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Ryuji Hata, Pengxiang Zhu, Masahito Ogasawara, Fang Cao, Kazutaka Maeyama, Masahiro Sakanaka
第３回トランスポーター研究会
２００８年６月７日（土）～８日（日）、京都、京都大学薬学部

１．特許修得
無し

２．実用新案登録
無し

３．その他
無し

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研究分担報告書

心原性脳塞栓症患者に対する細胞治療の臨床試験とその発展

分担研究者
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研究要旨

慢性腎臓病（CKD）自体が動脈硬化性疾患（脳血管障害・冠動脈疾患・閉塞性動脈硬化症）に対する危険因子となることが明らかとなっている。CKD 患者の中でも慢性維持透析患者は特にハイリスク群であり、この集団の解析は非透析患者の動脈硬化性疾患の診断・予防・治療に役立つ重要な情報が得られるものと期待される。我々はこれまでの前向き研究で、透析患者では①末梢血の CD34 陽性細胞の絶対数がコントロール群と比較して有意に減少していること、② CD34 陽性細胞数低値群では CD34 陽性細胞数高値群と比較して心血管事象の新規発症と全死亡率が有意に高いことを見出し報告してきた。本年度は、危険因子としての有用性を確認する目的で CD34 陽性細胞数の再現性および安定性を解析した。前向き研究に参加した慢性維持透析患者 20 名からエントリー後３年の時点で１か月の間隔で CD34 陽性細胞数を２回追加測定し、エントリー時の値とあわせ解析したところ、それぞれの数値の間に強い相関関係が認められた。

本研究の成果から、CD34 陽性細胞の絶対数は脳および心血管事象の発症の危険因子であり、その数値は３年の間隔をおいても再現性があることから、臨床的にも有用な指標となると考えられた。

A. 研究目的

我が国の透析患者数は 27 万人を超えなお増え続けている。透析に要する医療費は 1.2 兆円以上と言われており、慢性腎不全治療が我が国の医療財政に与える影響は多大である。さらに透析患者は高率に動脈硬化性疾患を合併してくることから、透析患者において脳血管障を確立することは臨床的な意義が大きい。さらに透析患者では心血管イベントや死亡が比較的多く発生することから、予後追跡研究に透析患者を用いることで比較的短い追跡期間でより確実な結果が得られるという研究上のメリットがある。本研究は、高い頻度で重篤な後遺症を残す心原性脳塞栓症患者に対し、強い微小循環保護(新生)作用を有する自己骨髄単核球の投与を行い、その治療効果および安全性に関する臨床試験を行うと共に、組織修復作用を有する細胞群を介した、全く新しい脳血管障害予防法の確立に向けた発展的な研究を行うものである。本分担研究は、脳血管障害予防法の確立に向けた探索的検討として、ハイリスク患者である透析患者における予後を幹細胞の観点から解析するとともに、CD34 陽性細胞数の意義及び臨床的有用性を明らかにすることを目的とする臨床研究を行う。

我々は、これまでに透析患者における血管事象に着目し各種の前向き研究を進めてき

た。これまでに「ハイリスク群である透析患者では幹細胞が減少していて、このことが血管事象の発生を促進し死亡率を上昇させる。」という仮説を確かめるための前向き研究を行ってきた。従来の報告では、非透析患者では内皮前駆細胞の数が少ない者では多い者に比較して予後が不良であることが報告されていた。透析患者では血中の血管内皮前駆細胞が減少するという報告と逆に増加するという報告があり、一定のコンセンサスが得られていなかった。さらに、慢性維持透析患者の幹細胞数と予後との関連をみる研究は存在しなかった。そこで我々は、慢性維持透析患者 216 名を登録し末梢血 CD34 陽性細胞数を測定した。その後 2 年間前向きに追跡し、CD34 陽性細胞数と患者の予後との関連を解析した。エンドポイントは血管イベントと全死亡とした。その結果、透析患者ではコントロール群および脳血管疾患を有する患者群と比較して CD34 陽性細胞数が著明に減少していることが明らかとなった。さらに、ROC 解析で求めたカットオフ値 0.37/μL（AUC 0.707）を用いて、エントリー時の CD34 陽性細胞数が多い群と少ない群の 2 群に分けたところ、血管イベントおよび全死亡ともに、CD34 陽性細胞低値群で有意に高率に発生することが判明した。Cox 多変量解析では、末梢血 CD34 陽性細胞数が低値（0.37/μL 以下）であることが、新規血管イベントの発生および全死亡に対する独立危険因子であることが判明した。本年度は、この登録患者を用いて CD34 陽性細胞数の測定値の安定性・再現性を明らかにすることを目的とした研究を行った。

