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European Journal of Pharmacology 562 (2007) 111–118
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Two types of circulating endothelial progenitor cells in patients receiving long term therapy by HMG-CoA reductase inhibitors

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Received 7 November 2006; received in revised form 9 January 2007; accepted 11 January 2007
Available online 1 February 2007

Abstract

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are widely used to decrease cholesterol synthesis and are well established to reduce vascular diseases. Recently, it has been proposed that statins mobilize endothelial progenitor cells from bone marrow during the first four weeks, which could help to prevent vascular diseases. However, in humans there are few data concerning the long term effects of statin treatment on these endothelial progenitor cells. We investigated whether endothelial progenitor cells can be detected and characterized in patients receiving long term statin therapy. Mononuclear cells from patients receiving or not receiving statin therapy were assessed for progenitor cell content by flow cytometry and were cultured in specific conditions to determine the number and the type of progenitors. Our results showed there were significantly more $CD34^+$, $CD34^+/CD144^+$ circulating progenitor cells in the $statin^{pos}$ group than in the $statin^{neg}$ group. In culture two types of endothelial progenitor cells were detected. Early endothelial progenitor cells gave colonies at day 5 comprising elongated cells whereas late endothelial progenitor cells generated cobblestone-like colonies with strong proliferation capacities. The number of circulating early endothelial progenitor cells was significantly higher in the $statin^{neg}$ group, while only late endothelial progenitor cells were detected in the $statin^{pos}$ group. Moreover, cells from cobblestones clearly had an endothelial phenotype $CD31^+$, $VEGF\text{-}R2^+$, $CD34^+$, $CD146^+$ in contrast to cells from colonies from early endothelial progenitor cells, which were $VEGF\text{-}R2^{low}$, $CD34^-$. These results strongly suggest that long term statin treatment specifically maintains late endothelial progenitor cells in circulation with a $CD34^+/CD144^+$ phenotype.

Keywords: Endothelial progenitor cell; Characterization; Progenitor marker; Statins

1. Introduction

In addition to lowering blood cholesterol by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, statins are used in primary and secondary prevention of ischemic heart disease. In addition to their pleiotropic effects, statins can promote neovascularization in ischemic tissue *via* the increasing of a cell population known as endothelial progenitor cells (EPCs). Indeed, it has been shown that statins activate protein kinase Akt (Kureishi et al., 2000) and the activation of PI3 kinase/Akt pathway has been shown to induce mobilization of EPCs from bone marrow (Dimmeler et al., 2001).

Since first described by Asahara and Shi, the role of EPCs in neovascularization and tissue repair has been under intense investigation (Asahara et al., 1997; Shi et al., 1998). Recent studies have shown the capacity of EPCs to promote new vessel formation and to improve myocardial function after

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doi:10.1016/j.ejphar.2007.01.045

administration in patients with acute myocardial infarction (for review see (Davani et al., 2005)). Moreover, Vasa et al. and Hill et al. have shown that the number and functional activity of cultured EPCs are inversely correlated with risk factors for coronary artery disease (Hill et al., 2003; Vasa et al., 2001a,b) and that the number of cultured EPCs can be increased in coronary artery disease patients treated with statins for 4 weeks (Vasa et al., 2001a,b). There is accumulating evidence to suggest that there are 2 different types of EPCs from circulating mononuclear cells according to their progeny observed in culture (Gulati et al., 2003; Hur et al., 2004). Indeed, early EPCs gave colonies consisting of spindle-shaped cells after 1 week with a limited proliferating potential. In contrast, colonies from late EPCs only appear after 3 weeks of culture and have been defined as being responsible for a strong growth capacity (Hur et al., 2004).

Taken together, there is a lack of data concerning the detection and the type of both early and late EPCs in the peripheral blood of patients treated by statin beyond 4 weeks.

The aims of this study were (i) to determine whether circulating EPCs can be detected in patients receiving long term statin therapy compared to those not receiving statin therapy, (ii) to determine their phenotype and to quantify them.

2. Methods

2.1. Study population

Two patient groups were recruited in this study. The first group consisted of 10 patients (statin^{pos} group) with clinically stable coronary artery disease, who were on chronic statin therapy (7 pravastatin, 40 mg/day and 3 simvastatin, 40 mg/day) for at least 4 weeks.

The second group consisted of 9 patients with clinically stable coronary artery disease, who did not receive statin (statin^{neg} group) because coronary artery disease was diagnosed subsequently. In this group statin was introduced after blood sampling.

Patients with trauma, infectious or malignant disease, diabetes type 1 or 2, anaemia (hemoglobin < 12 g/dl in women and < 13 g/dl in men), thrombocytopenia (platelets < 150,000 G/L), kidney or liver failure were excluded. Patients with myocardial infarction (< 3 months) or coronary revascularization were excluded from these groups. Clinical data for all groups are summarized in Table 1.

