Number and activity of endothelial progenitor cells from peripheral blood in patients with hypercholesterolaemia

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ABSTRACT

Hypercholesterolaemia contributes to atherosclerosis and coronary artery diseases by inducing endothelial cell injury and dysfunction. Recent studies have provided increasing evidence that EPCs (endothelial progenitor cells) participate in ongoing endothelial repair and postnatal neovascularization. However, the changes in EPCs in patients with hypercholesterolaemia have not been elucidated to date. Therefore we investigated the number and functional activity of EPCs in patients with hypercholesterolemia. Total MNCs (mononuclear cells) were isolated from 20 patients with hypercholesterolaemia and 20 matched control subjects. EPCs were characterized as adherent cells double-positive for DiI-LDL (1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanide percholate-labelled low-density lipoprotein) uptake and lectin binding by direct fluorescent staining under a laser scanning confocal microscope, and were characterized further by demonstrating the expression of KDR (kinase insert domain-containing receptor), CD34 and AC133 by flow cytometry. Proliferation, migration and in vitro vasculogenesis activity of EPCs were assayed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay, modified Boyden chamber assay and an in vitro vasculogenesis kit respectively. EPC adhesion assay was performed by replating cells on fibronectin-coated dishes and then counting the adherent cells. As a result, the number of EPCs was significantly reduced in patients with hypercholesterolaemia compared with that in control subjects (41.8 ± 8.7 compared with 64.5 ± 16.6 EPCs/× 200 field respectively; P < 0.05). The number of EPCs was inversely correlated with total cholesterol (r = −0.659, P < 0.001) and LDL-cholesterol (r = −0.611, P < 0.001) levels. In addition, the functional activities of isolated EPCs, such as proliferative, migratory, adhesive and in vitro vasculogenesis capacity, were also impaired. In conclusion, the results of the present study may state a novel pathophysiological mechanism of hypercholesterolaemia: the reduction of EPCs with decreased functional activity.

INTRODUCTION

Hypercholesterolaemia is a major risk factor for atherosclerosis and coronary artery disease. It is widely appreciated that endothelial dysfunction elicited by hypercholesterolaemia plays a critical role in the development of atherosclerosis [1]. Endothelial dysfunction ultimately represents a balance between the magnitude of...
injury and the capacity for repair [2]. A variety of evidence suggests that circulating EPCs (endothelial progenitor cells) constitute one aspect of this repair process [2,3]. EPCs are a cell population that have the capacity to circulate, proliferate and differentiate into mature endothelial cells, but have not yet acquired the characteristic mature endothelial markers and have not yet formed a lumen [4,5]. Laboratory evidence suggests that these precursors participate in postnatal neovascularization and re-endothelialization [2–4,6–11]. In addition, it has been shown recently [2,12] that patients at risk for coronary artery disease have decreased numbers of circulating EPCs with impaired activity.

We hypothesized that hypercholesterolaemia not only directly impairs endothelial cells, but also affects EPC number and function at the same time, thus influencing the endothelial repair process and disturbing the balance between the magnitude of injury and the capacity for repair, which leads to endothelial dysfunction and promotes the progression of CAD (coronary artery disease). To test this hypothesis, in the present study, we have measured the number and activity of EPCs from peripheral blood of patients with hypercholesterolaemia.

**MATERIALS AND METHODS**

**Characteristics of the patients**

Peripheral blood samples were collected from patients with hypercholesterolaemia ($n = 20$) or control subjects ($n = 20$). The diagnosis of hypercholesterolaemia was according to the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) [13]. Clinical data for patients with hypercholesterolaemia and control subjects are shown in Table 1. Volunteers were matched in terms of medications, and were all free of wounds, ulcers, retinopathy, recent surgery, inflammatory or malignant disease that may influence EPC kinetics. Patients with unstable angina or myocardial infarction within the previous 3 months were excluded. None of the patients was treated with statins. Coronary artery disease was defined angiographically ($> 50\%$ diameter reduction). Informed consent was obtained from all participants, and all of the procedures were done in accordance with national and international laws and policies.