B. 研究方法

① 患者登録

前向き追跡研究に参加した 216 名の慢性維持患者から無作為に 20 名を抽出した。研究についての十分な説明をし、全員から同意を得て今回の研究に登録した。

② 前向き予後追跡研究

20 名の慢性維持透析患者から 1 か月の間隔をあけて 2 回採血をし立行政法人国立病院機構大阪南医療センターに郵送した。3 年前の前向き研究エントリー時に測定したのと同様に、田口らが独自に開発した方法で、末梢血 CD34 陽性細胞数（絶対数）を測定した。今回の追加採血 2 回分については、SRL にも郵送し、CD34 と KDR の二重陽性細胞数を測定した。

③ 統計的解析

今回測定した 2 回の測定値（2 回目および 3 回目）の間の相関係数と P 値を求めた。続いて、今回測定した 2 回の CD34 陽性細胞数の平均値と求め、3 年前に測定したエントリー時の測定値との相関を求めた。

（倫理面への配慮）

臨床研究に関しては、名古屋共立病院の倫理委員会、名古屋大学倫理委員会、および国立循環器センター倫理委員会の承認を得ている。血液を提供して頂く際には十分な説明の上にご本人に文書で同意を得ている。またすべての検体は匿名化して扱い、個人情報が漏洩することがないよう最大限の注意を払っている。

C. 研究結果

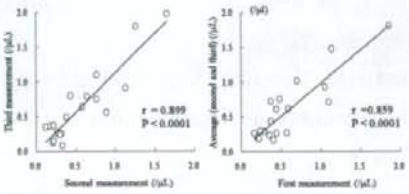
①1 か月間隔での CD34 陽性細胞数測定値の再現性

今回 1 か月の間隔で測定した 2 回の CD34 陽性細胞の絶対数を患者毎に比べたところ、両者の間には強い相関関係が認められた（r=0.899, P<0.0001）。つまり、慢性維持透析患者において、1 か月の間隔で測定した CD34 陽性細胞数の再現性は高いことが明らかになった。

②3 年間隔での CD34 陽性細胞数測定値の再現性

今回測定した2回のCD34陽性細胞数の平均値と求め、3年前に測定したエントリー時の測定値（初回測定値）との関連を解析したところ高い相関関係を認めた(r=0.859, P<0.0001)。

Figure 4



③ CD34とKDR陽性細胞数

今回20名の維持透析患者から2回採血を施行し、合計40検体についてCD34とKDRがともに陽性である細胞数を測定したが、いずれも検出感度以下であった。

D. 考察

田口らは独自にCD34陽性細胞の絶対数を正確に測定する技術を確立した。この方法を用いると、必ずしも他のマーカーを用いなくともCD34染色のみで血管内皮前駆細胞(EPC)もしくは血中の骨髄系幹細胞の数を反映するデータが導き出すことが可能である。日常的な臨床検査法としての応用を考えた場合、より単純な方法で意義のある結果が得られることは重要な点であると考える。一方、今回の検討ではCD34とKDRの両者が陽性である細胞（EPC）は全く検出されなかった。慢性維持透析患者においては内皮前駆細胞数が減少していることと関連している可能性も考えられる。

田口らが開発したCD34陽性細胞数の測定に関して、これまでに同じサンプルを用いた検討では測定値の安定性が高いことが確認されていた。しかし時間経過を追ったCD34陽性細胞数の再現性に関しては明らかでなかった。今回の検討で、1か月さらには3年の間隔をおいた測定値に関して高い相関が得られたことは、同一患者においては比較的高い再現性があることを示唆している。

昨年までの検討で慢性透析患者においてCD34陽性細胞数が予後規定因子となることを明らかにした。本年度の検討ではCD34陽性細胞数の再現性が明らかになり、予後規定因子としての臨床的有用性がさらに高まったものと考える。

