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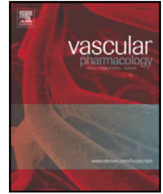

Short-term statin discontinuation increases endothelial progenitor cells without inflammatory rebound in type 2 diabetic patients

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ABSTRACT

Type 2 diabetes (T2D) is characterized by impaired vascular regeneration owing to reduced endothelial progenitor cells (EPCs). While statins are known to increase EPCs, the effects of statin withdrawal on EPCs are unknown. Herein, we evaluated the effects of statin discontinuation on EPCs, inflammation and in vivo angiogenesis. Thirty-four T2D patients were randomized to 5-day discontinuation or continuation of statin treatment. At baseline and at day 5, we determined lipid profile, EPC levels, monocyte–macrophage polarization, and concentrations of hsCRP, VEGF, SDF-1α, and G-CSF. Angiogenesis by human circulating cells was assessed in vivo. At day 5, patients who stopped statins showed raised total and LDL cholesterol and EPCs compared to baseline, while no changes were observed in patients who continued statins. No changes were observed in hsCRP, VEGF, SDF-1α, G-CSF, M1 and M2 macrophages and classical, intermediate and nonclassical monocytes in both groups. In vivo angiogenesis by circulating cells was increased in patients who stopped statin treatment. In vitro, cholesterol supplementation stimulated mobilizing signals in human bone marrow mesenchymal stem cells. In conclusion, a brief statin withdrawal increases circulating EPCs and functional proangiogenic cells in T2D. These findings identify statin-sensitive pathways as reverse target mechanisms to stimulate vascular repair in diabetes.

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1. Introduction

Type 2 diabetes (T2D) is associated with shortened life expectancy, mostly due to an increased risk of cardiovascular disease (CVD) [1]. Diabetic patients are particularly susceptible to aggressive CVD because of endothelial damage mediated by hyperglycemia, paralleled by impaired vascular repair. The latter is attributable to shortage of bone marrow (BM)-derived regenerative cells, especially endothelial progenitor cells (EPCs) [2]. EPCs contribute to endothelial turnover and support post-ischemic angiogenesis, thereby aiding cardiovascular protection [3]. As a consequence, pauperization of EPCs is believed to contribute to CVD development and progression. EPC depletion occurs early in the natural history of T2D [4] and further progresses in patients with long-standing T2D [4] and in those with severe vascular complications [5], reflecting a progressively impaired regenerative capacity. A low level of circulating progenitor cells is also an independent predictor of future cardiovascular events in different population of subjects [6]. For these reasons, ways to stimulate EPCs are actively pursued and several classes of drugs commonly used to treat T2D and CVD have been shown to be effective [7]. Statins raise EPC levels within days [8] and such short-term mechanism seems to be mediated by pleiotropic effects, namely activation of eNOS [9], rather than cholesterol lowering [10]. In fact, short term statin effects are considered to be mediated by pleiotropic activities, whereas the effects of cholesterol lowering emerge in the long-term [11]. However, the direct mobilizing effect of statins has been questioned [12], and cholesterol metabolism is a major regulator of stem cell trafficking [13]. In addition, it is unclear how sustained is the increase in EPC levels after initiation of statin therapy. Hristov et al. found that long-term statin-treated patients with CVD showed significantly reduced EPCs compared with matched controls, and that the beneficial effect of statin initiation onto EPC levels may be lost after 3 months [14].

Abbreviations: ACR, albumin/creatinine ratio; APC, allophycocyanin; CD, cluster of differentiation; CVD, cardiovascular disease; DIVAA, directed in vivo angiogenesis assay; eGFR, estimated glomerular filtration rate; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cells; FACS, fluorescence activated cell sorting; FITC, fluorescence isothiocyanate; G-CSF, granulocyte colony stimulation factor; HDL, high density lipoprotein; hsCRP, high sensitive C reactive protein; KDR, kinase insert domain receptor; LDL, low density lipoprotein; MACE, major adverse cardiovascular events; MSC, mesenchymal stem cells; PE, phycoerythrin; SDF, stromal derived factor; T2D, type 2 diabetes; VEGF, vascular endothelial growth factor.

