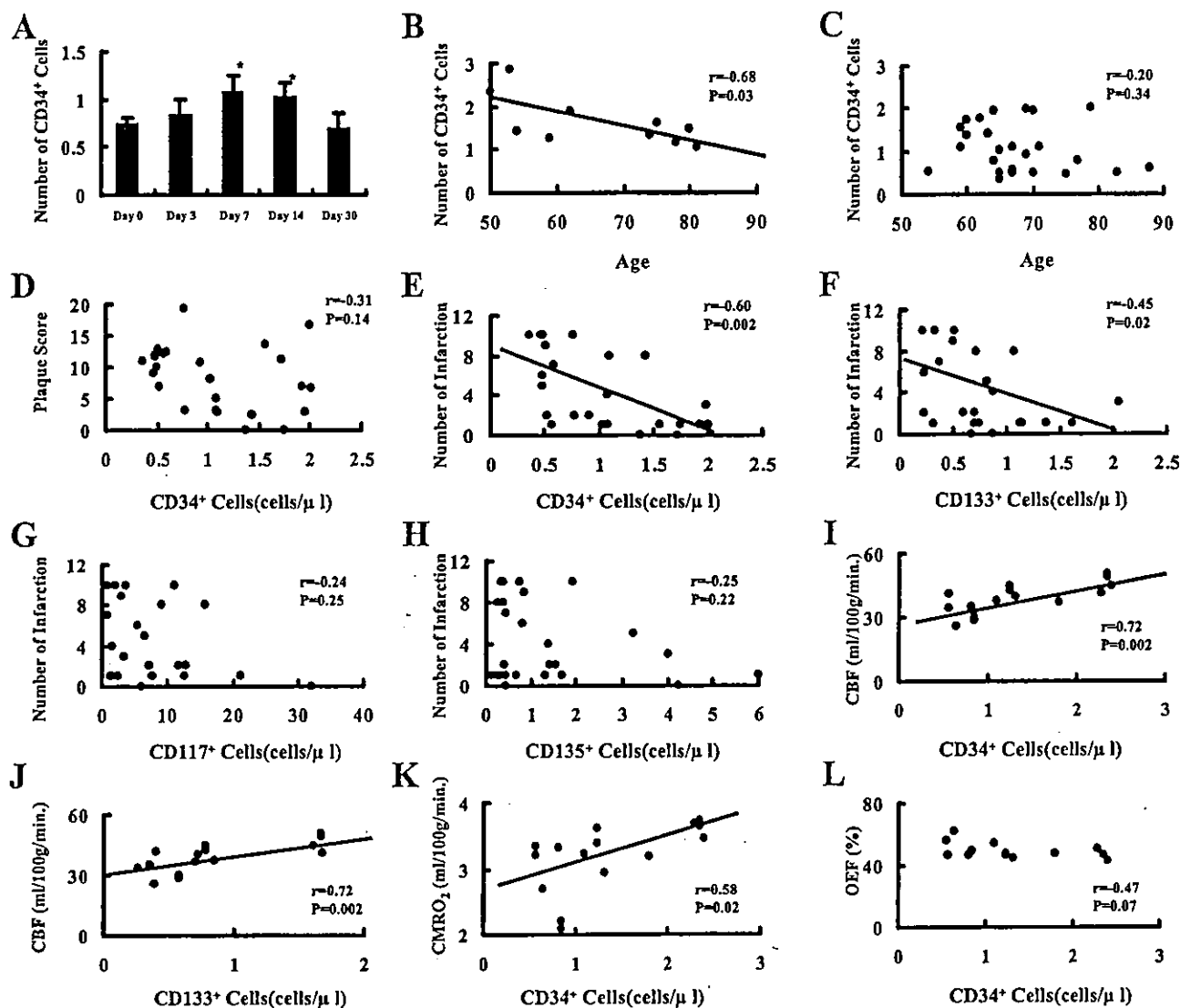


Figure 2. Levels of circulating CD34⁺ cells and stroke. Circulating CD34⁺ cells increased after the onset of stroke and peaked on day 7. A significant increase in circulating CD34⁺ cells was observed on days 7 and 14 (A). A decrease of circulating CD34⁺ cell was observed with aging in the control group (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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References

1. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275:964-967.
2. Taguchi A, Ohtani M, Soma T, et al. Therapeutic angiogenesis by autologous bone-marrow transplantation in a general hospital setting. *Eur J Vasc Endovasc Surg*. 2003;25:276-278.
3. Tateishi-Yuyama E, Matsubara H, Murohara T, et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet*. 2002;360:427-435.
4. Hamano K, Nishida M, Hirata K, et al. Local implantation of autologous bone marrow cells for therapeutic angiogenesis in patients with ischemic heart disease: clinical trial and preliminary results. *Jpn Circ J*. 2001;65:845-847.
5. Majka M, Janowska-Wieczorek A, Ratajczak J, et al. Numerous growth factors, cytokines, and chemokines are secreted by human CD34⁺ cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. *Blood*. 2001;97:3075-3085.
6. Beck H, Voswinckel R, Wagner S, et al. Participation of bone marrow-derived cells in long-term repair processes after experimental stroke. *J Cereb Blood Flow Metab*. 2003;23:709-717.
7. Sasaki T, Watanabe M, Nagai Y, et al. Association of plasma homocysteine concentration with atherosclerotic carotid plaques and lacunar infarction. *Stroke*. 2002;33:1493-1496.
8. Hirano T, Minematsu K, Hasegawa Y, et al. Acetazolamide reactivity on ¹²³I-IMP single photon emission computed tomography in patients with major cerebral artery occlusive disease: correlation with positron emission tomography parameters. *J Cereb Blood Flow Metab*. 1994;14:763-770.



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.

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).

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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)

