図２４：リンパ球・芽球の領域Fを設定

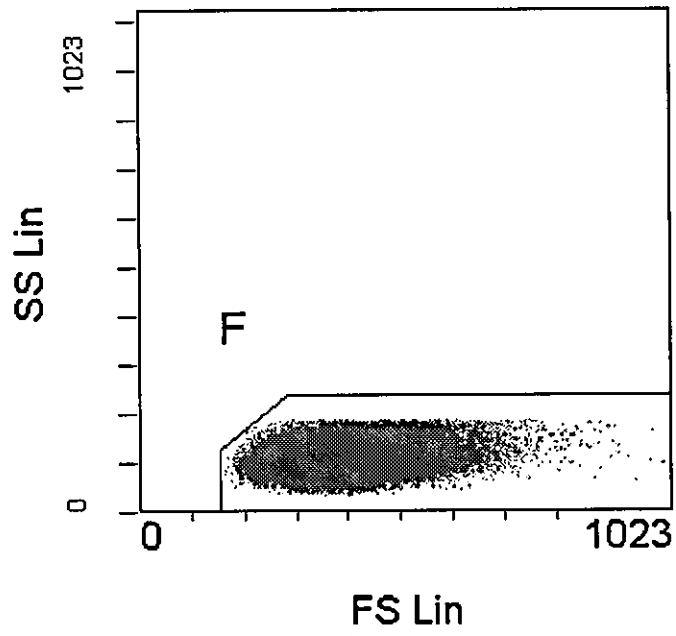


図２５：領域Fに存在する細胞のみ
CD34陽性HPCsとしてカウント

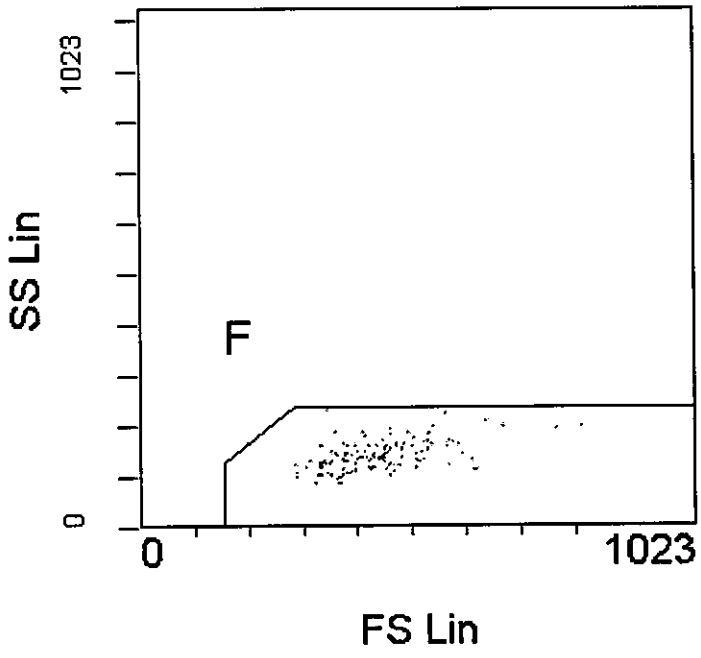


図２６：標準粒子コントロールビーズの領域Gの設定

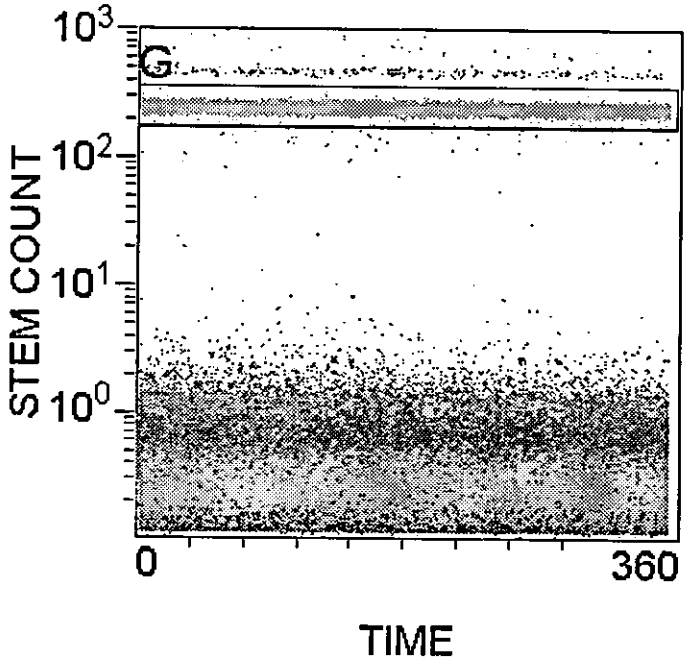
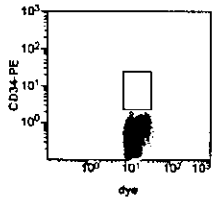
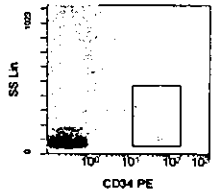
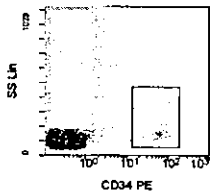