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In this randomized controlled study, we tested the effect of a brief statin discontinuation on EPC levels and in vivo angiogenesis by circulating cells in T2D patients. As statins are provided with anti-inflammatory effects [15], we also evaluated changes in inflammatory biomarkers.

2. Materials and methods

2.1. Patients and protocol

The study was approved by the Ethical Committee of the University Hospital of Padova (prot. n. 2616P) and the trial was registered in ClinicalTrials.gov (NCT01600690). All subject provided written informed consent and the study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. All consecutive T2D patients regularly followed up at the outpatient clinic of the University Hospital of Padova between May 2012 and June 2013 were eligible if they met inclusion/exclusion criteria. All the following inclusion criteria were to be met: diagnosis of T2D; male or female gender; age 35–80; on statin therapy from at least 6 months, with the following minimal dose: simvastatin 20 mg; pravastatin 40 mg; fluvastatin 80 mg; rosuvastatin 5 mg; and atorvastatin 10 mg. Such minimal doses were decided based on statin potency equivalence. The 5-day period was chosen as the shortest time needed for the statins with longest half-life to reach <10% of steady-state concentrations. Exclusion criteria were: type 1 diabetes; age <35 or >80; chronic renal failure (eGFR < 30 ml/min/1.73 mq); recent (within 1 month) acute diseases or trauma/surgery; chronic inflammatory diseases; active cancer; LDL cholesterol >160 mg/dl; carotid atherosclerosis with stenosis >30%; coronary artery disease; peripheral arterial disease (Leriche-Fontaine stages II–IV); therapy with ezetimibe, fibrates, niacin, or EP hormones; pregnancy or lactation; and inability to provide informed consent. Patients were randomized to statin discontinuation or continuation for 5 days, according to a balanced (1:1) randomly generated computer sequence available to the investigators. Assignment was unblinded. A fasting blood sample was drawn at day 0. Patients were then instructed to stop or continue statin assumption starting from the evening of day 0. On the morning of day 5, i.e. after 5 doses of statin withdrawal or continuation, fasting blood samples were again collected. During the 5 days, patients were asked to continue other chronic medications without changing dosage and schedule. Flow cytometry analyses were performed on fresh whole blood samples within 2 h after collection, whereas serum and plasma were stored at −80°. For all patients, we collected the following data: age, sex, height, weight, waist circumference, diabetes duration, HbA1c, and prevalence of the cardiovascular risk factors (smoke, hypertension and family history of CVD). Diabetic complications were diagnosed as follows. Retinopathy was defined based on digital retinal fundus examination scored by experienced ophthalmologists. Nephropathy was defined as eGFR <60 ml/min/1.73 mq or macroalbuminuria (urinary albumin/creatinine ratio [ACR] > 300 mg/g), while microalbuminuria was defined as ACR 30–300 mg/g. Neuropathy was defined based on symptoms, vibratory sensory threshold and monofilament examination, eventually confirmed by electromyography. Coronary artery disease was defined as a past history of myocardial infarction or unstable angina, or evidence of significant epicardial coronary artery stenosis, or a positive myocardial stress test suggestive of inducible ischemia. Peripheral arterial disease was defined as claudication or rest pain, with or without ischemic foot lesions. Cerebrovascular disease was as a past history of stroke or transient ischemic attack, or based on carotid ultrasound with determination of per cent stenosis. Data on anti-diabetic and other medications were also recorded.

The primary end-point was change in circulating $CD34^+KDR^+$ EPC levels, whereas changes in inflammatory markers and angiogenesis were secondary endpoints. The in vitro part of the study has to be considered exploratory.