E. 結論

ハイリスク群である透析患者における検討で、CD34陽性細胞の絶対数は3年間にわたって安定した再現性が得られた。幹細胞と動脈硬化性疾患との関係を明らかにする上で、CD34陽性細胞数の測定が臨床的にも有用であることが示された。本研究の成果は脳および心血管事象の新たな診断・予防・治療法の開発をめざす上で重要な知見であると考える。

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G. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

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Brief Communication

Circulating CD34-positive cells have prognostic value for neurologic function in patients with past cerebral infarction

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Increasing evidence points to a role for circulating endothelial progenitors, including populations of CD34-positive (CD34$^+$) cells present in peripheral blood, in vascular homeostasis and neovascularization. In this report, circulating CD34$^+$ cells in individuals with a history of cerebral infarction were correlated with changes in neurologic function over a period of 1 year. Patients with decreased levels of CD34$^+$ cells displayed significant worsening in neurologic function, evaluated by the Barthel Index and Clinical Dementia Rating. These results support the hypothesis that levels of circulating CD34$^+$ cells have prognostic value for neural function, consistent with their potential role in maintaining cerebral circulation.
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Introduction

Increasing evidence points to a role for circulating CD34-positive (CD34$^+$) cells in maintaining vascular homeostasis, both as a pool of endothelial progenitor cells (EPCs) and as a source of multiple growth/angiogenesis factors (Majka *et al*, 2001). Previously, we have shown accelerated neovascularization after administration of CD34$^+$ cells in an experimental model of stroke (Taguchi *et al*, 2004*b*), and observed a positive correlation between levels of circulating CD34$^+$ cells and neovascularization (Yoshihara *et al*, 2008) and regional blood flow (Taguchi *et al*, 2004*a*) in patients with chronic cerebral ischemia. In addition, we have delineated a contribution of circulating CD34$^+$ cells in support of neurologic function, presumably through their positive influence on the cerebral circulation in settings of ischemic stress (Taguchi *et al*, 2008). A role for circulating CD34$^+$ cells in vascular homeostasis has also been considered in other ischemic settings, such as myocardial (Okada *et al*, 2008) and peripheral vascular disease (Fadini *et al*, 2006*b*).

On the basis of these observations, we have hypothesized that circulating CD34$^+$ cells may contribute to the maintenance of neurologic function by enhancing cerebrovascular homeostasis in patients with a history of cerebral infarction. In this study, we have investigated the predictive value of the level of peripheral CD34$^+$ cells on neurologic function in patients with past cerebral infarction. Our results display a correlation between decreased levels of CD34$^+$ cells and diminished neurologic function over a study period of 1 year.

Methods

This study was approved by the institutional review board of the National Cardiovascular Center. All subjects provided written informed consent. A total of

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40 individuals with history of cerebral infarction (3 years or more from the last onset of stroke) were enrolled and followed for 1 year. Exclusion criteria included the following: patients who experienced a vascular event within 30 days of enrollment, patients with neurodegenerative diseases including Alzheimer's-type cognitive impairment, history of cerebral hemorrhage, cerebral infarction not classified according major causes (lacunar, atherothrombotic, or cardiogenic embolism), evidence of infection, malignant disease, and/or premenopausal women. On the day the first blood sample was obtained and 1 year after, all individuals were evaluated using the National Institutes of Health Stroke Scale (NIHSS), modified Rankin Scale (mRS), Barthel Index (BI), and Clinical Dementia Rating (CDR) by a single examiner masked to the experimental protocol and level of circulating CD34+ cells. Hypertension, hyperlipidemia, and diabetes mellitus were defined based on the need for oral anti-hypertensive, anti-hyperlipidemic, or oral anti-diabetic drug therapy (or insulin), respectively, prescribed by the primary care physician. Smoking was defined as a history of >2 years and/or smoking in the last year. Using a modification of the International Society of Hematotherapy and Graft Engineering (ISHAGE) Guidelines (Sutherland *et al*, 1996), the number of circulating CD34+ cells was quantified as described (Kikuchi-Taura *et al*, 2006) at the point of the entry and 1 year later. In brief, blood samples were incubated with phycoerythrin (PE)-labeled anti-CD34 antibody, fluorescein isothiocyanate (FITC)-labeled anti-CD45 antibody, 7-aminoactinomycin-D (7-AAD), and internal control (all of these reagents are in the Stem-Kit, BeckmanCoulter, Marseille, France). 7-AAD-positive dead cells and CD45-negative cells were excluded, and the number of cells forming a cluster characteristic of CD34+ cells (i.e., low side scatter and low-to-intermediate CD45 staining) was counted. The absolute number of CD34+ cells was calculated using the internal control. On the basis of our previous studies, the cumulative intraassay coefficient of variation of the measurement was 7.4% and test–retest intraclass correlation of the level of CD34+ cells is 0.88 (Taguchi *et al*, 2004*a*). For statistical analysis, JMP version 5.1J was used. Individual comparisons were performed using a Mann–Whitney's *U*-test, χ^2-test, or two-tailed unpaired Student's *t*-test. Pearson's correlation coefficient was used to evaluate the correlation of the levels of CD34+ cells between measurements. Mean ± s.e. is shown.