Written informed consent was obtained from patients before peripheral blood sampling. The protocol was approved by the local ethics committee.

2.2. Cell cultures

Twenty ml of blood was centrifuged on Hypaque-Ficoll density gradients and interface mononuclear cells were collected. After two washes in phosphate buffered saline (PBS) containing 0.1% (w/v) human serum albumin (LFFB, Courtaboeuf, France), these cells were counted using hemocytometry. The mononuclear cells were then cultured in 3 conditions to assess their potential for early and late EPCs and haematopoietic progenitor cells.

Table 1
Patient's characteristics

	Statin^{pos} (n=10)	Statin^{neg} (n=9)	P
Age (year)	68.4±3.5	68.7±4.3	0.59
Sex (% male)	72.7	100	0.22
Hypertension (%)	63.6	66.7	0.60
Smoking (%)	36.4	44.4	1.0
Diabetes (%)	0	0	
Hypercholesterolemia (%)	18.2	0	0.48
Current medications (%)			
Aspirin	90.1	66.7	0.30
Beta-blockers	72.7	33.3	0.18
ACE inhibitors	54.5	66.7	0.67
Calcium blocker	27.3	33.3	0.62
Diuretics	18.2	22.2	1.0
Clopidogrel	63.6	44.4	0.65

2.2.1. Early EPC conditions

Early endothelial progenitor potential was measured by placing 1×10^6 cells in a 24-well plate coated with fibronectin (Becton Dickinson Pharmingen, San Diego, CA) in Endocult™ liquid medium (Stemcell Technology Inc., Vancouver, Canada) containing human vascular endothelial growth factor (hVEGF), erythropoietin (hEPO), fibroblast growth factor basic (hFGFb), insulin-like growth factor-1 (hIGF-1) and 20% fetal calf serum. On days 4–7 the number of colonies cultured in the Endocult™ medium, comprising a central cluster of round cells surrounded by thin elongated cells was identified using an inverted microscope. This type of colony stopped growing after one week of culture. Adherent cells were then either cultured using conditions for late endothelial progenitors or harvested for immunophenotyping. The adherent cells from this type of colony were trypsinized and to avoid the deleterious effect of the trypsin, the cells were washed by trypsin inhibitors (PromoCell, Heidelberg, Germany) and PBS and used for flow cytometry studies.

2.2.2. Late EPC conditions

In the case of late endothelial progenitors, 10×10^6 cells were cultured as described previously (Gulati et al., 2003) in 25 cm^2 fibronectin coated flasks (Becton Dickinson Pharmingen) containing 5 ml of EGM-2 endothelial medium (PromoCell, Heidelberg, Germany). Cultures were maintained in a humidified atmosphere with 5% CO_2. According to the definition of outgrowth endothelial progenitors described by Lin et al (Lin et al., 2000), after 3 weeks, cobblestone-like colonies containing more than 5000 cells were counted. When culture dishes containing these "cobblestones" became near-confluent (week 5), cells were detached with 0.25% trypsin containing 1 mM EDTA (Gibco BRL) for 5 min at 37 °C, counted and subsequently replated with a 1/2 dilution in fibronectin coated flasks. As soon as the cultures were homogeneous (week 6–7), the cells were detached, washed and prepared for characterization. Human umbilical vein endothelial cells (HUVEC) from PromoCell were cultured in EGM-2 medium to serve as a control.

2.2.3. Haematopoietic progenitor cell conditions

Finally, mononuclear cell populations were also tested for their haematopoietic progenitor content. We plated 2×10^5 mononuclear cells in 35 mm Petri dishes in methylcellulose containing growth factors (stem cell factor, granulo-monocytic growth factor, interleukin 3 and hEPO) and 30% fetal calf serum (Stemcell Technology). After a period of 14 days at 37 °C, 5% CO_2, numbers of erythroid burst-forming units or BFU-e (multilobed compact colonies of red cells), granulo-monocytes colony-forming units or CFU-GM (round colonies containing small refringent cells) and mixed colonies or CFU-GEMM (refringent non-red cells contained within BFU-e) were determined.

2.3. Cell characterization

2.3.1. Characterization of circulating mononuclear cells

The mononuclear cells population of blood contains both haematopoietic and endothelial progenitors, which can be detected by specific markers. It is known that the CD45 membrane molecule is specific to haematopoietic cells, while CD34 sialomucin, CD117 (stem cell factor receptor) and CD133 proteins are restricted to their progenitors (Chan and Watt, 2001). Indeed, the circulating haematopoietic progenitor cells, which originate in bone marrow, can be characterized by the well-known $CD34^+/CD45^+$ phenotype (Rafii and Lyden, 2003). Circulating endothelial progenitors are thought to express CD34 and CD133 molecules, but also some other stem cell and endothelial markers, such as CD144 (VE-cadherin), CD146 (Muc18/S-endo) and VEGF-R2 (Chan and Watt, 2001).