表１：新しいプロトコールによる変動係数の減少

	ProCount	Stem-Kit	Improved Protocol
			
Actual CD34+ cells counts	16±1 (range: 10–31)	34±4 (range: 16–58)	174±18 (range: 88–404)
Percentage of CD34+ cells in leukocytes (%)	0.024±0.003 (range: 0.012–0.046)	0.021±0.001 (range: 0.011–0.032)	0.019±0.002 (range: 0.014–0.038)
Circulating CD34+ cells (cells/μl)	0.82±0.05	0.81±0.06	0.88±0.06
Cumulative intra-assay coefficient of variation (%)	30.3	25.7	7.4

慢性期脳血管障害患者における

末梢血中 CD34 陽性細胞数と患者予後に関する

経時的コホート研究

症例報告書

〈登録時評価用〉

医療機関名

1. □ 国立循環器病センター
2. □ 国立病院機構大阪南医療センター
3. □ 星丘厚生年金病院
4. □ 兵庫医科大学医学部

症例番号 _____

担当医師名 _____

研究協力者名 _____

CD34 陽性細胞採血日

平成　　年　　月　　日

患者背景

カルテ番号：

名前：　　性別：　□男・□女

生年月日：□明治　□大正　□昭和　　年　　月　　日　　年齢：　　歳

基礎疾患

糖尿病　　(有，無)
高血圧　　(有，無)
高脂血症　　(有，無)
喫煙　　(有，無，過去に禁煙)
虚血性心疾患　　(有，無)
（心筋梗塞，狭心症）
四肢動脈閉塞症　　(有，無)
腎機能障害　　(有，無)
（血中クレアチニン濃度 2.0 以上）

介護保険利用　　(有，無)

主な内服薬

降圧薬　　(Ca Blocker，β - Blocker，ACE，利尿薬、その他)
高脂血症治療薬（スタチン，その他）
糖尿病薬　　(SU 剤，インスリン、その他)
その他

①〈脳梗塞病型分類〉

アテローム血栓性梗塞，ラクナ梗塞，心原性脳塞栓，多発性脳梗塞、奇異性脳塞栓症、
動脈解離による脳梗塞、脳血管性痴呆症、その他：＿＿＿＿＿＿

②〈Barthel Index〉

項　目	点　数	記　述	判　定　基　準
1．食事	10	自立	皿やテーブルから自力で食物をとって、食べることができる。自助具を用いてもよい。食事を妥当な時間内に終える。
	5	部分介助	なんらかの介助・監視が必要（食物を切り刻む等）
	0	全介助・不能	
2．車椅子とベッド間の移乗	15	自立	すべての動作が可能（車いすを安全にベッドに近づける。ブレーキをかける。フットレストをもちあげる。ベッドへ安全に移る。臥位になる。ベッドの縁に腰かける。車椅子の位置を変える。以上の動作の逆)。
	10	最小限の介助	上記動作（１つ以上）で最小限の介助または安全のための指示や監視が必要。
	5	移乗の介助	自力で臥位から起きあがって腰かけられるが、移乗に介助が必要。
	0	全介助・不能	
3．整容	5	自立	手と顔を洗う。整髪する。歯を磨く。髭を剃る（道具は何でもよいが、引出しからの収納も含めて道具の操作・管理が介助なしにできる)。女性は化粧も含む（ただし髪を編んだり髪型を整えることは除く)。
	0	全介助・不能	
4．トイレ動作	10	自立	トイレの出入り（腰かけ、離れを含む)、ボタンやファスナーの着脱と汚れないための準備、トイレットペーパーの使用、手すりの使用は可。トイレの代わりに差し込み便器を使用する場合は便器の清浄管理ができる。
	5	部分介助	バランス不安定、衣服操作・トイレットペーパーの使用に介助が必要。
	0	全介助・不能	
5．入浴	5	自立	浴槽に入る、シャワーを使う、スポンジで洗う、このすべてがどんな方法でもよいが
	0	全介助・不能	