Results

To investigate the possible relationship between circulating CD34+ cells and changes in neurologic status over the 1-year-study period, individuals were divided into two groups according to the level of circulating CD34+ cells at the point of the entry. Baseline characteristics of the

Table 1 Baseline characteristic

	Total	*Group low*	*Group high*	P-*value for trend*
N	40	20	20	
At the point of entry				
No. of CD34+ cells (per μL)	0.65 ± 0.07	0.34 ± 0.03	0.93 ± 0.10	
Age (years)	73.1 ± 1.1	72.9 ± 1.4	73.4 ± 1.7	0.85
Male gender, *n* (%)	28 (70)	12 (60)	16 (80)	0.16
Time from last stroke (years)	4.5 ± 0.2	4.5 ± 0.3	4.6 ± 0.3	0.75
Etiology, n *(%)*				0.83
Lacuna	25 (63)	13 (65)	12 (60)	
Atherothrombotic	12 (30)	6 (30)	6 (30)	
Cardiogenic embolism	3 (8)	1 (5)	2 (10)	
Risk factor, n *(%)*				
Hypertension	24 (60)	12 (60)	12 (60)	1.00
Hyperlipidemia	15 (38)	8 (40)	7 (35)	0.74
Diabetes mellitus	6 (15)	4 (20)	2 (10)	0.37
Smoking	8 (20)	5 (25)	3 (15)	0.42
Other cardiovascular disease	9 (23)	3 (15)	6 (30)	0.26
Treatment, n *(%)*				
Ca-channel blockers	13 (33)	7 (35)	6 (30)	0.74
ARB	14 (35)	7 (35)	7 (35)	1.00
ACE inhibitor	3 (8)	2 (10)	1 (5)	0.54
Diuretic	2 (5)	1 (5)	1 (5)	1.00
Beta-blockers	0 (0)	0 (0)	0 (0)	NA
Aspirin	19 (48)	7 (35)	12 (60)	0.11
Ticlopidine	8 (20)	6 (30)	2 (10)	0.11
Statin	14 (35)	8 (40)	6 (30)	0.51
One year after				
No. of CD34+ cells (per μL)	0.69 ± 0.07	0.42 ± 0.05	0.97 ± 0.09	<0.001

ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; NA, not available.

