ORIGINAL ARTICLE

Endothelial Progenitor Cells in Patients with Acute Cerebrovascular Ischemia

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ABSTRACT: ***Objective:*** Statins have been shown to increase endothelial progenitor cells (EPCs) in patients with cardiovascular disease. However, there is no similar study that has been done on the patients recovering from cerebrovascular disease. We present the largest prospective study of statin therapy on EPC levels of patients recovering from stroke. ***Method:*** Our study subjects were treated with rosuvastatin (10 mg/day) over a period of 12 weeks. Blood was collected from these patients periodically and EPC levels were measured along with other biochemical parameters. ***Results and Conclusions:*** Our study shows that rosuvastatin treatment significantly reduces the low density lipoprotein (LDL) levels in the patients over the 12 weeks. However, we did not find any corresponding changes in the EPC levels during this time period. Earlier reports indicated that statin use could increase EPC proliferation. Our research, however, indicates that the *in-vivo* effects of rosuvastatin are not similar to those of previous reports. There may be several reasons for this lack of congruence between these two studies, including age of the study population, predominantly low high density lipoprotein (HDL) levels in our subjects and effects from other concomitant medications.

RÉSUMÉ: Cellules progénitrices endothéliales chez les patients atteints d'ischémie cérébrovasculaire aiguë. ***Objectif :*** Il a été démontré que les statines augmentent les cellules progénitrices endothéliales (CPE) chez les patients atteints de maladie cardiovasculaire. Cependant il n'existe pas d'étude similaire chez les patients en phase de récupération d'une maladie cérébrovasculaire. Nous présentons la plus grande étude prospective sur l'effet du traitement par les statines sur le niveau de CPE chez des patients en phase de récupération d'un accident vasculaire cérébral (AVC). ***Méthodologie :*** Les sujets ont reçu de la rosuvastatine (10 mg par jour) pendant 12 semaines et des prises de sang ont été faites périodiquement. Les niveaux de CPE ainsi que d'autres paramètres biochimiques ont été mesurés. ***Résultats et conclusions :*** Notre étude démontre que le traitement par la rosuvastatine a diminué significativement le niveau de LDL chez les patients au cours des 12 semaines de traitement. Cependant, nous n'avons pas observé de changement dans le niveau de CPE pendant cette période. Des études antérieures indiquaient que les statines pouvaient augmenter la prolifération des CPE. Cependant, notre étude indique que les effets de la rosuvastatine in vivo ne sont pas similaires à ceux observés dans une étude antérieure. Plusieurs raisons peuvent être invoquées pour expliquer cette divergence entre ces deux études, dont l'âge des sujets étudiés, un taux de HDL généralement bas chez nos sujets et les effets des médicaments concomitants.

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Stroke is one of the major vascular diseases affecting Western society and shares many risk factors with other vascular diseases[1]. The vascular endothelium found in blood vessels is a metabolically dynamic region; it secretes a number of cytokines that regulate vascular tone, and its dysfunction can promote atherosclerosis[2].

Endothelial dysfunction can be determined by a number of clinical tests, including anomalies in vessel wall reactivity to stress[3,4], measurement of molecules released into the blood[5], and by calculating the concentration of circulating endothelial progenitor cells (EPCs). Endothelial progenitor cells belong to a class of adult stem cells that are committed to mature into endothelial cells and are believed to have both vasculogenic and arteriogenic properties[6]. Under ischemic stress or with increasing risk to ischemic insult such as atherosclerosis, the critical of role of EPC is compromised. This results in one of the following two situations: 1) lack of mature endothelial cells in the affected area and/or 2) lack of EPC supply to replenish the vascular endothelium.

Therefore, any strategy that can increase the level of circulating EPCs can be potentially beneficial. High EPC levels have been found in subjects who have fewer vascular risk factors and EPC's are inversely related to brachial artery reactivity, a marker of vascular tone[7]. The statin class of cholesterol lowering drugs has been shown to increase EPCs *in-vitro*[8]. Clinically, treatment of patients with stable cardiovascular disease with statin therapy leads to an increase in the level of EPCs[8-10]. However, the study by Vasa et al looked at the effects of statin therapy in a small number (N=15) of stable cardiovascular patients[10].