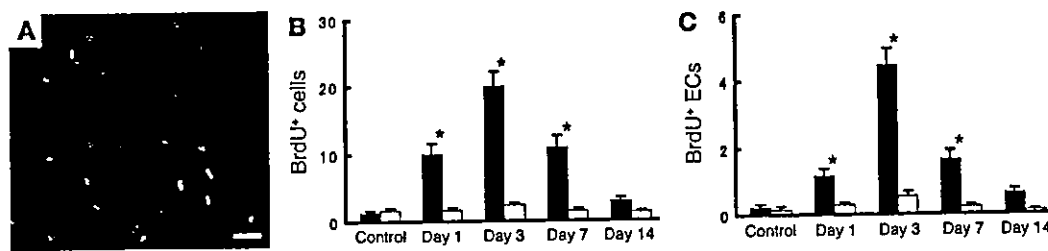


Figure 1

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⁺

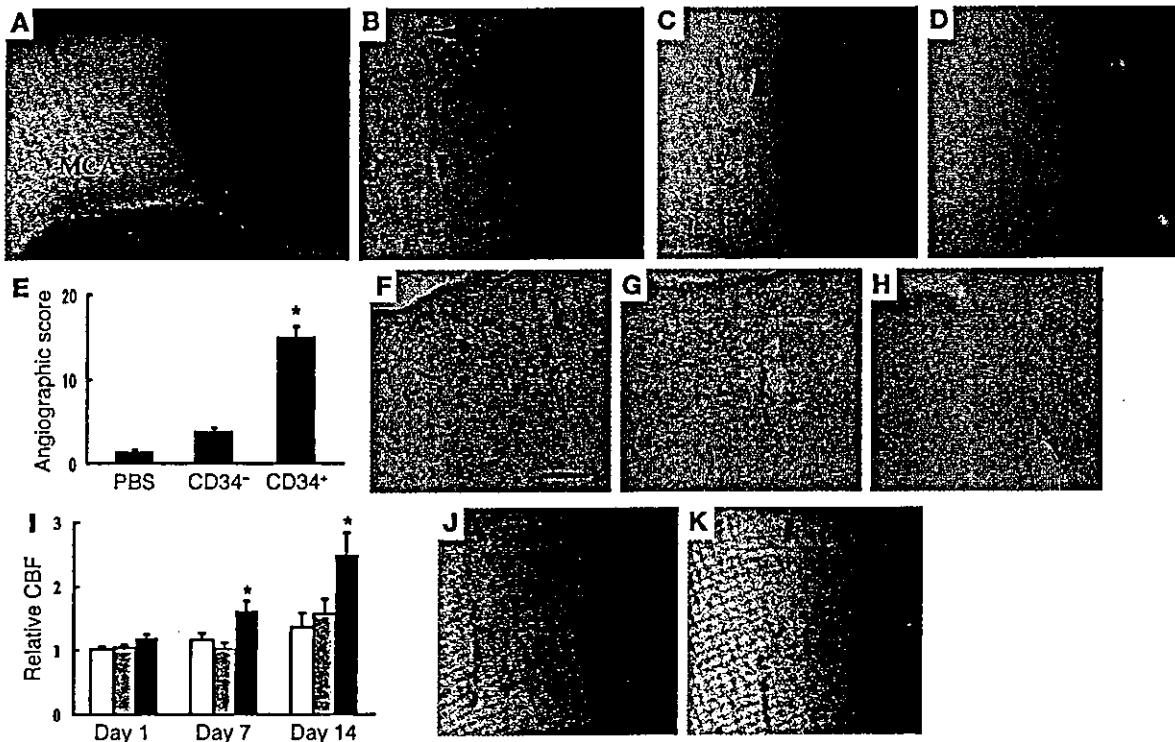


Figure 2

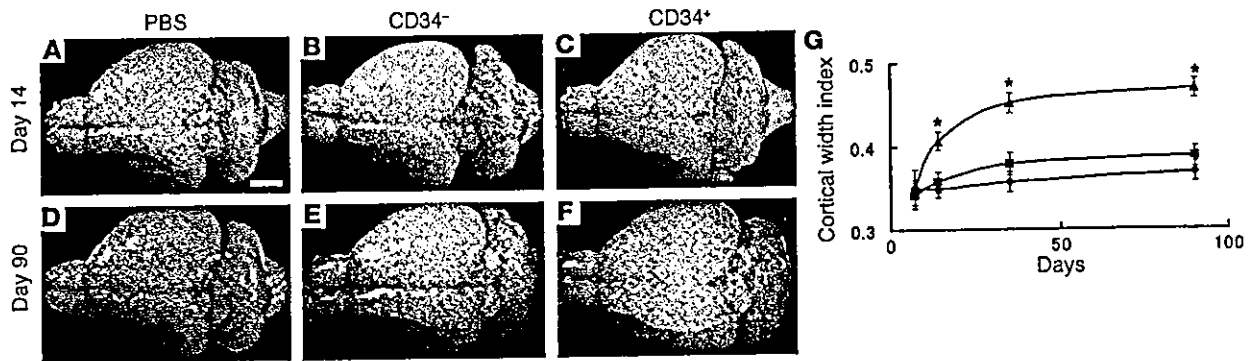
Transplantation of CD34⁺ cells after stroke accelerates neovascularization. (A–D) Mice subjected to stroke received CD34⁺ cells (A and B), CD34⁻ cells (C), or PBS alone (D) on day 2. Animals were infused with carbon black ink and killed at 24 hours after cell transplantations. Sections were stained with 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 neovascularization at the border of the MCA and ACA cortex in animals treated with CD34⁺ cells (J) and CD34⁺/Fik-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.