6．移動	15	自立	介助や監視なしに45m以上歩ける。義肢・装具や杖・歩行器（車つきを除く）を使用してよい。装具使用の場合には立位や坐位でロック操作が可能なこと。装着と取り外しが可能なこと。
	10	部分介助	上記事項について、わずかな介助や監視があれば45m以上歩ける。
	5	車椅子使用	歩くことはできないが、自力で車椅子の操作ができる。角を曲がる、方向転換、テーブル、ベッド、トイレ等への操作など、45m以上移動できる。患者が歩行可能なときは採点しない。
	0	全介助・不能	
7．階段昇降	10	自立	介助または監視なしに安全に階段の昇降ができる。手すり、杖、クラッチの使用可。杖を持ったままの昇降も可能。
	5	部分介助	上記事項について、介助や監視が必要。
	0	全介助・不能	
8．更衣	10	自立	通常着けている衣服、靴、装具の脱着（こまかい着かたまでは必要条件としない；実用性があればよい）が行える。
	5	部分介助	上記事項について、介助を要するが、作業の半分以上は自分で行え、妥当な時間内に終了する。
	0	全介助・不能	
9．排便自制	10	自立	排便の自制が可能で失敗がない。座薬や浣腸の使用を含む。
	5	部分介助	座薬や浣腸の使用に介助を要したり、ときどき失敗する。
	0	全介助・不能	
１０．排尿自制	10	自立	昼夜とも排尿自制可能。
	5	部分介助	ときどき失敗がある。トイレに行くことや尿器の準備が間に合わなかったり、集尿バッグの操作に介助が必要。
	0	全介助・不能	

合計　　点

③〈modified Rankin Scale〉

□　０．全く障害なし
□　１．症状はあるが特に問題となる障害はない。日常生活および活動は可能
□　２．軽度の障害。以前の活動は障害されているが、介助なしに自分のことができる
□　３．中程度の障害。何らかの介助を要するが、介助なしに歩行可能
□　４．比較的高度の障害。歩行や日常生活に介助が必要
□　５．高度の障害。ベッド上の生活、失禁、常に介助が必要
□　６．死亡

④〈NIHSS〉

1a. 意識水準	□0: 完全覚醒　□1: 簡単な刺激で覚醒 □2: 繰り返し刺激、強い刺激で覚醒　□3: 完全に無反応
1b. 意識障害－質問 〈今月の月名及び年齢〉	□0: 両方正解　□1: 片方正解　□2: 両方不正解
1c. 意識障害－従命 （開閉眼、「手を握る・開く」）	□0: 両方可　□1: 片方可　□2: 両方不可
2. 最良の注視	□0: 正常　□1: 部分的注視麻痺　□2: 完全注視麻痺
3. 視野	□0: 視野欠損なし　□1: 部分的半盲 □2: 完全半盲　□3: 両側性半盲
4. 顔面麻痺	□0: 正常　□1: 軽度の麻痺 □2: 部分的麻痺　□3: 完全麻痺
5. 上肢の運動〈右〉 *仰臥位のときは45度右上肢 □9: 切断、関節癒合	□0: 90度*を10秒間保持可能（下垂なし） □1: 90度*を保持できるが、10秒以内に下垂 □2: 90度*の挙上または保持ができない □3: 重力に抗して動かない □4: 全く動きが見られない
上肢の運動〈左〉 *仰臥位のときは45度左上肢 □9: 切断、関節癒合	□0: 90度*を10秒間保持可能（下垂なし） □1: 90度*を保持できるが、10秒以内に下垂 □2: 90度*の挙上または保持ができない □3: 重力に抗して動かない □4: 全く動きが見られない
6. 下肢の運動〈右〉 *仰臥位のときは45度右上肢 □9: 切断、関節癒合	□0: 30度*を5秒間保持可能（下垂なし） □1: 30度*を保持できるが、5秒以内に下垂 □2: 重力に抗して動きが見られる □3: 重力に抗して動かない □4: 全く動きが見られない
下肢の運動〈左〉 *仰臥位のときは45度左上肢 □9: 切断、関節癒合	□0: 30度*を5秒間保持可能（下垂なし） □1: 30度*を保持できるが、5秒以内に下垂 □2: 重力に抗して動きが見られる □3: 重力に抗して動かない □4: 全く動きが見られない
7. 運動失調 □9: 切断、関節癒合	□0: なし　□1: 1肢　□2: 2肢
8. 感覚	□0: 障害なし　□1: 軽度から中等度　□2: 重度
9. 最良の言語	□0: 失語なし　□1: 軽度から中等度 □2: 重度の失語　□3: 無言、全失語
10. 構音障害 □9: 挿管または身体的障壁	□0: 正常　□1: 軽度から中等度　□2: 重度
11. 消去現象と注意障害	□0: 異常なし □1: 視覚、触覚、聴覚、視空間、または自己身体に対する不注意、あるいは1つの感覚様式で2点同時刺激に対する消去現象 □2: 重度の半側不注意あるいは2つ以上の感覚様式に対する半側不注意