In an earlier study conducted by us we saw that transient ischemic attack (TIA) or stroke had a measurable effect on endothelial function[11], where the level of EPCs significantly correlated with Framingham risk score in patients with

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cerebrovascular disease. Therefore, following Vasa et al[10] and our own study[11], we hypothesized that treatment with statins in patients with cerebrovascular disease would produce a similar increase in EPC levels. The results presented here are a prospective randomized study on the effects of rosuvastatin in EPCs and other vascular parameters in stable patients with TIA or ischemic cerebrovascular disease.

Methodology

Patient Recruitment

All procedures were approved by the Health Research Ethics Board of the University of Alberta, Edmonton, Canada. The subjects were recruited from the AH Owen & Family Stroke Prevention Clinic and Alberta Stroke Unit, and informed consent was obtained from them. Patients who had low density lipoprotein (LDL) cholesterol level of > 2.5 mmol per liter were considered hyperlipidemic and were invited to participate in the study. Patients were enrolled between two weeks to three months after onset of the stroke or TIA. Our previous study had shown that EPC levels remain stable in patients with acute stroke for up to three months after the initial event[11]. The study was started prior to announcement of the results of the SPARCL trial[12,13]. None of the patients had any history of cardiac disease and we considered the short six week period of non-statin treatment to be relatively safe. Statin treatment was continued in all patients who were able to tolerate it once the study was completed. We believed that this six week period of no statin therapy was important to appreciate the effects of the medication on EPC function. This part of the protocol was also discussed and approved by the Ethics Committee for Human Research at the University of Alberta.

Patients were enrolled in the study if they fulfilled all inclusion criteria; and were adult male or post-menopausal female with TIA or stroke. Only patients with ischemic stroke were approached to participate in the trial. Patients with ischemic stroke either secondary to large vessel disease or 'lacunar' sub-cortical disease were enrolled into the trial. Patients where the underlying diagnosis was suspected to be secondary to a cardiac etiology were not approached for enrollment. The diagnosis of TIA was entertained where the underlying diagnosis was similar to the above mentioned etiologies but where the patient recovered completely within less than 24 hours. Subjects had not taken statins at any time in the past. The subjects who had any of the following conditions were excluded from the study: premenopausal women, patients with hemorrhagic stroke, patients with any condition in which neovascularization might be present including hemorrhage, cancer, retinopathy, inflammatory diseases, patients with unstable angina or who had myocardial infarction in the last three months, or who had undergone any surgical procedure in the last three months prior to their participation in the study. Patients who were already enrolled in any interventional study were also excluded from this study.

Groups

The study patients were randomized into A and B arms of rosuvastatin treatment (10 mg/day dose). The patients in Group A were treated with the drug for 12 weeks, whereas the patients in Group B were treated for only six weeks, with an initial lag period of six weeks. Clinical evaluation and EPCs were assessed at three times in both groups (day 1, 6 weeks and 12 weeks). Patients maintained a diary of their daily medication and reported to the clinic every six weeks. During their visit to the clinic, their blood was collected and used for EPC culture and biochemical evaluation. We used rosuvastatin for the study as the research was in part funded by AstraZeneca and the medications were a generous gift from the company.

Thirty-six patients had TIAs, divided between the two treatment groups (22 in arm A and 14 in arm B). In patients who had an ischemic stroke, the majority of these were secondary to large vessel atherosclerosis and again there were no significant differences in the distribution between the two groups.

Cell Culture

Cells were isolated and cultured following the well established technique in our lab[11]. Briefly, 25 ml of blood was withdrawn from patients. Blood was fractionated on Ficoll (Sigma) density gradient centrifugation at 400xg for 30 minutes. Buffy coat obtained was washed twice with Phosphate Buffered Saline, and final wash was done with M199 media (Gibco) (supplemented with 20% Fetalk Bovine Serum). The cells were re-suspended in M199 and plated on fibronectin coated plates (BD Sciences) for 48 hrs. Following 48 hr incubation the non-adherent cells were collected, and re-plated (final plating) on fibronectin coated 24- well plates at the concentration of one million cells per well. The cells were allowed to culture for seven days with regular media change.