2.3.2. Characterization of cultured mononuclear cells

The mononuclear cells from the patients groups were cultured according to the experimental procedure described previously (Gulati et al., 2003; Massa et al., 2005). Since progenitors were defined as cells with a strong capacity to proliferate and to differentiate, we only examined the CFU obtained *in vitro* in these appropriate culture conditions. Throughout 6 weeks of culture, CFUs were thus counted and then the cultured cells were assessed for the endothelial phenotype using flow cytometry.

Both mononuclear cells before culture and cells from cultures were examined using flow cytometry. The following antibodies conjugated to phytoerythrin (PE) or fluorescein isothiocyanate (FITC) were used: mouse monoclonal antibodies (Mabs) anti-human CD34, Platelet-Derived Growth Factor Receptor (CD140b) (Becton Dickinson Pharmingen), Mabs anti-human CD31, CD45, Stem Cell Factor Receptor (CD117) (Diaclone, Besançon, France), Mabs anti-human CD146 (Chemicon, Temecula, CA), Mabs anti-human CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany), Mabs anti-human VEGF-R2 (vascular endothelial growth factor-type 2 receptor) (RD, Minneapolis, MN) and rabbit polyclonal anti-human CD144 (VE-cadherin) (Valbiotech, Paris, France). Cells were washed in PBS/HSA, and primary antibodies at the appropriate concentrations were added. Antibodies of the same isotype were used as negative controls (Diaclone, Immunotech Becton Dickinson and Chemicon). The cells were passed through the Becton Dickinson FACSort (Mountain View, CA) equipped with the Cellquest software program. The fluorescence histogram for each monoclonal antibody was displayed along with that of the corresponding control antibody.

2.4. Statistical analysis

Values are given as mean±S.E.M. Comparisons between groups were made using a repeated measures analysis of variance (ANOVA) test or a nonparametric Mann–Whitney *U* test. Comparisons of categorical variables were analyzed using the Pearson X^2 test and Fishers exact test. The difference was statistically significant when $P<0.05$.

3. Results

3.1. Phenotypic studies on circulating mononuclear cells

In order to compare the $statin^{pos}$ to the $statin^{neg}$ group for their content in both haematopoietic and endothelial progenitors, we quantified the positive cells for these markers using flow cytometry among circulating mononuclear cells.

Among these mononuclear cells, there were significantly more $CD34^+$ cells in $statin^{pos}$ patients compared to $statin^{neg}$ patients. The number of $CD133^+$ cells increased in $statin^{pos}$ patients comparing to the $statin^{neg}$ group, whether it was used alone or in combination with both CD144 and CD34 proteins (Table 2). Moreover, double staining showed that the scarce populations of $CD34^+/CD144^+$ and $CD34^+/CD117^+$ were significantly greater in the $statin^{pos}$ group than in $statin^{neg}$ patients. A specific study of the endothelial antigens on mononuclear cell allowed us to identify an increase in CD146 expression within the $statin^{neg}$ group. Whatever their origin ($statin^{pos}$ or $statin^{neg}$), a large majority of $CD146^+$ cells were also negative for CD144 (Fig. 1A). Interestingly, the study of the haematopoietic progenitor phenotype CD34/CD45 showed no difference between the groups. Furthermore, these $CD45^+$ cells were also found to be negative for the endothelial specific marker VEGF-R2 (Fig. 1B).

Table 2
Progenitor phenotypic assessment on mononuclear cells from $statin^{neg}$ and $statin^{pos}$ groups

Antigens	$Statin^{neg}$	$Statin^{pos}$	$Statin^{neg}$ *vs.* $statin^{pos}$
CD34	0.7±0.1	1.3±0.1	<0.0005
CD133	0.4±0.06	0.8±0.1	NS
CD117	1.4±0.3	1.1±0.4	NS
CD146	2.1±0.5	0.5±0.1	<0.001
CD144	6.3±0.6	7.7±0.6	NS
CD34/CD45	0.3±0.1	0.9±0.2	NS
CD34/CD133	0.1±0.04	0.3±0.1	NS
CD34/VEGF-R2	0.1±0.2	0.3±0.1	NS
CD34/CD144	0.1±0.05	0.9±0.2	<0.001
CD34/CD117	0.1±0.02	0.5±0.2	<0.005
CD144/CD133	0.01±0.01	0.2±0.1	NS

NS: nonsignificant.
Values are shown as percent of positive cells (mean±S.E.M.).