⑤〈MMSE〉

	質問内容	回答	得点
1（5点）	今日は何年ですか.	年	
	今の季節は何ですか.		
	今日は何曜日ですか.	曜日	
	今日は何月何日ですか.	月	
		日	
2（5点）	ここはなに県ですか.		
	ここはなに市ですか.		
	ここはなに病院ですか.		
	ここは何階ですか.		
	ここはなに地方ですか.（例：関東地方）		
3（3点）	物品名3個〈例：りんご，電車，着物〉 検者は物の名前を1秒間に1個ずつ言う．その後，被験者に繰り返させる. 正答1個につき1点を与える．3個すべて言うまで繰り返す.〈6回まで〉 繰り返した回数＿＿回		
4（5点）	100から順に7を引く（5回まで）.		
5（3点）	3で提示した物品名を再度復唱させる.		
6（2点）	（時計を見せながら）これは何ですか.		
	（鉛筆を見せながら）これは何ですか.		
7（1点）	次の文章を繰り返す・ 「みんなで，力を合わせて網を引きます」		
8（3点）	（3段階の命令） 「右手にこの紙を持ってください」 「それを半分に折りたたんでください」 「机の上に置いてください」		
9（1点）	（次の文章を読んで，その指示に従ってください） 「眼を閉じてください」		
10（1点）	（なにか文章を書いてください）		
11（1点）	（次の図形を書いてください）		
		得点合計	

⑥〈Clinical Dementia Rating〉

	健康 (CDR 0)	痴呆の疑い (CDR 0.5)	軽度痴呆 (CDR 1)	中等度痴呆 (CDR 2)	重度痴呆 (CDR 3)
記憶	□ 記憶障害なし。時に若干もの忘れ	□ 一貫した軽いもの忘れ。出来事を部分的に思い出す良性健念	□ 中等度記憶障害、とくに最近の出来事に関するもの日常活動に支障	□ 重度記憶障害。高度に学習した記憶は保持、新しいものはすぐに忘れる	□ 重度記憶障害。断片的記憶のみ残存
見当識	□ 見当識障害なし	同左	□ 時間に対しての障害あり。検査では場所，人物の失見当なし。しかし時に地理的失見当あり	□ 常時，時間の失見当，時に場所の失見当	□ 人物への失見当識もあり
判断力と問題解決	□ 適切な判断力，問題解決	□ 問題解決の障害が疑われる	□ 複雑な問題解決に関する中程度の障害。社会的判断力は保持	□ 重度の問題解決能力の障害。社会的判断力の障害	□ 判断不能。問題解決不能
社会適応	□ 仕事，買い物，ビジネス，金銭の取り扱い，ボランティアや社会的グループで，普通の自立した機能	□ 左記の活動の軽度の障害もしくはその疑い	□ 左記の活動のいくつかにかかわっていても，自立した機能が果たせない	□ 家庭外（一般社会）では独立した機能は果たせない	同左
家庭状況および趣味，関心	□ 家での生活趣味，知的関心が保持されている	□ 同左，もしくは若干の障害	□ 軽度の家庭生活の障害。複雑な家事は障害。高度の趣味・関心の喪失	□ 単純な家事のみ限定された関心	□ 家庭内不適応
介護状況	□ セルフケア安全	同左	□ ときどき注意が必要	□ 着衣，衛生管理など身の回りのことに介助が必要	□ 日常生活に十分な介護を要する。しばしば失禁