**Isolation and cultivation of EPCs**

EPCs were cultured according to techniques described previously [2,14,15]. Briefly, total MNCs (mononuclear cells) were isolated from blood of patients with hypercholesterolaemia or control subjects by Ficoll density gradient centrifugation. Cells were plated on to culture dishes coated with human fibronectin (Chemicon) and maintained in medium 199 (Sigma) supplemented with 20 % FCS (fetal calf serum), 10 ng/ml VEGF (vascular endothelial growth factor; Chemicon), 100 units/ml penicillin and 100 μg/ml streptomycin. After 4 days in culture, non-adherent cells were removed by washing with PBS, fresh media was added, and the culture was maintained until 7 days.

**Cellular staining**

Fluorescent chemical detection of EPCs was performed on attached MNCs after 7 days in culture. Direct fluorescent staining was used to detect dual binding of FITC-labelled UEA-1 (*Ulex europaeus* agglutinin; Sigma) and DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchorlate)-labelled acetylated LDL (low-density lipoprotein; Molecular Probes). Cells were incubated with DiI-LDL at 37 °C and then fixed with 2 % paraformaldehyde for 10 min. After washing, cells were treated with UEA-1 (10 μg/ml) for 1 h. After staining, samples were viewed with an inverted fluorescent microscope (Leica) and demonstrated further by laser scanning confocal microscope (Leica). Cells demonstrating double-positive fluorescence were identified as differentiating EPCs [12,14–16]. Two or three independent investigators evaluated the number of EPCs/well by counting 15 randomly selected high-power fields ($\times 200$) with an inverted fluorescent microscope.

**Flow cytometry analysis**

FACS detection of EPCs was performed on attached MNCs after 7 days in culture. MNCs were detached

<table>
<thead>
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<th>Characteristics</th>
<th>Patients with hypercholesterolaemia</th>
<th>Control subjects</th>
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<tr>
<td>$n$</td>
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<tr>
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<td>Diabetes (%)</td>
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<td>Total cholesterol (mmol/l)</td>
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<td>$5.05 \pm 0.35$</td>
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<td>LDL-cholesterol (mmol/l)</td>
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<td>$3.31 \pm 0.24$</td>
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<td>HDL-cholesterol (mmol/l)</td>
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<tr>
<td>Nitrates (%)</td>
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Table 1 Clinical data for patients with hypercholesterolaemia and control subjects

Values are means ± S.D., or percentages; *P < 0.05 compared with control subjects. HDL, high density lipoprotein; ACEI, angiotensin-converting enzyme inhibitor.
with 0.25 % trypsin, followed by repeated gentle flushing through a pipette tip. Cells (2 × 10^5) were incubated for 30 min at 4 °C with anti-VE-cadherin (vascular endothelium-cadherin; Chemicon) and phycoerythrin-conjugated monoclonal antibodies against KDR (kinase insert domain-containing receptor; R&D), CD34 and AC133 (Miltenyi Biotec). A FITC-conjugated anti-mouse antibody (Vector) was added for staining with VE-cadherin. Isotype-identical antibodies served as controls. After treatment, cells were fixed in 1 % paraformaldehyde. Quantitative FACS was performed on a FACStar flow cytometer (Coulter) [12,14].

**Migration assay**

EPC migration was evaluated by using a modified Boyden chamber assay (Jiangsu Qilin Medical Equipment Factory). Briefly, isolated EPCs were detached using 0.25 % trypsin, harvested by centrifugation, resuspended in 500 µl of M199 and counted. EPCs (2 × 10^4 cells) were placed in the upper chamber of a modified Boyden chamber. M199 and human recombinant VEGF (50 ng/ml) were placed in the lower compartment of the chamber. After 24 h of incubation at 37 °C, the lower side of the filter was washed with PBS and fixed with 2 % paraformaldehyde. For quantification, cells were stained with Giemsa solution. Cells migrating into the lower side of the filter was washed with PBS and fixed 1 % paraformaldehyde. Quantitative FACS was performed on a FACStar flow cytometer (Coulter) [12,14].