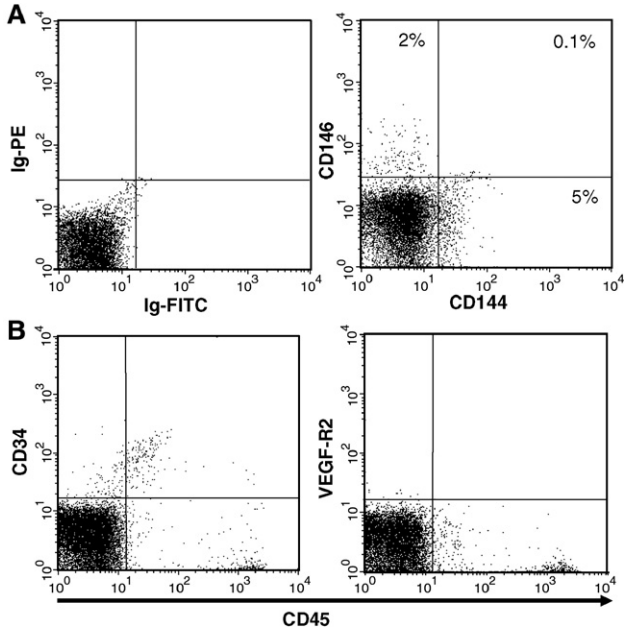

Fig. 1. Haematopoietic and endothelial antigen expression by circulating mononuclear cells from the statin^{pos} group. The specific markers of haematopoietic (CD34, CD45) and endothelial (VEGF-R2, CD144, CD146) progenitors were under particular investigation using flow cytometry. Very few endothelial CD144^{+} cells were found to be CD146^{+} (A). In addition, the haematopoietic marker CD45 was found to be expressed (B) by some CD34^{+} haematopoietic progenitors (CD34^{+}/CD45^{+}), but all CD45^{+} haematopoietic cells were also found negative for the endothelial specific marker VEGF-R2.

In summary, in circulating mononuclear cells from statin^{pos} patient blood, there were significantly more cells with a bone marrow progenitor phenotype (CD34^{+} and CD34^{+}/CD117^{+}), and endothelial progenitor phenotype (CD34^{+}/CD144^{+}), compared to the statin^{neg} group. Nevertheless, the circulating mononuclear cells population from the statin^{neg} group contained more CD146^{+} cells than found in statin^{pos} patients. In contrast, no difference was observed for the haematopoietic progenitor phenotype CD34^{+}/CD45^{+}.

3.2. Cultured endothelial progenitor detection

Using a microscope (Fig. 2A), two types of CFU were observed in culture from the mononuclear cells of the statin^{pos} patients group (given as a frequency of observed colonies per cells seeded). Spindle cells developed after 1 week, and were characterized by a central cluster of round cells surrounded by radiating thin flat cells ($10\times10^{-6}\pm6$ spindle colonies per mononuclear cells seeded) (Fig. 2B). The second type appeared only after 3 weeks and formed a typical cobblestone monolayer ($3.4\times10^{-6}\pm1$ cobblestones per mononuclear cell seeded) (Fig. 2C, D). Interestingly, mononuclear cells from statin^{neg} patients only generated the spindle colonies in culture. In the statin^{neg} group we detected significantly more spindle colonies than in the statin^{pos} ($25\pm10\times10^{-6}$ *vs.* $10\times10^{-6}\pm6$ respectively; $P<0.05$).

The cultures were then followed up to 4 weeks. Spindle cell proliferation was limited and only cells from cobblestones were