⑦DSM-ⅢR

□	A.	記憶（短期、長期）の障害
□	B.	次のうち少なくとも１項目以上
	□	（1） 抽象的思考の障害
	□	（2） 判断の障害
	□	（3） 高次皮質機能の障害（失語、失行、失認、構成障害）
	□	（4） 性格変化
□	C.	A，Bの障害により、仕事、社会活動、人間関係が損なわれる
□	D.	意識障害のときには判断しない（せん妄の除外）
□	E.	病歴や検査から脳器質性因子の存在が推測できる

（上記A～E全てを満たしたとき痴呆有りと診断する）

判定：痴呆症　有、　なし

＜糖尿病患者におけるサブ解析＞

＜糖尿病病型分類＞

Ⅰ型糖尿病、Ⅱ型糖尿病、その他：＿＿＿＿＿＿

＜眼底所見＞　最新の検査結果（CD34陽性細胞測定より１年未満の検査）

福田分類＿＿＿＿＿

＜腎機能＞

血中クレアチニン濃度　　：＿＿＿mg/dl
血中BUN濃度　　：＿＿＿mg/dl

＜介護保険利用患者におけるサブ解析＞

記入日　平成　　年　　月　　日　（CD34陽性細胞測定より６ヶ月以内）

診断名１：　　診断名２：　　診断名３：

〈日常生活の自立度〉

・　障害老人の日常生活自立度（寝たきり度）　正常　J1　J2　A1　A2　B1　B2　C1　C2

・　痴呆性老人の日常生活自立度　正常　Ⅰ　Ⅱa　Ⅱb　Ⅲa　Ⅲb　Ⅳ　M

〈理解及び記憶〉

・　短期記憶　問題なし　問題あり

・　日常の意志決定を行うための認知能力　自立　いくらか困難　見守り必要　判断できない

・　自分の意志の伝達能力　伝えられる　いくらか困難　具体的要求に限られる　伝えられない

・　食事　自立ないし何とか自分で食べられる　全面介助

〈問題行動の有無〉　有　無

有の場合・・・幻想・幻聴　妄想　昼夜逆転　暴言　暴行　介護への抵抗　徘徊　火の不始末
不潔行為　異食行動　性的問題行動　その他（　　）

〈医学的管理の必要性〉

訪問診療　短期入所療養介護　訪問栄養食事指導
訪問看護　訪問歯科診療　その他（　　）
訪問リハビリテーション　訪問歯科衛生指導
通所リハビリテーション　訪問薬剤管理指導

過去の記録（CD34陽性細胞測定より７ヶ月以降18ヶ月以内）

記入日　平成　　年　　月　　日　（CD34陽性細胞測定より６ヶ月以内）

診断名１：　　診断名２：　　診断名３：

〈日常生活の自立度〉

・　障害老人の日常生活自立度（寝たきり度）　正常　J1　J2　A1　A2　B1　B2　C1　C2

・　痴呆性老人の日常生活自立度　正常　Ⅰ　Ⅱa　Ⅱb　Ⅲa　Ⅲb　Ⅳ　M

〈理解及び記憶〉

・　短期記憶　問題なし　問題あり

・　日常の意志決定を行うための認知能力　自立　いくらか困難　見守り必要　判断できない

・　自分の意志の伝達能力　伝えられる　いくらか困難　具体的要求に限られる　伝えられない

・　食事　自立ないし何とか自分で食べられる　全面介助

〈問題行動の有無〉　有　無

有の場合・・・幻想・幻聴　妄想　昼夜逆転　暴言　暴行　介護への抵抗　徘徊　火の不始末
不潔行為　異食行動　性的問題行動　その他（　　）

〈医学的管理の必要性〉

訪問診療　短期入所療養介護　訪問栄養食事指導
訪問看護　訪問歯科診療　その他（　　）
訪問リハビリテーション　訪問歯科衛生指導
通所リハビリテーション　訪問薬剤管理指導