groups are shown in Table 1. Comparing these groups, there were no significant differences in age, gender, etiology of cerebral infraction, hypertension, hyperlipidemia, diabetes mellitus, smoking, and drug treatments. In univariate analysis, each cerebrovascular risk factor, including hypertension ($P=0.46$), hyperlipidemia ($P=0.35$), diabetes mellitus ($P=0.12$), and smoking ($P=0.35$), was not significantly correlated with a decrease in the number of circulating CD34+ cells. Treatment with a Ca-channel blocker ($P=0.73$), angiotensin-converting enzyme (ACE) inhibitor ($P=0.053$), angiotensin II receptor blocker (ARB) ($P=0.53$), diuretics ($P=0.52$), statins ($P=0.47$), aspirin ($P=0.86$), and/or ticlopidine ($P=0.80$) also did not correlate with a consistent difference in the number of circulating CD34+ cells. Each cerebrovascular risk factor and particular drug treatment was also not associated with a significant difference in neurologic function in 1 year, based on NIHSS, mRS, BI, and CDR (data not shown). At the point of entry, there were no significant differences in neurologic or cognitive function between groups (Figures 1A–1D). Compared with levels of circulating CD34+ cells in non-stroke control subjects presented in our previous report (0.81 ± 0.06 cells/μL; age, 74.2 ± 0.7; $n=32$) (Taguchi *et al*, 2008), the level of circulating CD34+ cells was significantly reduced in patients in the CD34+ cell low group in the current study ($P<0.001$). There was no significant difference between the level of circulating CD34+ cells in the CD34+ cell high group (in the current study) and the previously reported value ($P=0.20$; Taguchi *et al*, 2008). During the period of our observation, no patients had special exercise training, other than intensive rehabilitation in patients who had recurrent strokes.

During the 12-month-study period, 5 patients had recurrent strokes (3 patients in the lower CD34+ and 2 in the higher CD34+ group, respectively; $P=0.63$ between groups). After 12 months, neurologic and cognitive functions of all patients were reexamined, and changes in each score were recorded. Although there was no significant difference in the NIHSS score between groups (Figure 1E, $P=0.28$), there was significant worsening in neurologic function, based on BI in patients with decreased levels of CD34+ cells versus the group with increased levels (Figure 1F, $P=0.04$). Similarly, a trend towards worsening of mRS occurred in patients with decreased levels of CD34+ cells versus the group with increased levels, although these results did not achieve statistical significance (Figure 1G, $P=0.65$). In terms of cognitive function, a significant worsening in the CDR score was observed in patients with decreased levels of CD34+ cells, compared with the higher CD34+ cell group (Figure 1H, $P=0.002$). It is notable that no individual in the highest quartile ($n=10$) for levels of CD34+ cells displayed worsening of the CDR or BI score over the 1-year-study period. In the analysis of the patients without a recurrent stroke, a similar trend was observed (Figures 1I–1L), although the change of BI did not achieve statistically significant ($P=0.08$). Analysis of the correlation coefficient of the levels of CD34+ cells between at the point of the entry and 1 year later revealed significant strong correlation in patients without recurrence ($P<0.001$, $R^2=0.68$).