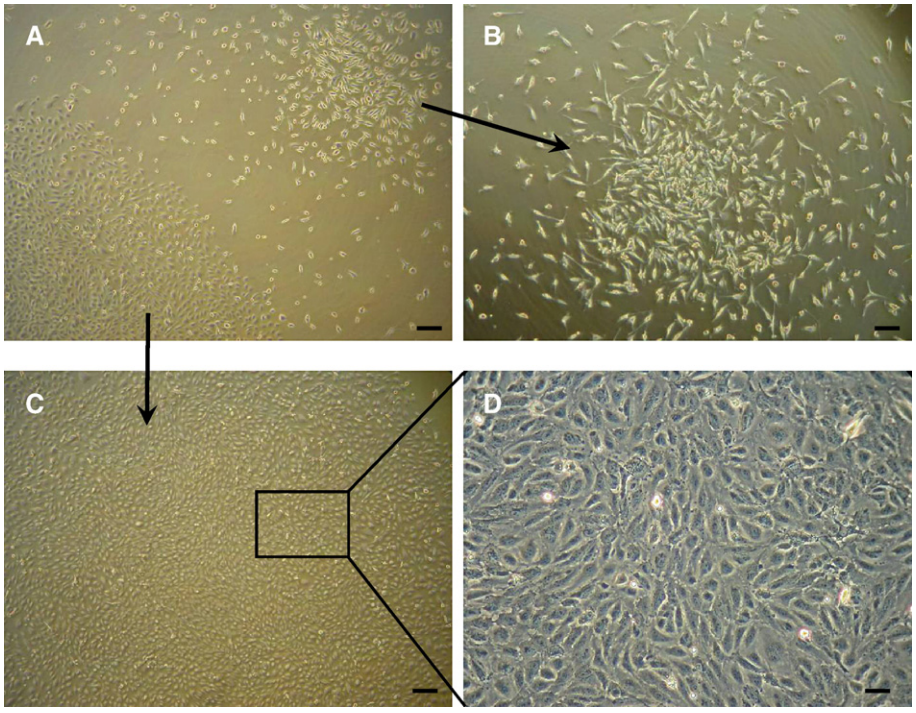

Fig. 2. Endothelial cells colonies *in vitro*. Two types of colonies were detected in cultures (A) from statin^{pos} patients: a colony of round (in the center) and spindle cells (on the periphery) (B) and a cobblestone-like colony (C and D). The colony consisted of spindle cells appearing earlier (days 4–7) than cobblestones (week 3). Magnification ×40 for A and C (scale bars=200 μm); ×100 for B and D (scale bars=50 μm).