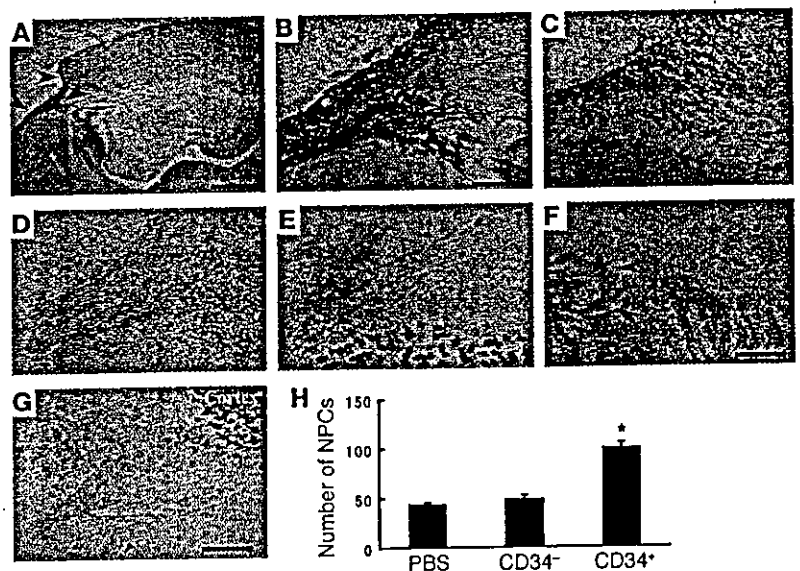
for PSA-NCAM (Figure 4, A and B, corresponding to lower and higher magnifications, respectively), as well as the neuronal stem cell marker, Musashi-1 (Figure 4C) (19). Expression of doublecortin (DCX), a marker of immature, or early, neurons (20), was principally restricted to NPCs close to the SVZ (Figure 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.

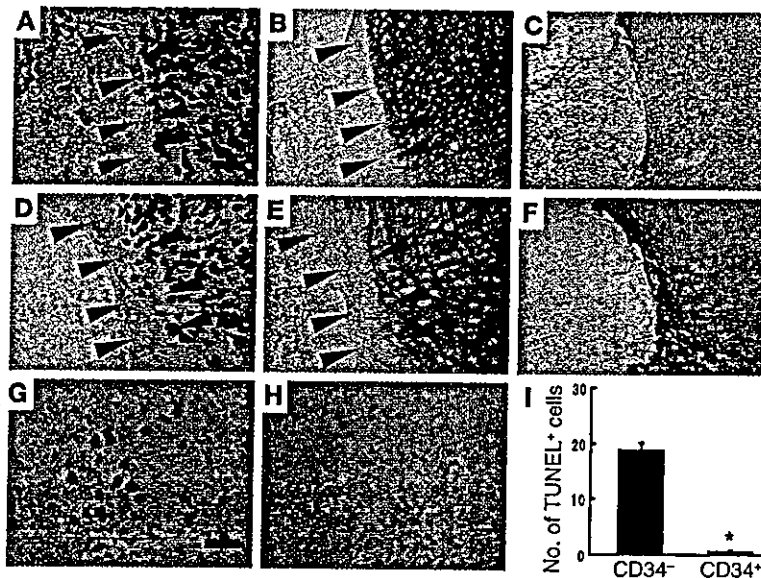


Figure 5 Therapeutic neovascularization, due to CD34⁺ cell transplantation after stroke, enhances neurogenesis. (A–F) On day 14 after CD34⁻ cell transplantation, mature cortical neurons were observed up to the edge of the ischemic region displaying neuronal markers, NeuN (A) and MAP-2 (B), whereas only 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% ± 3% and 22% ± 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 post-stroke cortex was less in mice treated with CD34⁺ cells + Endostatin (1,469 ± 53), compared with animals treated with CD34⁺ cells + PBS (2,213 ± 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 regulat-

ing 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 ± 1,267 and 1,333 ± 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-