過去の記録（CD34陽性細胞測定より19ヶ月以降30ヶ月以内）

記入日　平成　　年　　月　　日　（CD34陽性細胞測定より6ヶ月以内）

診断名１：　　診断名２：　　診断名３：

〈日常生活の自立度〉

- 障害老人の日常生活自立度（寝たきり度）　正常　J1　J2　A1　A2　B1　B2　C1　C2
- 痴呆性老人の日常生活自立度　正常　Ⅰ　Ⅱa　Ⅱb　Ⅲa　Ⅲb　Ⅳ　M

〈理解及び記憶〉

- 短期記憶　問題なし　問題あり
- 日常の意志決定を行うための認知能力　自立　いくらか困難　見守り必要　判断できない
- 自分の意志の伝達能力　伝えられる　いくらか困難　具体的要求に限られる　伝えられない
- 食事　自立ないし何とか自分で食べられる　全面介助

〈問題行動の有無〉　有　無

有の場合・・・幻想・幻聴　妄想　昼夜逆転　暴言　暴行　介護への抵抗　徘徊　火の不始末
不潔行為　異食行動　性的問題行動　その他（　　）

〈医学的管理の必要性〉

訪問診療	短期入所療養介護	訪問栄養食事指導
訪問看護	訪問歯科診療	その他（　　）
訪問リハビリテーション	訪問歯科衛生指導	
通所リハビリテーション	訪問薬剤管理指導	

過去の記録（CD34陽性細胞測定より31ヶ月以降42ヶ月以内）

記入日　平成　　年　　月　　日　（CD34陽性細胞測定より6ヶ月以内）

診断名１：　　診断名２：　　診断名３：

〈日常生活の自立度〉

- 障害老人の日常生活自立度（寝たきり度）　正常　J1　J2　A1　A2　B1　B2　C1　C2
- 痴呆性老人の日常生活自立度　正常　Ⅰ　Ⅱa　Ⅱb　Ⅲa　Ⅲb　Ⅳ　M

〈理解及び記憶〉

- 短期記憶　問題なし　問題あり
- 日常の意志決定を行うための認知能力　自立　いくらか困難　見守り必要　判断できない
- 自分の意志の伝達能力　伝えられる　いくらか困難　具体的要求に限られる　伝えられない
- 食事　自立ないし何とか自分で食べられる　全面介助

〈問題行動の有無〉　有　無

有の場合・・・幻想・幻聴　妄想　昼夜逆転　暴言　暴行　介護への抵抗　徘徊　火の不始末
不潔行為　異食行動　性的問題行動　その他（　　）

〈医学的管理の必要性〉

訪問診療	短期入所療養介護	訪問栄養食事指導
訪問看護	訪問歯科診療	その他（　　）
訪問リハビリテーション	訪問歯科衛生指導	
通所リハビリテーション	訪問薬剤管理指導	

〈脳梗塞による入院歴のある患者におけるサブ解析〉

退院年月日
昭和、平成　　年　　月　　日

入院時

Barthel Index　　:
NIHSS　　:
modified Rankin Scale :

退院時

Barthel Index　　:
NIHSS　　:
modified Rankin Scale :
MMSE　　:

Circulating CD34-Positive Cells Provide an Index of Cerebrovascular Function

Akihiko Taguchi, MD; Tomohiro Matsuyama, MD; Hiroshi Moriwaki, MD; Takuya Hayashi, MD; Kohei Hayashida, MD; Kazuyuki Nagatsuka, MD; Kenichi Todo, MD; Katsushi Mori; David M. Stern, MD; Toshihiro Soma, MD; Hiroaki Naritomi, MD