**Cell adhesion assay**

Human EPCs were washed with PBS and gently detached with 0.25 % trypsin. After centrifugation and resuspension in M199/5 % (v/v) FCS, identical cell numbers were replated on to fibronectin-coated culture dishes and incubated for 30 min at 37 °C. Adherent cells were counted by independent investigators [3,15].

**EPC proliferation assay**

EPC proliferation was determined by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay. After being cultured for 7 days, EPCs were digested with 0.25 % trypsin and cultured in serum-free medium in 96-well culture plates (200 µl of medium/well). EPCs were supplemented with 10 µl of MTT (5 mg/ml) and incubated for a further 6 h. The supernatant was then discarded by aspiration and 200 µl of DMSO was added to the EPC preparation and shaken for 10 min. After shaking, A was measured at 490 nm [15].

**In vitro vasculogenesis assay**

*In vitro* vasculogenesis assay was performed using an In Vitro Angiogenesis Assay Kit (Chemicon), according to the manufacturer’s instructions. Briefly, ECMatrix™ solution was thawed on ice overnight, mixed with 10 × ECMatrix™ Diluent and placed in a 96-well tissue culture plate at 37 °C for 1 h to allow the matrix solution to solidify. EPCs were harvested as described above and replated (1 × 10^4 cells/well) on top of the solidified matrix solution. Cells were incubated at 37 °C for 24 h. Tubule formation was inspected under an inverted light microscope at × 200 magnification. Tubule formation was defined as a structure exhibiting a length four times its width [15,17,18]. Five independent fields were assessed for each well, and the mean number of tubules/× 200 field was determined.

**Statistical analysis**

All data are presented as percentages or means ± S.D. Differences between group means were assessed by Student’s t test for single comparisons. Categorical variables were compared using the χ^2 test. Linear regression analysis was used to compare bivariate correlation. The interaction between LDL-cholesterol and EPC number and activity was examined by multivariate analysis using the multiple stepwise logistic regression model. A value of P < 0.05 was considered significant. All statistical analyses were performed with SPSS 9.0.

**RESULTS**

**Characteristics of human EPCs**

Total MNCs isolated and cultured for 7 days resulted in a spindle-shaped endothelial cell-like morphology (Figure 1). EPCs were characterized as adherent cells double-positive for Dil-LDL uptake and lectin binding under a laser scanning confocal microscope (Figure 2). These cells were characterized further by demonstrating the expression of VE-cadherin (76 ± 8.6 %), KDR (78 ± 7.8 %), CD34 (28.7 ± 6.9 %) and AC133 (17.1 ± 8.1 %) by flow cytometry (Figure 3).
Figure 2  Characterization of EPCs by confocal microscopy
Mononuclear cells were cultured for 7 days and adherent cells were assessed under a laser scanning confocal microscope for lectin binding (A1 and B1; green; excitation wavelength, 477 nm) and Dil-LDL uptake (A2 and B2; red; excitation wavelength, 543 nm). Double-positive cells, appearing in yellow in the overlay (A3 and B3), were identified as differentiating EPCs. Magnification, ×400. A1–A3, EPCs from patients with hypercholesterolaemia; B1–B3, EPCs from control subjects. The number of EPCs was significantly lower in subjects with hypercholesterolaemia compared with control subjects.

Figure 3  Flow cytometric analysis of EPCs from patients with hypercholesterolaemia and control subjects
Flow cytometric analyses of adherent cells at day 7 of culture (n = 6). Adherent cells were positive for KDR (78 ± 7.8 %), CD34 (28.7 ± 6.9 %), and AC133 (17.1 ± 8.1 %). Data are percentage of positive cells. The left-hand curve represents the isotype control.

Influence of hypercholesterolaemia on EPC number
The number of EPCs was significantly lower in subjects with elevated serum cholesterol levels compared with age-matched control subjects (41.8 ± 8.7 compared with 64.5 ± 16.6 EPCs/×200 field respectively; P < 0.05; see Figure 2). The number of EPCs was inversely correlated with total cholesterol (Figure 4A) and LDL-cholesterol (Figure 4B) levels, whereas no correlation between the number of EPCs and triacylglycerols (r = −0.239, P > 0.05) or HDL-cholesterol levels (r = 0.237, P < 0.05) was observed. By multivariate analysis, total cholesterol (standard coefficient = −0.530, P < 0.001) and LDL-cholesterol (standard coefficient = −0.417, P < 0.01) levels remained independent predictors of lower EPC numbers.