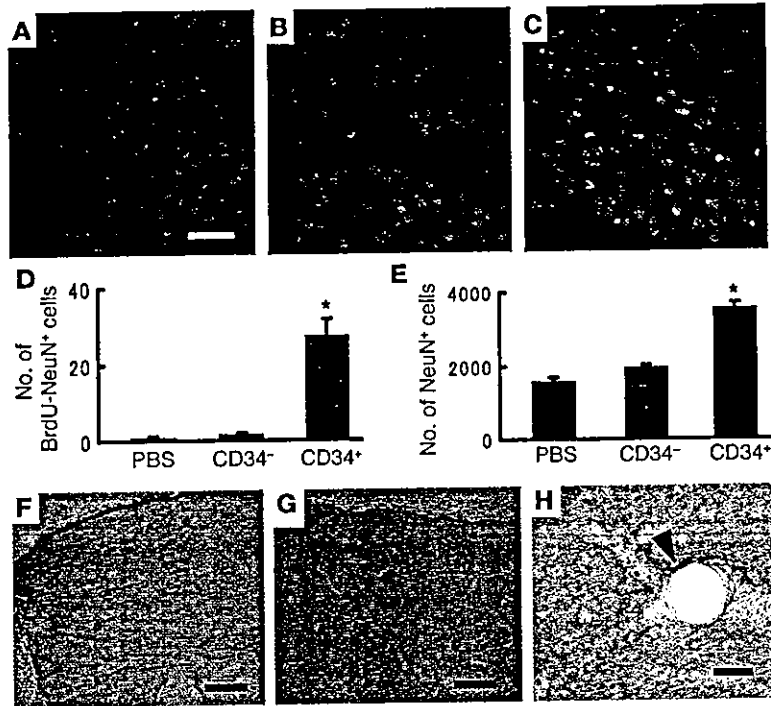


Figure 6 Therapeutic neovascularization supports survival of regenerating neurons. (A–C) Animals treated with PBS alone (A), CD34⁻ cells (B), or CD34⁺ cells (C) were infused with BrdU, killed, and studied immunohistochemically with antibody to BrdU (red), NeuN (green), or both (yellow). (D) The average 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-

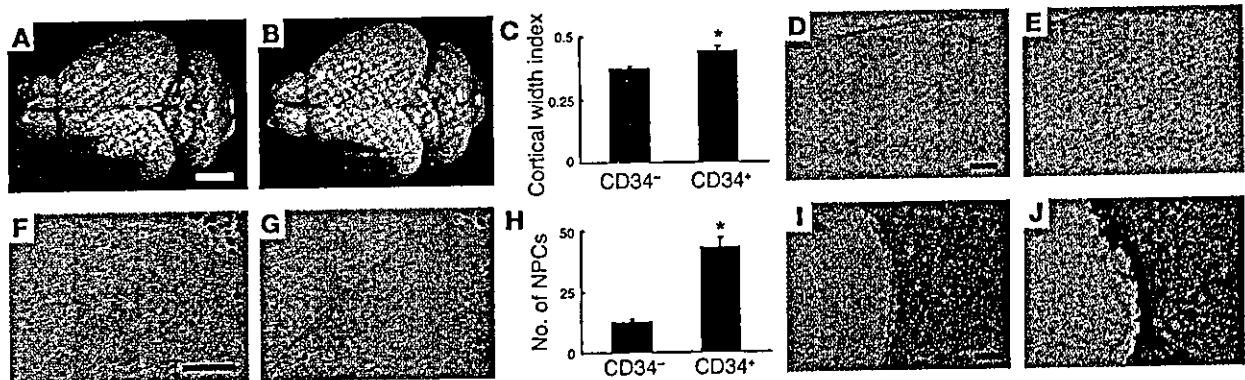


Figure 7 CD34⁺ cell transplantation in 24-week-old animals. (A–C) On day 35 after cell transplantation, the brains were evaluated. Compared with post-stroke mice treated with CD34⁻ cells (A), animals transplanted with CD34⁺ cells (B) showed an increase in area occupied by the left cortex. Significant 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.



research article

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/6J 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 μ g/ml) were excluded from further analysis.