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 ischemia[4] 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 μL 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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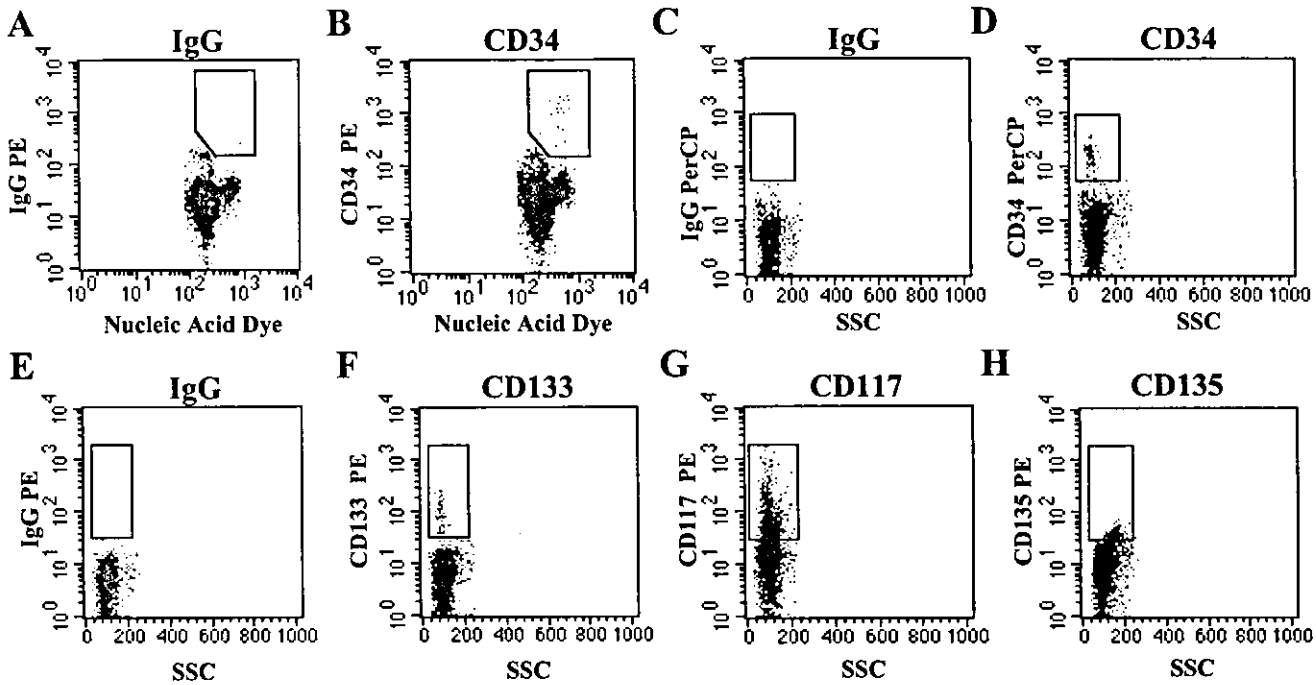

Figure 1. Quantification of circulating immature cells in patients with stroke. Nucleic acid dye versus CD34 PE dot plot was gated on lymphocytes and CD45 dim leukocytes. A, Results with an isotype-matched control antibody. B, Results with anti-CD34 antibody. Enriched immature mononuclear cells were 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 ($CMRO_2$) were quantified by conventional steady-state ^{15}O 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.

Univariant 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 Students' *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,