Effects of hypercholesterolaemia on EPC proliferation
The effect of hypercholesterolaemia on EPC proliferation was determined using the MTT assay. Hypercholesterolaemia decreased the proliferative activity of EPCs.
Figure 4 Correlation between the number of EPCs from patients with hypercholesterolaemia and total cholesterol (A) and LDL-cholesterol (B) levels

Figure 5 Correlation between the proliferation of EPCs from patients with hypercholesterolaemia and total cholesterol (A) and LDL-cholesterol (B) levels

Figure 6 Correlation between the migratory activity of EPCs from patients with hypercholesterolaemia and total cholesterol (A) and LDL-cholesterol (B) levels

Effects of hypercholesterolaemia on EPC migration

The effect of hypercholesterolaemia on EPC migration was analysed in a modified Boyden chamber assay. Hypercholesterolaemia profoundly impaired cell migration compared with control subjects (15.1 ± 3.8 compared with 6.3 ± 2.9 cells/×400 fields respectively; P < 0.05). EPC migratory activity was inversely correlated with total cholesterol (Figure 6A) and LDL-cholesterol (Figure 6B) levels, whereas neither triacylglycerols (r = −0.254, P > 0.05) nor HDL-cholesterol levels (r = 0.311, P > 0.05) correlated with EPC migratory activity. Multivariate analysis revealed that total cholesterol (standard coefficient = −0.625, P < 0.001) and LDL-cholesterol (standard coefficient = −0.642, P < 0.001) levels were associated with a significant reduction in EPC migration.
Effects of hypercholesterolaemia on EPC adhesiveness

To study the possibility that hypercholesterolaemia altered the adhesiveness of cultured human EPCs, EPCs were detached with 0.25% trypsin, and then replated on fibronectin-coated dishes. As a result, EPCs from patients with hypercholesterolaemia exhibited a significant decrease in the number of adhesive cells at 30 min compared with control subjects (20.2 ± 4.6 compared with 34.8 ± 9.7 respectively; \( P < 0.05 \)). The number of adhesive cells was inversely correlated with total cholesterol (Figure 7A) and LDL-cholesterol (Figure 7B) levels, whereas neither triacylglycerols (\( r = -0.242, P > 0.05 \)) nor HDL-cholesterol levels (\( r = 0.213, P > 0.05 \)) were correlated with the number of adhesive cells. By multivariate analysis, total cholesterol (standard coefficient = −0.496, \( P < 0.001 \)) and LDL cholesterol (standard coefficient = −0.517, \( P < 0.001 \)) levels were associated with a significant reduction in the adhesiveness of EPCs.

Effects of hypercholesterolaemia on EPC vasculogenesis

Recent studies have demonstrated [4,11] that circulating EPCs home into sites of neovascularization and differentiate into endothelial cells \textit{in situ} in a manner consistent with a process termed vasculogenesis. An \textit{in vitro} vasculogenesis assay was used to simulate this process and was used to investigate the ability of EPCs to participate in neovascularization, which is the most important activity of EPCs. An example of vasculogenesis in this assay, determined by means of tubule formation of EPCs, is shown in Figure 8(A). The number of tubules formed from EPCs from patients with hypercholesterolaemia was less than those from control subjects (14.2 ± 4.6 compared with 25.8 ± 5.1 tubules/×200 field respectively; \( P < 0.05 \)). Tubule number was inversely correlated with total cholesterol (Figure 8B) and LDL-cholesterol (Figure 8C) levels, whereas neither triacylglycerols (\( r = -0.304, P > 0.05 \)) nor HDL-cholesterol levels (\( r = 0.291, P > 0.05 \)) correlated with tubule number. Moreover, tubules in the hypercholesterolaemia group were qualitatively different and less complex than those in the control subjects. Multivariate analysis revealed that total cholesterol (standard coefficient = −0.584, \( P < 0.001 \))
and LDL-cholesterol (standard coefficient = \(-0.603\), \(P < 0.001\)) levels were associated with a significant reduction in EPC vasculogenesis.