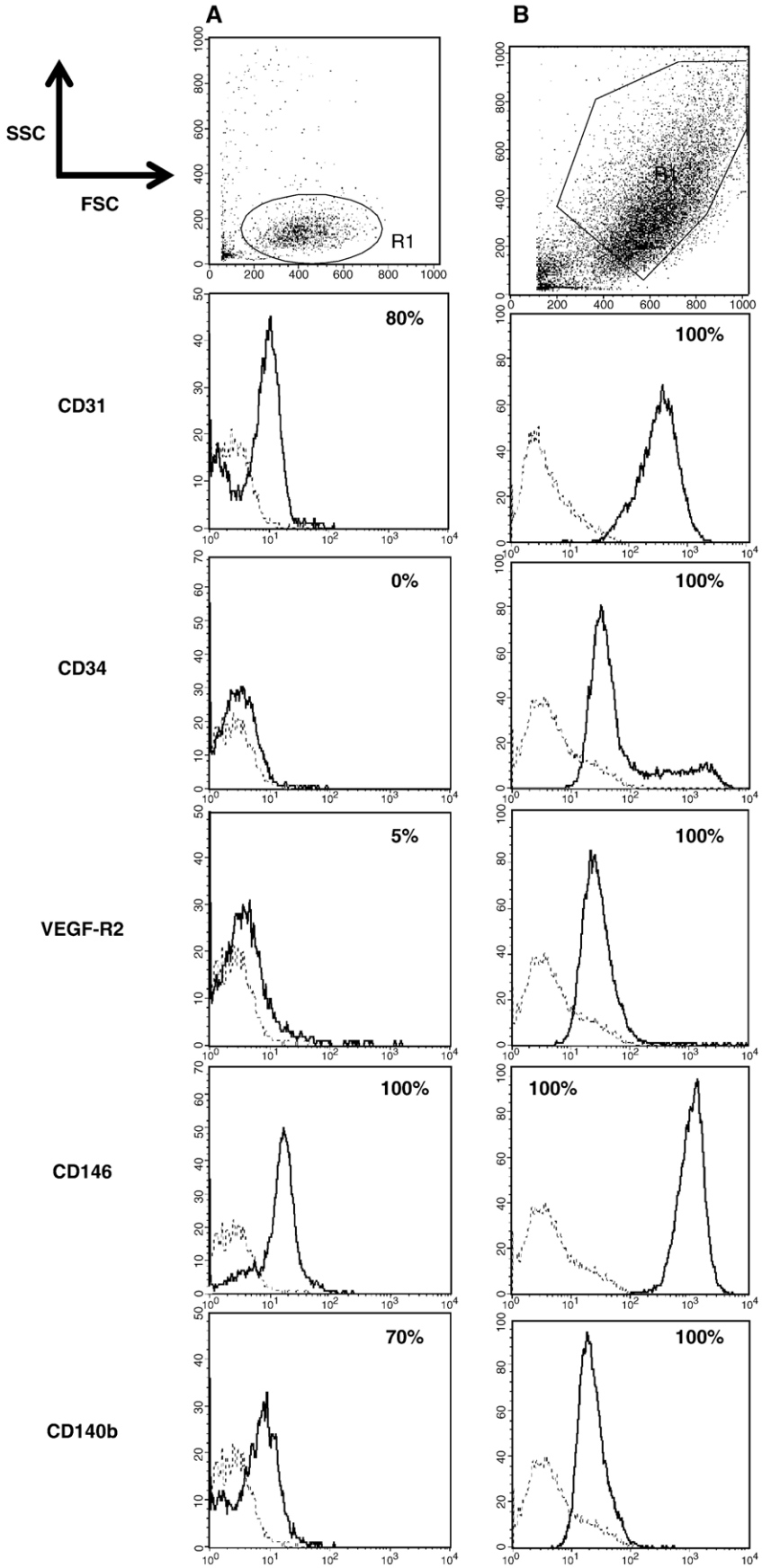

Fig. 3. Endothelial phenotype evaluation of cells from cultures. Cell populations from early (A) and late (B) colonies, appearing clearly in FSC *vs.* SSC scatter plot, were studied for the expression of endothelial specific molecules. Positivity was determined by comparing the fluorescence given by negative controls (discontinuous line) to that obtained for the targeted markers (continuous line). The mean of percentages of positive cells was determined and noted on histograms.

detected and grown. After two weeks of expansion one cobblestone generated more than 2×10^5 cells ($2.1\times10^5\pm0.9$) confirming their outgrowth potential.

The early EPC colonies were also evaluated for their late EPC content by culturing them in the same conditions as used for the growth of late EPCs. After week 3, cobblestones were identified only in cultures which gave the same type of colonies when circulating mononuclear cells were directly seeded in late EPC culture conditions. Furthermore, these cobblestones appeared in different sites of the flask where early EPCs were previously detected (Fig. 2A), which could signify a different cell origin.

Cells from cobblestones or spindle cells were harvested and phenotyped using flow cytometry. The SSC and FSC values (Fig. 3) were significantly lower for spindle cells than for cobblestone-derived cells (FSC/SSC: 400/120 *vs.* 600/250 respectively). Therefore, spindle cells were both smaller and more agranular than cells from cobblestones. In addition, cells from both types of colonies were positive for endothelial markers including CD146, CD31, CD140b and VEGF-R2 (Fig. 3), but negative for CD133 (not shown). Interestingly, CD34 and VEGF-R2 were strongly expressed by cobblestone cells (Mean fluorescence intensity or MFI=255 and 35 respectively), while they were either barely perceptible or even absent on spindle cells (MFI=8 and 20). Cells from cobblestones also showed a higher level of expression for the CD31 and CD146 endothelial-specific markers than spindle cells (MFI=372 and 1088 *vs.* 11 and 20 respectively). Finally, spindle cells were $CD45^+$ contrary to cells from cobblestones. The phenotype found for the latter cell type was similar to that observed for HUVEC (data not shown).

As described above, we found that CD146 and CD144 were expressed differently by mononuclear cells. In order to investigate their origin, cells from early EPC colonies, cobblestones and HUVEC were tested for both CD146 and CD144 expression.

Cells from early EPC colonies were either $CD144^+/CD146^-$ or $CD144^-/CD146^+$ (Fig. 4A), while a large proportion of both HUVEC and cells from cobblestones were found to be $CD146^+/CD144^+$ (Fig. 4B). We then investigated the effect of statin on HUVEC for the expression of these molecules. HUVEC were treated by 1 μM simvastatin and we observed that this expression was not affected (data not shown).

In summary, cells from cultured late colonies have i) strong proliferation capacities and ii) a more marked endothelial phenotype like those found for HUVEC, than cells from early colonies. Contrary to HUVEC, circulating mononuclear cells initiating early colonies expressed either CD146 or CD144. As the CD146 protein was significantly more expressed by circulating mononuclear cells from $statin^{neg}$ patients, who also gave more early EPC colonies, CD146 may be a marker of early EPCs. Moreover, their culture in late EPC conditions suggested that cells initiating cobblestones were different to those initiating early EPC colonies. Moreover, all cells from these early colonies in culture were of haematopoietic origin as previously reported by Rehman et al. who defined these cells as being monocytic angiogenic (Rehman et al., 2003).

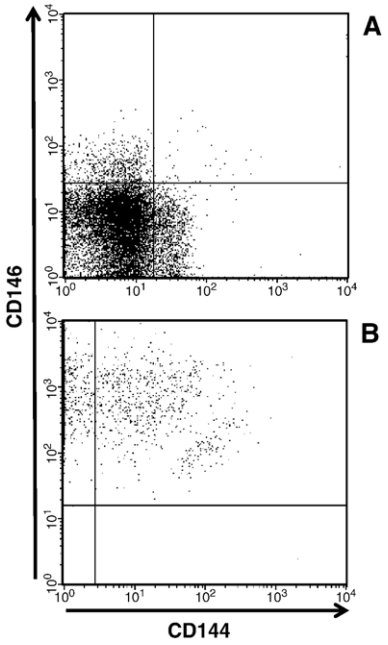

Fig. 4. The CD146 and CD144 expression by early endothelial progenitor cells (EPCs) and human umbilical vein endothelial cells (HUVEC). Cells from cultured early EPCs (A) and HUVEC (B) were incubated with monoclonal antibodies anti-endothelial markers CD146 and CD144. Cells were passed through the cytometer after washing.