Immunohistochemistry. Mice brains were removed and fixed in paraformaldehyde. Coronal sections (20 μ m) were prepared using a vibratome (Leica Microsystems Inc., Wetzlar, Germany). To assess cell proliferation and angiogenesis, fresh frozen sections (20 μ m) 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- μ m-diameter circles arranged in rows spaced 100 μ m 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 μ g; subcutaneous; Calbiochem-Novabiochem, Darmstadt, Germany) once daily for 14 days. To stimulate mobilization of EPCs originating in the host, EPO (1,000 μ 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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- Asahara, T., et al. 1997. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 275:964-967.
- Asahara, T., et al. 1999. Bone marrow origin of endothelial progenitor cells responsible for post-natal vasculogenesis in physiological and pathological neovascularization. *Circ. Res.* 85:221-228.
- Kawamoto, A., et al. 2001. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation*. 103:634-637.
- Majka, M., et al. 2001. Numerous growth factors, cytokines, and chemokines are secreted by human CD34⁺ cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. *Blood*. 97:3075-3085.
- Taguchi, A., Ohtani, M., Soma, T., Watanabe, M., and Kinoshita, N. 2003. Therapeutic angiogenesis by autologous bone-marrow transplantation in a general hospital setting. *Eur. J. Vasc. Endovasc. Surg.* 25:276-278.
- Tateishi-Yuyama, E., et al. 2002. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet*. 360:427-435.
- Hamano, K., et al. 2001. Local implantation of autologous bone marrow cells for therapeutic angiogenesis in patients with ischemic heart disease: clinical trial and preliminary results. *Jpn. Circ. J.* 65:845-847.
- Chen, J., et al. 2001. Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke*. 32:2682-2688.
- Hess, D.C., et al. 2002. Bone marrow as a source of endothelial cells and NeuN-expressing cells after stroke. *Stroke*. 33:1362-1368.
- Nakatomi, H., et al. 2002. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell*. 110:429-441.
- Drago, J., Murphy, M., Carroll, S.M., Harvey, R.P., and Bartlett, P.F. 1991. Fibroblast growth factor-mediated proliferation of central nervous system precursors depends on endogenous production of insulin-like growth factor I. *Proc. Natl. Acad. Sci. U. S. A.* 88:2199-2203.
- Jin, K., et al. 2002. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 99:11946-11950.
- Solovey, A., et al. 1997. Circulating activated endothelial cells in sickle cell anemia. *N. Engl. J. Med.* 337:1584-1590.
- Bhagwat, S.V., Petrovic, N., Okamoto, Y., and Shapiro, L.H. 2003. The angiogenic regulator CD13/APN is a transcriptional target of Ras signaling pathways in endothelial morphogenesis. *Blood*. 101:1818-1826.
- Van Dam, D., et al. 2003. Age-dependent cognitive decline in the APP23 model precedes amyloid deposition. *Eur. J. Neurosci.* 17:388-396.
- Farkas, T., et al. 2003. Peripheral nerve injury influences the disinhibition induced by focal ischaemia in the rat motor cortex. *Neurosci. Lett.* 342:49-52.
- Tamatani, M., et al. 2001. ORP150 protects against hypoxia/ischemia-induced neuronal death. *Nat. Med.* 7:317-323.
- Iwai, M., et al. 2003. Temporal profile of stem cell division, migration, and differentiation from sub-ventricular zone to olfactory bulb after transient forebrain ischemia in gerbils. *J. Cereb. Blood Flow Metab.* 23:331-341.
- Sakakibara, S., et al. 1996. Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell. *Dev. Biol.* 176:230-242.
- Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., and Lindvall, O. 2002. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat. Med.* 8:963-970.
- O'Reilly, M.S., et al. 1997. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*. 88:277-285.
- Capillo, M., et al. 2003. Continuous infusion of endostatin inhibits differentiation, mobilization, and clonogenic potential of endothelial cell progenitors. *Clin. Cancer Res.* 9:377-382.
- Bahlmann, F.H., et al. 2004. Erythropoietin regulates endothelial progenitor cells. *Blood*. 103:921-926.