**DISCUSSION**

The present study has demonstrated that the number of EPCs was significantly reduced in patients with hypercholesterolaemia, and was inversely correlated with total cholesterol and LDL-cholesterol levels. In addition, the functional activities of isolated EPCs, such as proliferative, migratory, adhesive and *in vitro* vasculogenesis capacity, were also impaired. Moreover, LDL-cholesterol in the patients was the most powerful predictor of EPC changes. These data are consistent with those reported recently by Hill et al. [2]. In that study, these workers examined EPCs in 45 men without known cardiovascular disease and observed an inverse correlation between the number of circulating EPCs and serum cholesterol levels with logistic analysis. They also observed an inverse correlation between LDL-cholesterol levels and number of circulating EPCs. However, this relationship was not statistically significant in their study [2]. The difference in characteristics of study subjects between the present study and that of Hill et al. [2] may account for this difference. Vasa et al. [12] used an analogous technique to examine circulating EPC levels and migratory activity in patients with coronary artery disease. In that study, similar trends were found, but did not reach statistical significance. The greater number of patients with hypercholesterolaemia enrolled in the present study, as well as the longer culture period (7 days compared with 4 days), may have produced this difference.

Endothelial damage ultimately represents the balance between the magnitude of injury and the capacity for repair [2]. A variety of evidence suggests that hypercholesterolaemia induces endothelial injury and that impaired endothelial function reflects this ongoing injury. However, the mechanisms by which the vessel wall undergoes repair remain unclear. Local migration and proliferation of endothelial cells adjacent to the site of injury had been regarded as the principal mechanism of endothelial repair until Asahara et al. [4], in 1997, described circulating EPCs [4]. These cells are bone-marrow-derived and have the capacity to target to sites of endothelial injury, where they incorporate into the endothelium and thereby repair the defects [19]. More recently, studies have shown that EPCs contribute up to 25% of endothelial cells in newly formed vessels [20,21].

However, our present findings, together with other investigators [2,12], have documented that hypercholesterolaemia can decrease EPC number and activity. Given the well-established role of EPCs in participating in neovascularization and re-endothelialization, our findings, together with those of others [2,12], may establish a novel pathophysiological mechanism of hypercholesterolaemia: namely, hypercholesterol not only impairs endothelial cells directly, but also affects EPC number and function at the same time. Thus hypercholesterolaemia may influence the endothelial repair process and disturb the balance between the magnitude of injury and the capacity for repair, which leads to endothelial dysfunction, and promote the progression of coronary artery disease.

There is evidence to suggest that smoking, hypertension, CAD and diabetes affect EPC number and function [2,12,17]. Because the patients with hypercholesterolaemia and the control subjects in the present study included these kinds of patients, one would expect our data to overestimate the difference in number and activity between the two groups. However, these factors in the two groups were almost identical. Moreover, smoking and diabetes had no significant influence on EPC number and activity in our present study (results not shown). The fewer number of these two kinds of patients enrolled in the present study might produce this result. Patients with CAD or hypertension exhibited a reduction in EPCs with decreased functional activity (results not shown), which is consistent with previous studies [2,12].

The mechanisms by which hypercholesterolaemia decreases EPC number and activity remain to be determined. There are several possible scenarios by which hypercholesterolaemia could have this effect. First, this might be due to increased apoptosis of premature progenitor cells, as CD34-positive EPCs have been shown [22] to be very sensitive to apoptosis induction. Moreover, ox-LDL (oxidized-LDL) is known to induce apoptotic cell death [23]. Secondly, hypercholesterolaemia may interfere with the signalling pathways regulating EPC differentiation or mobilization. Thirdly, the continuous endothelial damage or dysfunction may lead to an eventual depletion or exhaustion of a presumed finite supply of EPCs.

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