2.2. Flow cytometry

2.2.1. Enumeration of endothelial progenitor cells

Circulating progenitor cells were quantified using flow cytometry as previously described [16]. After red blood cell lysis, 150 μl of peripheral blood was stained with 10 μl of FITC-conjugated anti-human CD34 mAb (Becton Dickinson), 10 μl of PE-conjugated anti-human KDR mAb (R&D Systems) and 10 μl of APC-conjugated anti-CD133 mAb (Miltenyi Biotech). The frequency of peripheral blood cells positive for the above reagents was determined by a 2D side scatter-fluorescence dot plot analysis, after appropriate gating. We gated $CD34^+$ or $CD133^+$ peripheral blood cells in the mononuclear cell fraction and then examined the resulting population for the dual expression of KDR. At the intersection of the CD34 and CD133 gates we identified $CD34^+CD133^+$ cells, which were examined for KDR expression. In separate analyses, CD45 co-staining was performed and $CD45^{neg}CD34^+$ cells were also quantified, as they have been reported to contain a population enriched in endothelial colony forming cells (ECFC) [17]. For FACS analysis, 5×10^5 cells were acquired and scored using a FACS Calibur (BD). Data were processed using the Macintosh CELLQuest software program (BD). The same trained operator, blind to the clinical status of the patients, performed the tests throughout the study.

2.2.2. Enumeration of monocyte and macrophage subsets

Identification of monocyte subsets was performed using multiparameter flow cytometry. For analysis of classical, intermediate, and non classical monocytes, cells were stained with a FITC or PE-labeled anti-CD14 mAb (BD) and a FITC- or PE-Cy5-labeled anti-CD16 mAb (Beckman-Coulter). The analysis was performed according to standardized gating strategy [18]. For more definite monocyte–macrophage subsets, we stained with FITC-conjugated anti-CD68 mAb (Dako) and PE- or AlexaFluor-647 conjugated anti-CCR2 mAb (R&D Systems) for identification of M1 cells and with FITC-conjugated anti-CX3CR1 (Biolegend), PE-conjugated anti-CD163 (BD) and APC-conjugated anti-CD206 (BD) mAbs for M2. M1 were defined as $CD68^+CCR2^+$ cells and M2 were defined as $CX3CR1^+CD163^+/CD206^+$, as previously described [19]. The relative frequency of these monocyte subsets was expressed as % of the monocyte gate, whereas the relative frequency of monocytes, neutrophils and lymhocytes was expressed as % of the total live gate.

2.3. Biochemical measures

Commercially available human ELISA kits from R&D Systems were used for the quantification of VEGF (cat. DVE00), G-CSF (cat. DCS50) and SDF-1α (cat. DSA00), according to the manufacturer's instructions. High sensitive C-reactive protein (hsCRP) was measured using a commercially available kit (Dbc-lab, cat. CAN-CRP-4360). Serum total cholesterol, HDL cholesterol, and triglyceride concentrations were measured using a Roche automated modular analyzer COBAS 8000.

2.4. Reverse cholesterol transport

Cholesterol efflux capacity was quantified using a slightly modified method designed to increase throughput [20]. J774 macrophages, cultured in RPMI 1640 medium containing 10% FBS, 2 mM glutamine, 1% Penicillin/Streptomycin at 37 °C and 5% CO_2, were seeded onto a 24 well plate at a density of 3 × 105 cells. The following day, the medium was removed and cells were incubated in 1% FBS RPMI 1640 medium radiolabeled with 1 μCi of 3H-cholesterol/ml (Perkin Elmer), for 24 h. Then, medium was collected and the radioactivity was assayed in a β-counter Packard 1600TR in order to quantify 3H cholesterol uptaken from cells. ABCA1 (ATP binding cassette transporter A1) was up-regulated by adding 0.3 mM 8-(4-chlorophenylthio)-cyclic AMP and 0.2% bovine serum albumin for 16 h. Subsequently, the medium was removed and the radioactivity was measured. Cells were washed with

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