3.3. Cultured haematopoietic progenitor detection

As shown above, there was an increase in progenitor cells displayed by the increase of $CD34^+$ cells in the peripheral blood of $statin^{pos}$ patients. However, the number of $CD34^+/CD45^+$ was not significantly different between $statin^{neg}$ and $statin^{pos}$ groups suggesting no increase in haematopoietic progenitor cells phenotype in patients receiving statin treatment. We assessed both the quality and quantity of haematopoietic progenitor cells using a standardized *in vitro* method.

After 10 days of culture, the numbers of different types of haematopoietic colonies — erythro-granulo-monocytic or mixed (CFU-GEMM), granulo-monocytic and erythroid colonies (CFU-GM, BFU-E and CFU-E) — were counted under an inverted microscope. No difference was observed between groups.

4. Discussion

The results of our study show that (i) EPCs can be found in the peripheral blood of patients receiving long term statin therapy, (ii) long term statin therapy raises EPC levels by increasing late EPC populations without affecting early population levels, (iii) early EPCs are different to late EPCs, and (iv) long term statin therapy did not increase levels of haematopoietic progenitor cells.

The capacity of statins to increase EPC numbers in the peripheral blood of coronary artery disease patients up to 4 weeks has already been reported (Vasa et al., 2001a,b). However, the exact type of EPCs and the capacity of statins to increase EPC levels beyond 4 weeks are poor known.

We and other investigators (Gulati et al., 2003; Hur et al., 2004) consider endothelial progenitors to be characterized by their capacity to generate a colony in appropriate conditions. This definition was taken into account in the detection, quantification and characterization of both early and late EPCs. Furthermore, as described by Lin et al. (Lin et al., 2000), late or outgrowth EPCs have to generate cobblestone-like colonies containing more than 1000 cells. In our study we detected 2 types of EPCs in culture in accordance with the findings of Hur et al. (Hur et al., 2004).

In early EPCs, a significant difference in quantity was observed between the statin^{pos} and statin^{neg} groups suggesting that chronic statin therapy decreases the mobilization of early EPCs. However, it has been previously reported that statin treatment can affect the mobilization of EPCs in patients with stable coronary artery disease (Dimmeler et al., 2001; Vasa et al., 2001a,b) although these authors did not assess the CFU potential of early EPCs. They used phenotypic methods on EPCs on day 4 of culture uptaking the dioctadecyl-tetramethy-lindo-carbocyanin-labeled acetylated low-density lipoprotein (Di-LDL) and expressing lectin (Vasa et al., 2001a,b), VEGF-R2, CD31 and Von Willebrand Factor (Dimmeler et al., 2001). The different techniques used to detect early EPCs may explain this discrepancy. Elsewhere, Dimmeler et al. have evaluated the circulation of EPCs in mice after 3 weeks of treatment by statin and Vasa et al. at 4 weeks in humans (Dimmeler et al., 2001; Vasa et al., 2001a,b). In a recent report, Hristov et al. showed that early EPCs decreased in long term statin (>8 weeks) treated patients (Hristov et al., in press). They also phenotyped them as CD34$^+$/VEGF-R2$^+$, a feature of population of cells which did not increase in our study. Thus, the increase in the number of only circulating early EPCs may therefore be transient and begin to decrease after the third week of treatment when compared to control values.

This notion is supported by that fact that the number of circulating early EPCs phenotyped on day 4 was found to stop increasing at week 4 of treatment (Vasa et al., 2001a,b; Walter et al., 2002). Kinetics studies should be performed in order to validate this hypothesis.