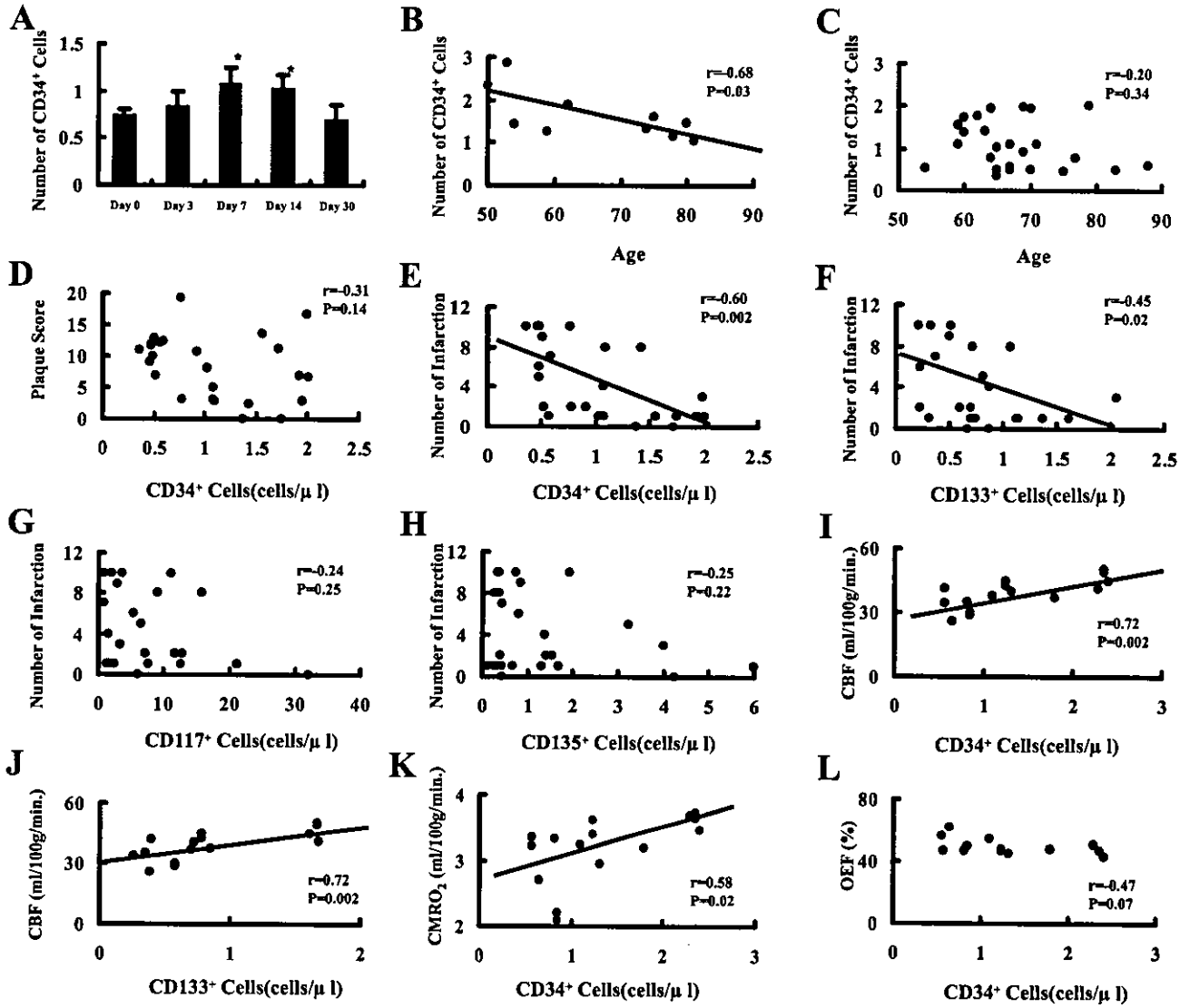

Figure 2. Levels of circulating CD34+ cells and stroke. Circulating CD34+ cells increased after the onset of stroke and peaked on day 7. A significant increase in circulating CD34+ cells was observed on days 7 and 14 (A). A decrease of circulating CD34+ cell was observed with aging in the control group (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 $CMRO_2$ (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/μL; $P=0.02$).

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 $CMRO_2$, 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.

Acknowledgments

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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); chloromethylbenzamido (CM-DiI); 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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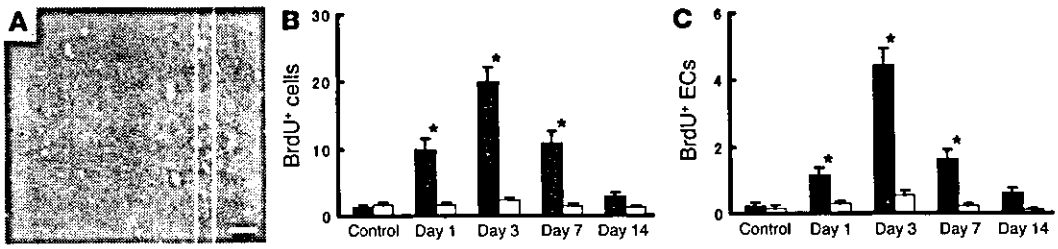

Figure 1
Endothelial proliferation in situ after stroke. On days 1, 3, 7, and 14 after stroke, the number of proliferating cells (BrdU+) and proliferating endothelial cells (co-staining for BrdU and CD31) was determined in the left cortical area of 1–1.5 mm distal from the midline. (**A**) Immunohistological 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% ± 0.1% and 0.9% ± 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 neovascularization. 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 neovascularization 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+