Colony Counting

Following seven days of incubation, the cells were viewed at low magnification (40X) and regular shaped colonies were counted. Average value from five wells was recorded and reported[11].

Blood Biochemistry

Blood biochemistry including lipid profile, glucose, HbA1c, homocysteine, creatinine and C reactive protein were collected as standard of care on subjects at their scheduled visits to the Stroke Prevention Clinic. These were sent to the routine clinical lab in the University of Alberta Hospital.

Statistics

Results were expressed as mean ± standard deviation, median with range for quantitative variables and number (percentages) for qualitative variables. Repeated measure of ANOVA was used to see the trend over time for all clinical characteristics. Univariate analyses were performed by using the independent sample t-test, and Mann-Whitney-U test, Pearson's chi-square test, and Fisher exact tests were used whenever appropriate to compare demographic and clinical factors between Group A vs. Group B. Repeated measure of ANOVA was also used to compare the EPC within and between the two groups A vs. B at three time points (Baseline, 4-6 weeks, and 12 week). Endothelial progenitor cells were divided by tertile and clinical characteristics, and were compared among the three groups (High count >10, Intermediate cell count 4.2 – 10, and Low cell count <4.2) by using ANOVA and Kruskal-Wallis H test if

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Table 1: Univariate comparison of Rosuvastatin A and Rosuvastatin B arms

Factor	Rosuvastatin A (n=63)	Rosuvastatin B (n=40)	P-value
Age in years	63.4 ± 11.5	63.4 ± 9.5	0.98
Gender			
Male	25 (39.7%)	26 (65%)	
Female	38 (60.3%)	14 (35%)	0.012
Systolic blood pressure	141.8 ± 24.6	134.5 ± 18.3	0.24
History of hypertension			
Yes	44 (70%)	22 (55%)	
No	19 (30%)	18 (45%)	0.13
History of diabetes			
Yes	14 (22%)	5 (13.2%)	
No	49 (78%)	35 (86.8%)	0.27
History of hyperlipidemia			
Yes	61 (96.5%)	32 (81.1%)	
No	2 (3.5%)	8 (18.9%)	0.03
History of hyperhomocystienemia			
Yes	8 (30.8%)	4 (17.4%)	
No	18 (69.2%)	19 (82.6%)	0.23
Atrial Fibrillation			
Yes	3 (3.9%)	1 (2.7%)	
No	61(96.1%)	37(97.3%)	0.98
History of smoking			
Yes	39 (62.1%)	22 (55.9%)	
No	24 (37.9%)	18 (44.1%)	0.56
Number of risk factors			
None	1 (4.3%)	3 (16.7%)	
One	2 (8.7%)	4 (22.2%)	
Two	12 (52.2%)	5 (27.8%)	0.28
Three	6 (26.1%)	4 (22.2%)	
Four to five	2 (8.7%)	2 (11.2%)	
Use of medications			
ACE/ARB		18 (17%)	
ARB		8 (8%)	
ASA		65 (63%)	
Aspirin/extended-release dipyridamole 25 mg/200 mg capsules		8 (8%)	
Clopidogrel		20 (19%)	
Thaizide		25 (24%)	
Vit B/Folate		11(11%)	

ACE/ARB=Angiotensin Converting Enzyme/Angiotensin II Receptor Bockers; ARB=Angiotensin II Receptor Blockers; ASA=Acetyl Salicylic Acid (Aspirin)

appropriate. A P-value of <0.05 was considered statistically significant and all p-values reported were two sided. All analyses were performed in SAS 9.1 TS Level 1M3 for Windows.

Results

A total of 103 subjects were recruited in the study with a mean age of 63.6 ± 10.7 years, evenly divided between males and females. Of the total patients recruited, 63 patients (stroke: 41 patients and TIA: 22 patients) 61% were treated with rosuvastatin (10 mg/day) for a period of 12 weeks (Group A); 40 patients (stroke: 26 patients and TIA: 14 patients) (39%) were treated with rosuvastatin (10 mg/day) for 6 weeks (Group B). Ninety-three patients (91%) had at least one or more risk factor at the beginning of the study (Table 1). The majority (67%) had hypertension, 19% had diabetes and 90% had a history of hyperlipidemia. Only a few subjects had a history of hyperhomocysteinemia or atrial fibrillation (Table 1). More than 60% of the patients were on aspirin. Other medications used by some patients before the start of the study included ACE inhibitors (17%), Aspirin/extended-release dipyridamole 25 mg/200 mg capsules (8%), Clopidogrel (19%), Folate (11%) and Thiazide (24%).