- Jaquet, K., Krause, K., Tawakol-Khodai, M., Geidel, S., and Kuck, K.H. 2002. Erythropoietin and VEGF exhibit equal angiogenic potential. *Microvasc. Res.* 64:326-333.
- Gage, F.H. 2000. Mammalian neural stem cells. *Science*. 287:1433-1438.
- Yagita, Y., et al. 2001. Neurogenesis by progenitor cells in the ischemic adult rat hippocampus. *Stroke*. 8:1890-1896.
- Ross, M.A., Sander, C.M., Kleeb, T.B., Watkins, S.C., and Stolz, D.B. 2001. Spatiotemporal expression of angiogenesis growth factor receptors during the revascularization of regenerating rat liver. *Hepatology*. 34:1135-1148.
- Toda, S., et al. 1999. Immunohistochemical expression of growth factors in subacute thyroiditis and their effects on thyroid folliculogenesis and angiogenesis in collagen gel matrix culture. *J. Pathol.* 188:415-422.
- Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M., and McKay, R.D. 1996. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev.* 10:3129-3140.
- Leventhal, C., Rafii, S., Rafii, D., Shahar, A., and Goldman, S.A. 1999. Endothelial trophic support of neuronal production and recruitment from the adult mammalian subependyma. *Mol. Cell. Neurosci.* 13:450-464.
- Araujo, D.M., and Cotman, C.W. 1993. Trophic effects of interleukin-4, -7 and -8 on hippocampal neuronal cultures: potential involvement of glial-derived factors. *Brain Res.* 600:49-55.
- Valable, S., et al. 2003. Angiopoietin-1-induced PI3-kinase activation prevents neuronal apoptosis. *FASEB J.* 17:443-445.
- Sun, W., Funakoshi, H., and Nakamura, T. 2002. Localization and functional role of hepatocyte growth factor (HGF) and its receptor c-met in the rat developing cerebral cortex. *Brain Res. Mol. Brain Res.* 103:36-48.
- Louissaint, A., Jr., Rao, S., Leventhal, C., and Goldman, S.A. 2002. Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. *Neuron*. 34:945-960.
- Lo, E.H., Dalkara, T., and Moskowitz, M.A. 2003. Mechanisms, challenges and opportunities in stroke. *Nat. Rev. Neurosci.* 4:399-415.
- Beck, H., et al. 2003. Participation of bone marrow-derived cells in long-term repair processes after experimental stroke. *J. Cereb. Blood Flow Metab.* 23:709-717.
- Zhang, Z.G., Zhang, L., Jiang, Q., and Chopp, M. 2002. Bone marrow-derived endothelial progenitor cells participate in cerebral neovascularization after focal cerebral ischemia in the adult mouse. *Circ. Res.* 90:284-288.
- Kondziolka, D., et al. 2000. Transplantation of cultured human neuronal cells for patients with stroke. *Neurology*. 55:565-569.
- Hoehn, M., et al. 2002. Monitoring of implanted stem cell migration in vivo: a highly resolved in vivo magnetic resonance imaging investigation of experimental stroke in rat. *Proc. Natl. Acad. Sci. U. S. A.* 99:16267-16272.
- Abe, K. 2000. Therapeutic potential of neurotrophic factors and neural stem cells against ischemic brain injury. *J. Cereb. Blood Flow Metab.* 20:1393-1408.
- Matsushita, K., et al. 1998. Marked, sustained expression of a novel 150-kDa oxygen-regulated stress protein, in severely ischemic mouse neurons. *Brain Res. Mol. Brain Res.* 60:98-106.
- Matsuyama, T., et al. 1983. Why are infant gerbils more resistant than adults to cerebral infarction after carotid ligation? *J. Cereb. Blood Flow Metab.* 3:381-385.
- Takeshita, S., et al. 1994. Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J. Clin. Invest.* 93:662-670.
- Kimble, D.P. 1968. Hippocampus and internal inhibition. *Psychol. Bull.* 70:285-295.
- Sasaki, H., Iso, H., Coffey, P., Inoue, T., and Fukuda, Y. 1998. Prepulse facilitation of auditory startle response in hamsters. *Neurosci. Lett.* 248:117-120.
- Balschun, D., et al. 2003. Does cAMP response element-binding protein have a pivotal role in hippocampal synaptic plasticity and hippocampus-dependent memory? *J. Neurosci.* 23:6304-6314.
- Mereu, G., et al. 2003. Prenatal exposure to a cannabinoid agonist produces memory deficits linked to dysfunction in hippocampal long-term potentiation and glutamate release. *Proc. Natl. Acad. Sci. U. S. A.* 100:4915-4920.
- Gratama, J.W., et al. 1999. Comparison of single- and dual-platform assay formats for CD34⁺ haematopoietic progenitor cell enumeration. *Clin. Lab. Haematol.* 21:337-346.