It has also been previously reported that early EPCs express the monocytic marker CD14 protein (Gulati et al., 2003). The CD14 positive cells from mononuclear cells were cultured and were CD31$^+$, VEGF-R2$^+$, Tie-2$^+$ cells binding lectin and incorporating Di LDL, but were CD144$^-$. However, these cells failed to generate cobblestones and their neovessel formation capacity was dramatically low. In an attempt to better identify the origin of early EPCs, Rehman et al. phenotyped cultured cells for haematopoietic markers and found that they were of monocytic origin with co-expression of the endothelial markers CD62E (E-selectin), CD144 and CD31 (Rehman et al., 2003). Another recent report described a population of cells from ovarian carcinoma known as “vascular leukocytes”, co-expressing the CD45 molecule and CD146, CD34, CD144, CD31 proteins (Conejo-Garcia et al., 2005). Therefore, both fresh and cultured early EPCs express endothelial markers, albeit of haematopoietic origin. We tested cells from early EPC colonies for CD45 expression and all were positive, which is consistent with the previous data. Therefore, it will be difficult to clearly separate or detect early EPCs using haematopoietic markers. Since it is well-known that CD144 and CD146 are specific to endothelial lineage, we assessed their expression before and after culture. Our phenotypic studies showed that mononuclear cells before the culture and cells from cultured early EPCs were either CD144$^-$/CD146$^+$ or CD144$^+$/CD146$^-$, while HUVEC were CD144$^+$/CD146$^+$. So, it seems that CD14 is not a relevant marker, and we propose CD144$^-$/CD146$^+$ and CD144$^+$/CD146$^-$ as a specific phenotype of circulating early EPCs.

We also showed that long term statin therapy maintains the number of late EPCs in the statin^{pos} group above those of the statin^{neg} group. Contrary to early EPCs, we found that late EPCs had strong proliferation capacities, which is compatible with previous studies (Hur et al., 2004; Ingram et al., 2004; Lin et al., 2000). Late or outgrowth EPCs have been shown to express both haematopoietic and fully differentiated endothelial specific markers. We found here an increase in the number of circulating CD34$^+$ and CD34$^+$/CD144$^+$ cells in the statin^{pos} group. The CD34$^+$/CD144$^+$ phenotype excludes the haematopoietic cells whereas it can be a marker of fully differentiated endothelial cells. Nevertheless, our results suggest that late EPCs would be different from fully differentiated endothelial cells in terms of proliferation. In addition, we confirmed that circulating EPCs are both CD34$^+$ and CD117$^+$, as found elsewhere (Shintani et al., 2001). Others have also shown that circulating endothelial progenitors expressed the CD133 molecule, whereas mature cells were negative (Peichev et al., 2000). Thus, CD133 marker can be used to discriminate these two types of cells.

Here, we observed an increase in circulating (before culture) CD133$^+$, CD133$^+$/CD34$^+$and CD133$^+$/CD144$^+$ cells, but without significant differences between patients. Whatever the initial expression of CD133, we found here that the cultured cells were CD133$^-$ according to Peichev et al. (Peichev et al., 2000). In light of these results, we would suggest that the phenotype CD34$^+$/CD144$^+$ is a more specific marker than CD34$^+$/VEGF-R2$^+$ for the detection of late circulating EPCs in blood.

Results obtained from flow cytometry as well as the number of haematopoietic colonies observed in semi-solid cultures show that long term statin therapy does not modify the number of circulating haematopoietic progenitor cells. These results are consistent with those of Vasa et al. (Vasa et al., 2001a,b).

Chronic statin therapy can be argued to specifically increase EPCs for neoangiogenesis without the adverse events caused by the mobilization of haematopoietic progenitor cells in contrast to G-CSF treatment that has induced stent re-stenosis, probably caused by an increase in white blood cells (Kang et al., 2004).

In summary, our data did not allow us to explore the functional properties of late EPCs. Nevertheless, Hur et al. have hypothesized that late EPCs are involved in neovasculogenesis (Hur et al., 2004), and here we confirmed that they possess a strong proliferation capacity which is crucial for tissue regeneration. Long term statin therapy could thus play an important role in maintaining late EPCs in the peripheral blood without raising the number of other progenitors, particularly early EPCs. The latter type of cells were reported to be highly angiogenic

because of their cytokine expression potential (VEGF, HGF, G-CSF and GM-CSF), their endothelial-like phenotype (Rehman et al., 2003) and their capacities to home to sites of limb ischemia improving neovascularization (Chavakis et al., 2005). We can suggest that early EPCs are haematopoietic cells capable of homing to all sites which have to be revascularized and then of building a specific "niche" for the real endothelial progenitors. Thus, there are sequential events: after the circulation of early EPCs, late EPCs can be induced to circulate as sentinels until they are attracted by an angiogenic "niche". This hypothesis has yet to be demonstrated, but strengthens the clinical improvement of statins.

In addition, our results provide information about the characterization of regenerative EPCs, crucial for establishing a standardized method for detecting EPCs in research and for their use in clinical settings.

5. Limitations

The principal limitations of this study were the low number of patients in the groups and the fact that the patients from statin^{pos} and statin^{neg} groups were not matched for different parameters. However, since there were no significant differences between these two groups, we considered that the differences observed between groups were relative to statin therapy.

Acknowledgments

ZS was supported by a grant from Conseil Scientifique de l'Etablissement Français du Sang. This work was supported by grants from the Ligue Contre le Cancer (Doubs) and the Fondation pour la Transplantation (ET-040615).

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