Univariate comparison between Rosuvastatin A and Rosuvastatin B arms of the study

There was no significant difference between A and B arms of the study in most of the descriptive parameters determined by univariate analyses method, including blood pressure, hypertension, diabetes, hyperlipidemia, hyperhomocysteemia and atrial fibrillation (Table 1). In the lipid profile, although there was a difference in the total cholesterol level between both arms of the study, P=0.02, there was no difference in the LDL, high density lipoprotein (HDL) and triglyceride between the Rosuvastatin A and B groups.

Effect of treatment over a 12 week time period

There was a decrease in the total cholesterol and LDL during 12 weeks of treatment (P<0.0001), Table 2. This response to the rosuvastatin treatment was expected and indicated the efficacy of the drug i.e. lowering cholesterol. However, there was no change in the HDL with Rosuvastatin treatment, (Table 3). Other biochemical parameters including triglycerides, fasting glucose, homocysteine and C reactive protein also remained unchanged (Table 3). There was slight but significant increase in HbA1c level in the patients during the course of the study.

Effects of Rosuvastatin treatment on EPC colonies

After 12 weeks of rosuvastatin treatment, EPC counts and other biochemical parameters showed no significant change (P=0.37) in the number of EPC colonies during this period both in Group A (n=63).

Effect of treatment over a six week time period

In the second arm (n=40) of the study there was a six week

Table 2: Comparison between rosuvastatin A and rosuvastatin B (Lipid values)

Factor	Rosuvastatin A (n=63)	Rosuvastatin B (n=40)	P-value
Total Cholesterol (mmol/L)	6.2 ± 1.0	5.6 ± 0.71	0.002
LDL(mmol/L)	3.8 ± 0.8	3.5 ± 0.7	0.07
HDL(mmol/L)	1.4 ± 0.5	1.3 ± 0.4	0.33
Triglyceride(mmol/L)	1.9 ± 0.9	1.8 ± 1.2	0.64
Ratio	4.6 ± 1.2	4.5 ± 0.8	0.84

Results are expressed as mean ± standard deviation

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Table 3: Lipid Values over a period of 12 weeks of Rosuvastatin A treatment

Factors	Baseline	4-6 Weeks	12 Weeks	P Value
Total Cholesterol(mmol/L)	6.1 ± 1.3	4.6 ± 1.2	4.1 ± 0.84	<0.0001
LDL(mmol/L)	3.7 ± 0.82	2.6 ± 1.1	2.1 ± 0.7	<0.0001
HDL(mmol/L)	1.4 ± 0.46	1.32 ± 0.36	1.32 ± 0.42	0.09
Triglyceride(mmol/L)	1.85 ± 0.99	1.58 ± 0.66	1.48 ± 0.73	0.003
Ratio	4.57 ± 1.02	3.64 ± 1.1	3.32 ± 0.90	<0.0001
Other blood parameters in patients over period of 12 weeks of Rosuvastatin A treatment				
HbA1c (%age)	3.72 ± 3.1	4.23 ± 2.94	4.79 ± 2.31	0.01
Fasting glucose(mmol/L)	5.9 ± 1.32	5.85 ± 2.02	5.62 ± 0.84	0.14
Fasting homocysteine (µmol/L)	12.4 ± 4.1	13.7 ± 11.5	11.96 ± 7.4	0.25
Creatinine (µmol/L)	85.7 ± 24.9	84.1 ± 22.6	81.45 ± 26.44	0.32
C. Reactive protein(mg/L)	2.81 ± 1.78	3.25 ± 2.72	3.34 ± 3.86	0.42

Results are expressed as mean ± standard deviation

lag period of statin treatment followed by six weeks of treatment period (Figure 1). We also did not find any differences in the patients presenting with large atherosclerosis related stroke, subcortical lacunar stroke or patients with TIAs.