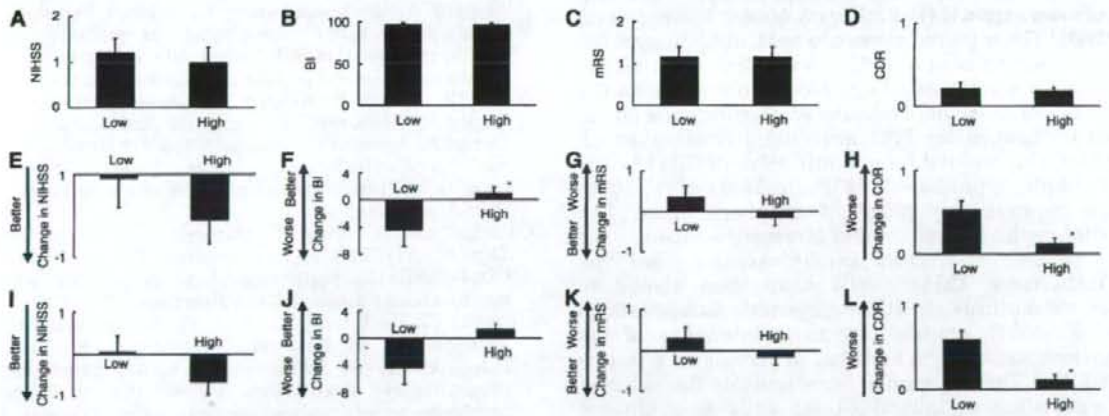

Figure 1 The level of circulating CD34+ cells and neurologic function in the study group after 1 year. (**A–D**) At the point of entry, there were no significant differences in the level of neurologic function, including NIHSS (**A**), BI (**B**), mRS (**C**), and CDR (**D**). (**E–H**) There was a trend suggesting accelerated worsening of neurologic function, evaluated by NIHSS, in patients with decreased levels of circulating CD34+ cells, although this did not achieve statistical significance (**E**). Compared with BI scores in patients with increased levels of circulating CD34+ cells, significant worsening was observed in patients with decreased levels of CD34+ cells (**F**). There was a trend of worsening of mRS in patients with decreased levels of circulating CD34+ cells, although this did not achieve statistical significance (**G**). Significantly poorer CDR scores were observed in patients with decreased levels of CD34+ cells, compared with those with increased levels of CD34+ cells (**H**). (**I–L**) Analysis of patients without recurrent strokes showed nonsignificant differences, but a similar trend was observed in changes in NIHSS (**I**), BI (**J**), and mRS (**K**). Poorer CDR scores were observed in patients with decreased levels of CD34+ cells, compared with those with increased levels of CD34+ cells (**L**), and this difference achieved statistical significance. *$P<0.05$ versus patients with decreased levels of circulating CD34+ cells.

Discussion

In this study, we have found that the level of circulating CD34+ cells has prognostic value for neural function in support of activities of daily living (BI) and cognitive function (CDR) in patients with a history of cerebral infarction. This result is potentially consistent with a role of CD34+ cells in maintenance of cerebral vasculature.

Similar to the correlation between mobilization of CD34+ cells and improved myocardial function after a coronary ischemic event (Wojakowski *et al*, 2006), mobilization of circulating CD34+ cells has been shown to correlate with functional recovery during the acute phase of cerebral infarction (Dunac *et al*, 2007; Yip *et al*, 2008). Our report herein shows a relationship between increased levels of CD34+ cells and improved functional outcome even in the extensive phase after stroke. These observations may reflect a close relationship between angiogenesis and neurogenesis under physiologic (Louissaint *et al*, 2002), as well as pathologic (Taguchi *et al*, 2004*b*) conditions.

The level of EPCs can be quantified using an assay for endothelial colony formation or fluorescence-activated cell-sorting analysis with multiple markers, including CD34 and kinase insert domain receptor (KDR) (Werner *et al*, 2005). Although the population of CD34+ cells is enriched in EPCs, it comprises multiple and heterogeneous subpopulations, indicating the possible advantage of selectively quantifying EPCs. However, measurement of EPCs is quite inexact, as large variations in their levels have been reported (i.e., by ~100-fold between reports) (Fadini *et al*, 2006*a*; Werner *et al*, 2005). Thus, there appears to be a need to standardize measurement of EPCs, in addition to a requirement for a relatively large blood volume to do the assay (for example, Loomans *et al* collected a 60 mL blood sample for EPC analysis) (Loomans *et al*, 2004). Our method for quantification of CD34+ cells is simple, reproducible (Kikuchi-Taura *et al*, 2006) and requires only 200 μL of peripheral blood. The latter method is suitable for screening a broad group of patients at risk for cerebrovascular disorders. Furthermore, CD34+ cells have been shown to secrete multiple growth/angiogenesis factors (Majka *et al*, 2001), contributing to maintenance of the microvasculature in addition to serving as a source of EPCs. These considerations indicate the value of quantitating peripheral CD34+ cells as a clinical biomarker in patients with vascular disease, not only as a substitute for quantifying EPCs.

In conclusion, our results indicate that circulating CD34+ cells in patients with cerebral ischemia have a positive impact on the course of disease, in terms of maintenance of neurologic function. In contrast, decreased levels of circulating CD34+ cells, possibly because of 'exhaustion' of the bone marrow or inability to mount an increase in cell counts, are associated with deterioration of neurologic status. Taken together with our previous results indicating that the level of circulating CD34+ cells can be correlated with cerebral blood flow and cerebral metabolic rate in patients with chronic cerebral hypoperfusion (Taguchi *et al*, 2004*a*), our present findings provide further support for a contribution of circulating CD34+ cells in maintenance of neurologic function in settings of ischemic stress. Although further basic and clinical studies will be required, we speculate that treatments with the goal of increasing levels of circulating CD34+ cells have the possibility of improving neurologic outcome in patients with impaired cerebral microcirculation.

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Conflict of interest

We declare that we have no conflicts of interest.