資料 1

脳血管性痴呆症および虚血性脳血管障害における末梢血幹細胞の研究 再生医療研究を目的とする血液の採取とその利用についてのお願い

研究の概要

血液の中に含まれている細胞（幹細胞：様々な細胞になることのできる細胞）が脳梗塞や心筋梗塞等の改善に関連している可能性があり、当病院ではそれらの細胞を使った、新しい予防法や治療法（再生医療）の実現を目的とした研究を行っております。

研究への協力について

あなた(または、提供者本人の代わりに同意の意思を決定されるあなた)に、この研究への協力をお願いするため説明を行います。

この研究への協力の同意はあなたの自由意志で決めてください。強制ではありませんし、また、この協力の同意しなくてもあなたの不利益になることは一切ありません。また、一旦同意した場合でもあなたは不利益を受けることなく、いつでもその同意を取り消すことができ、その場合は採取した血液は廃棄され、それ以降の研究目的に使用されることもありません。

研究目的

血液に含まれている細胞（幹細胞）が脳梗塞等の病気に関連している可能性が考えられています。脳梗塞の患者さん、および脳梗塞のない患者さんの血液を比較することにより、それらの細胞の持つ働きについて研究します。これらの研究を通じて、新しい予防法や治療法の開発ができると考えています。

研究方法

血液中の幹細胞は新しく血管を作ったり、血管を修復したりする能力がありますが、この研究ではこれらの能力と、病気や病状の関連について研究します。

具体的にはあなたの血液を採取させて頂き、その中に含まれる幹細胞等の量や質に関して検査を行うと共に、本来の治療に必要な検査結果（脳 CT 撮影など）との比較検討をします。これらの研究のため静脈あるいは点滴ルートより採血を 1 度行いますので、よろしくお願いたします。

あなたにもたらされる利益および不利益について

本研究によりあなたに直接有益な情報をもたらす可能性は低いですが、解析が進めば病気になる医学情報が進歩し、結果的に臨床上有益な情報が得られる可能性はあります。また研究の成果は今後の医学の発展に寄与すると考えています。不利益は静脈血採取に伴う疼痛および血液の物理的損失がありますが、通常の採血と同じで採血量も少なくなるように配慮しています。

個人情報(プライバシー)の保護について

あなたの本研究に関する個人情報は、他の医療情報と同様に厳重に保護されます。

費用負担等について

この研究に係る費用については兵庫医科大学診療研究費等で負担します。また、当該研究により発生した知的財産権は、兵庫医科大学および研究者に帰属します。

担当医への連絡

この研究に関して問題点や分からないこと、心配なことなどがありましたら、いつでも御遠慮なく担当医に申し出てください。

平成 年 月 日

兵庫医科大学病院

担当医師（主治医） 氏名 _____

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資料 2

脳血管性痴呆症および虚血性脳血管障害患者における末梢血幹細胞の研究への協力に関する同意書

兵庫医科大学病院 病院長 殿

私は、「脳血管性痴呆症および虚血性脳血管障害患者における末梢血幹細胞の研究」（主任研究者 芳川浩男）に関して、その目的、内容、利益及び不利益を含む下記の事項について担当者から説明文書を用いて説明をうけ、理解しました。

また、同意した後であっても、いつでも同意を撤回できること、そのことによって何ら不利益を生じないこと、疑問があればいつでも質問できることについても説明を受け納得しました。

ついては、私自身の自由意思により研究への協力を同意いたします。

（御理解頂いた項目に をいれてください。）

- 研究の概要
- 研究への協力について
- 研究目的
- 研究方法（採血を1度行います）
- 私にもたらされる利益および不利益について
- 個人情報(プライバシー)の保護について
- 研究成果の公表について
- 費用負担等について
- 担当医への連絡

平成 年 月 日

氏名 _____

(署名または記名、押印)

(代理人の場合)

氏名 _____ (患者との関係 _____)

(署名または記名、押印)

住所 _____

電話番号 _____ () _____