Other observations of the study

To look further into the correlation of EPCs with other biochemical parameters, the EPC numbers were distributed in tertiles and clinical characteristics were compared among these three groups (High count >10, Intermediate cell count 4.2 – 10, and Low cell count <4.2), Table 4. The subjects who had a higher cell count had a lower mean age (59.8 years) compared to those who had a lower cell count (66.6 years). A positive correlation between HDL and EPC counts was also noticed. However these numbers were not significant.

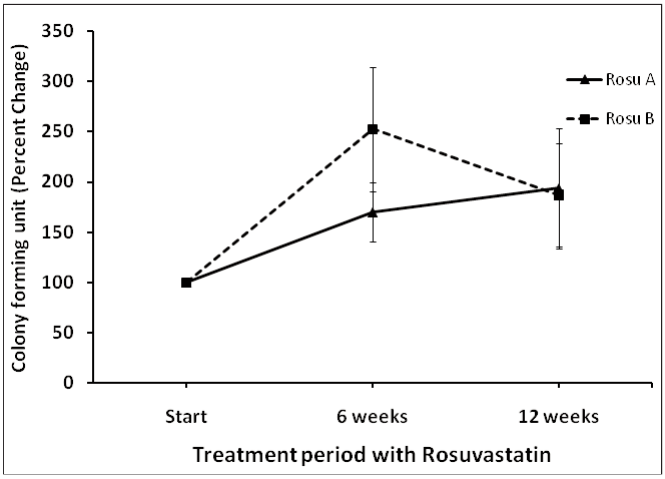

Figure 1: *Percent change in EPC colony forming units (CFU) over a period of 12 weeks in the blood of stroke patients: (Rosu A = Rosuvastatin treatment for 12 weeks. N=63; Rosu B = Rosuvastatin treatment for 6 weeks with initial lag period of 6 weeks N=40).*

DISCUSSION

We have reported earlier that cerebrovascular disease can result in decreased EPC levels[11]. Following the initial study we wanted to see if there was any way to reverse the decline in the EPCs in cerebrovascular patients. We came across a similar study done by Dimmeler's group suggesting that statins can increase EPCs in a different cohort, coronary-arterial disease (CAD), of patients[10]. The current study was therefore designed to see if the decline in EPC numbers in stroke patients can be reversed by statin treatment. Two treatment arms (6 and 12 weeks) were set-up so that the temporal efficacy of the drug could be determined. In our study we found that total cholesterol and LDL decreased significantly (P<0.0001) over a period of 12 weeks (Table 3) indicating clinical efficacy of statin treatment. Having established an important index of lowering cholesterol and LDL, we used blood samples at same time points to measure the EPCs in these patients. We used the colony counting method to determine the EPC numbers (EPC-CFU) as described by Hill and associates[14]. However we found no significant change in the EPCs over the same period of time (Figure). Arm B, where the subjects went without treatment for six weeks before the statin treatment, showed an unexpected increasing trend in EPC colonies during the first six weeks, which disappeared after the subjects were put on statin. This difference was not significant, although this trend may indicate internal compensatory mechanism towards ischemia[15].

A number of earlier studies have indicated that EPCs play an important role in reversing the effect of vascular dysfunction[6,16-23]. Dimmler et al reported that statin treatment led to an increase

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Table 4: Comparisons of clinical characteristics with tertile distribution of EPC levels