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Pioglitazone treatment stimulates circulating CD34-positive cells in type 2 diabetes patients

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ABSTRACT

Circulating bone marrow derived immature cells, including CD34-positive ($CD34^+$) cells, contribute to maintenance of the vasculature, not only as a pool of endothelial progenitor cells (EPCs), but also as a source of growth/angiogenesis factor. We hypothesized that the thiazolidineone compound pioglitazone could stimulate the circulating $CD34^+$ cells in diabetic patients. Thirty-four patients with type 2 diabetes received 15–30 mg pioglitazone for 24 weeks. The number of circulating $CD34^+$ cells significantly increased at 12 and continued this effect for 24 weeks (1.08 ± 0.39, 1.34 ± 0.34 and 1.32 ± 0.28 cells/μl at 0, 12 and 24 weeks, respectively). The change of $CD34^+$ cell levels ($\Delta CD34^+$ cells) between 0 and 12 weeks was significantly correlated with the change of high sensitive C reactive protein levels (Δhs-CRP) and change in adiponectin levels (Δadiponectin) ($r = -0.412$, $r = 0.359$, respectively). Our study demonstrated that pioglitazone treatment increased circulating $CD34^+$ cells, suggesting that this effect may at least partly contribute to the anti-atherosclerotic action of pioglitazone.

1. Introduction

Endothelial dysfunction plays a pivotal role in the progression of the atherosclerosis. Circulating EPCs contribute to the maintenance of vascular homeostasis and repair. They also play an important role in the maintenance of vascular endothelial function [1,2]. In diabetic patients, both a decrease in number and function of circulating EPCs are reported, suggesting that circulating EPCs participate in diabetic vascular complications [3].

Recent studies have identified circulating bone marrow derived immature cells, including $CD34^+$ cells, contribute to maintenance of the vasculature, not only as a pool of EPCs, but also as a source of growth/angiogenesis factor [4]. In fact, one recent report indicates that circulating $CD34^+$ cells are more strongly correlated with cardiovascular risk than circulating $CD34^+$/kinase insert domain receptor $(KDR)^+$ cells generally regarded as EPCs [5]. We have also reported that circulating $CD34^+$ cell levels are associated with cerebral infarction [6]. These findings indicate that persistent stimulation of $CD34^+$ cells may be a useful method to repair endothelial injury and microcirculation, and to suppress the progression of atherosclerotic disease at least theoretically. Recent experimental and clinical studies demonstrate that thiazolidinediones, peroxisome-proliferator-activated receptor γ (PPARγ) agonists, has the effects on the prevention of atherosclerosis including the maintenance of vascular endothelial function [7–9]. Therefore, we hypothesized that the thiazolidineone

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compound pioglitazone could stimulate the circulating CD34$^+$ cells in diabetic patients.

2. Methods

2.1. Study subjects

All subjects gave a written informed consent. The study was approved by the local ethics committee. Thirty-four patients with type 2 diabetes (age 60 ± 10, M/F; 18/16, HbA1c 9.3 ± 1.4%) received 15 or 30 mg pioglitazone for 24 weeks (15 mg; 31 patients, 30 mg; 3 patients). Other medications for diabetes, hypertension and hyperlipidemia were unchanged throughout the study. Insulin was given to 9 patients. Sulfonylurea was given to 15 patients. Biguanide was given to 21 patients. Alpha glucosidase inhibitor was given to 10 patients. Angiotensin converting enzyme inhibitor and/or angiotensin receptor blocker was given to 21 patients. Statin was given to 18 patients. Sixteen patients afflicted with cardiovascular diseases (CVD). Eighteen patients afflicted with nephropathy, 14 patients afflicted with retinopathy, and 15 patients afflicted with neuropathy.

2.2. Measurement of CD34$^+$ cells

Three milliliters of heparinized peripheral blood were obtained after 12-h fasting and measured CD34$^+$ cells. The precise number of circulating CD34$^+$ cells was quantified as we described previously [10]. We evaluated circulating CD34$^+$ cells with Stem-Kit™ (BeckmanCoulter, Marseille, France) according to manufacturers' protocols. These protocols are based on International Society of Hematotherapy and Graft Engineering (ISHAGE) Guidelines [11], and are frequently used for quantification of CD34$^+$ cells mobilized into peripheral blood. To increase the reproducibility of CD34$^+$ cell counts, the protocol of Stem-Kit was modified as follows: the blood sample volume, antibodies and lysing solution were doubled. After adding 30 μl of internal control (Stem count: BeckmanCoulter), samples were centrifuged for 5 min at 450 × *g* and 3860 μl of supernatant was removed carefully with a pipet. Samples were analyzed by Coulter CYTOMICS™ FC500 & XL-system II software (BeckmanCoulter) for 6 min each.

2.3. Other laboratory analysis

Blood samples were taken after 12-h fasting to measure adiponectin and, high sensitive C-reactive protein (hs-CRP) concentrations. Serum adiponectin and concentration was measured by enzyme-linked immunosorbent assay (SRL, Tokyo, Japan). Serum hs-CRP concentration was measured by latex nephelometry method (SRL, Tokyo, Japan). We also measured HbA1c, total cholesterol, HDL cholesterol and triglyceride levels.

2.4. Statistical analysis

Data was expressed using the mean ± S.D. The Student's *t*-test was used to compare parameter changes over time. The strength of correlation between variables was performed using Spearmann's correlation coefficient.

3. Results

3.1. Effects of pioglitazone on glucose and lipid metabolism

Treatment of pioglitazone significantly decreased HBA1c levels (9.3 ± 1.4, 7.4 ± 1.2 and 7.5 ± 1.7% at 0, 12 and 24 weeks, respectively). Systemic blood pressure levels did not change throughout the study period. BMI did not change throughout the study period (26.8 ± 3.2, 27.5 ± 3.0 and 27.9 ± 3.3 at 0, 12 and 24 weeks, respectively). Total cholesterol and triglyceride levels did not change throughout the study, whereas HDL cholesterol levels significantly increased at 12 and 24 weeks (1.08 ± 0.39, 1.34 ± 0.34 and 1.32 ± 0.28 mmol/l at 0, 12 and 24 weeks, respectively).

3.2. Effects of pioglitazone on adiponectin and inflammatory marker

The inflammatory marker, hs-CRP significantly decreased at 12 and 24 weeks (1518 ± 2350, 840 ± 975, and 838 ± 904 ng/ml at 0, 12, and 24 weeks, respectively). Serum adiponectin levels significantly increased at 12 and 24 weeks (5.0 ± 2.2, 13.5 ± 6.7 and 13.8 ± 8.4 μg/ml at 0, 12 and 24 weeks, respectively). The change in adiponectin levels between 0 and 12 weeks (Δadiponectin) of 30 mg pioglitazone was significantly larger than 15 mg of pioglitazone (15 mg; 7.9 ± 4.7 vs. 30 mg; 19.6 ± 2.5, $p < 0.05$), whereas there was no significant difference in the change in hs-CRP levels (Δhs-CRP) between 15 mg and 30 mg of pioglitazone (15 mg; 267 ± 322 vs. 30 mg; 480 ± 1883).

3.3. Effects of pioglitazone on circulating CD34$^+$ cell level

The number of circulating CD34$^+$ cells significantly increased at 12 and 24 weeks (0.90 ± 0.48, 1.10 ± 0.50, and 1.10 ± 0.57 cells/μl at 0, 12, and 24 weeks, respectively (Fig. 1). This effect was found in both patients with CVD and without CVD (patients with CVD; 0.81 ± 0.51, 1.05 ± 0.46 and 1.04 ± 0.50 cells/μl at 0, 12 and 24 weeks, respectively, $n = 16$, patients without CVD; 0.98 ± 0.41,

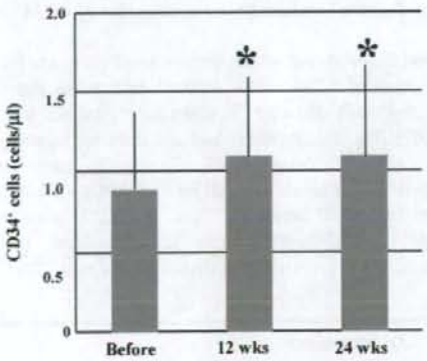

Fig. 1 – CD34+ cell level at 0, 12 and 24 weeks, *$p < 0.05$ vs. 0 week.