Factors	Endothelial Progenitors cell count			P-value
	High Cell Count > 10	**Intermediate Cell Count** 4.2 - 10	**Low Cell Count < 4.2**	
Age (years)	59.8 ± 11.4	63.5 ± 11.4	66.6 ± 9.4	0.06
Systolic BP (mm Hg)	135 ± 21.4	136.2 ± 19.8	140 ± 21.5	0.67
Total Cholesterol (mmol/L)	6.14 ± 0.92	5.9 ± 0.98	6.4 ± 1.9	0.36
LDL(mmol/L)	3.87 ± 0.75	3.6 ± 0.78	3.9 ± 0.61	0.15
HDL(mmol/L)	1.2 ± 0.3	1.5 ± 0.45	1.4 ± 0.34	0.07
Triglyceride(mmol/L)	2.2 ± 0.85	1.6 ± 0.67	1.86 ± 1.4	0.15
Ratio	5.15 ± 1.03	4.1 ± 0.95	4.6 ± 0.9	0.001
HbA1c (% age)	3.4 ± 3.5	3.5 ± 3.4	4.2 ± 2.7	0.62
Fasting glucose(mmol/L)	5.97 ± 1.4	5.7 ± 0.88	5.7 ± 1.1	0.61
Fasting homocysteine (μmol/L)	12.9 ± 4.5	10.7 ± 3.4	12.3 ± 3.3	0.26
Creatinine (μmol/L)	78.8 ± 10.2	89.8 ± 28.8	85.1 ± 30.5	0.18
C. Reactive protein(mg/L)	2.5 ± 1.8	3.1 ± 1.1	2.8 ± 2.3	0.74

Results are expressed as mean ± standard deviation. BP=blood pressure

in EPC proliferation[8]. We anticipated a similar effect of statin treatment on the cerebrovascular patients. However, our study indicates that statin treatment does not increase EPC levels in patients recovering from stroke (Figure 1), despite the fact that statin treatment successfully lowered the blood cholesterol/LDL levels in these patients (Table 3). We believe that there may be a number of reasons why we did not see an increase in EPCs following statin treatment in the study.

Firstly, HDL levels remained unchanged in the subjects during the course of the study (Table 3). This is important because we know from our earlier study that HDL decreases apoptosis in the EPC[24] which, in turn, may help increase the EPC numbers. This may be a contributing factor in the lack of response to rosuvastatin treatment on EPC numbers. Secondly, the lack of response to the treatment in these patients may be due to the age of the subjects recruited for the study. The mean age of the study subjects was about 64 years and it was reported earlier that the apoptosis rate increases with increasing age[25]. Interestingly, the mean age of the subjects in the CAD study was also 64 years. Other important factors such as the treatment effects of concomitant medications cannot be ruled out, (Table1) and may also be a reason for such a large variability in the colony count in this population (SEM = 29-61%), with possible outcome effects. There were also some limitations in the techniques that were used to measure EPCs. Hill and associates were the first to show the relationship between EPC colonies and cardiovascular disease[14].We have used the same colony counting method in this study[14], which is a good representation of the functional study although it may not truly represent the number of EPCs in the blood[26]. Interestingly, there are no specific markers available for the identification of EPCs[23]. In order to better quantify the EPCs, we included an additional assay using FACS (in the later stages of the study - result not shown). We used CD34 and CD31 double positive cells (N=8-10) and CD34 and KDR double positive (N=5-6) for the quantification of the EPCs. However, we did not see any change in the outcome of the results. We stopped our FACS study along with the CFU method because we believed that it would be unethical to continue the study just to increase the N values. Moreover, under normal circumstances, the naturally low numbers of EPCs restricts our ability to quantify the changes in the numbers of EPCs[23]. Interestingly, we also saw an increase in the glycated hemoglobin (HbA1c) in the patients over the statin treatment period of 12 weeks, Table 3. Similar findings have been reported from JUPITER trials[27], and this might be an interesting cause and effect relationship to be looked into. More studies that specifically look at the relationship between statin treatment and glycated hemoglobin will certainly be able to address this issue. A recent report also suggests that long-term high dose statin (40 mg/day) treatment of patients with coronary artery disease can result in reducing circulating EPCs[28].This is in stark contrast to earlier reports of increased EPC levels[10], after only four weeks of 40 mg/day atorvastatin therapy. Therefore the information about the effects of statins on EPC levels is still not clear. A more extensive study which includes both clinical and mechanistic components will be helpful in establishing the exact role of statins in EPC regulation.

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Conclusions

Our study shows that statin treatment does not increase EPC levels in patients with a history of cerebrovascular disease. It also indicates that the earlier reports of increased EPC levels with statin therapy may be restricted to a different cohort. However a clearer picture may emerge with the advent of newer techniques or improvement in the technical facilities available.

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