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Atorvastatin induces T cell proliferation by a telomerase reverse transcriptase (TERT) mediated mechanism

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ABSTRACT

Statins are one of the most potent drugs in delaying age-related inflammatory changes in the arterial vessel wall, slowing down the progression of atherosclerosis. Statins have also been shown to abrogate telomere-attributed cardiovascular risk. The goal of our study was to explore a potential effect of atorvastatin on telomerase activity in peripheral blood mononuclear cells (PBMCs) and T-lymphocytes (T cells).

Methods and results: Treatment with pharmacologically relevant concentrations (0.1–0.3 μM) of atorvastatin resulted in a 6-fold increase of telomerase activity (TA) ($p < 0.0001$) in human and mouse PBMCs and CD4 T cells, translating into moderate proliferation of T lymphocytes. In contrast, high doses of atorvastatin (2–5 μM) or the addition of LDL cholesterol completely inhibited proliferation, thereby abrogating telomerase activity. The proliferative effect of atorvastatin was ablated by the absense of the catalytic subunit of telomerase, telomerase reverse transcriptase (TERT). Using transgenic GFP-*mTert* reporter mice, we observed a decrease in telomerase-positive lymphocytes from 30% to 15% during the first 5 months of age ($p < 0.01$). This suggests that the decrease in immune cell turnover during normal development and maturation is mirrored by a reduction in telomerase activity in lymphocytes in-vivo.

Conclusion: Atorvastatin and cholesterol have opposing effects on telomerase in mononuclear cells and T-lymphocytes. Our study suggests a link between cholesterol metabolism and telomere-related cardiovascular risk.

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1. Introduction

Telomerase is a ribonucleoprotein composed of an RNA subunit (TERC), serving as a template for telomere repeat addition, and a reverse transcriptase (TERT) subunit that facilitates the replication of telomeres, the ends of chromososmes [1,2]. TERT also has additional functions beyond telomere maintenance. These include the control of gene expression, chromatin organisation and mitochondria shuttling [3–5].

Multiple studies so far have provided evidence for an association of short telomeres with conditions of increased oxidative stress, including smoking, obesity and coronary heart disease (CHD) [6–11]. In a cohort of 1500 patients the WOSCOPS substudy identified that the telomere-attributed risk of developing coronary heart disease was attenuated by treatment with pravastatin [12]. Atorvastatin has been demonstrated to reduce oxidative stress in various clinical studies, including patients with atherosclerosis, hypercholesterolemia, rheumathoid arthrits, chronic kidney disease and polycystic ovarian syndrome [13–18]. However, it remains unclear whether short telomere length is only a bystander in atherosclerosis and whether statins can exert a direct effect on telomere length. We previously published that telomere length shortening in CHD patients, when compared to age-matched controls, is more pronounced in T-lymphocytes (T cells) than in myeloid cells, suggesting that T cells could play an important role in ageing- and telomere-mediated atherogenesis [19]. T cell mediated immune responses play important roles at all stages of atherosclerotic lesion development [20] with the majority of T cells in an atherosclerotic lesion being CD4+ T-helper cells (Th1) that produce interferon-γ [21–23].

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In vitro, atorvastatin has been shown to inhibit T cell activation and proliferation, thereby exerting an anti-inflammatory effect [24,25]. However, these and similar studies used drug concentrations of up to 10 μM, 30 times higher than in the plasma of atorvastatin-treated patients [26]. In pharmacologically relevant dosages, statins have been shown to induce the Akt pathway and promote proliferation in endothelial cells [27]. We have also shown that statins induce telomere-repeat binding factor TRF2 in endothelial progenitor cells [28]. Telomere length was found to be longer in patients under statin therapy compared to those without [29]. A recent study of 230 subjects showed that statin therapy was associated with higher telomerase activity independently of multiple covariates, including age, gender, cardiovascular risk factors and systemic inflammation [30]. In the same study, subjects on statin treatment also showed significant lower telomere erosion along with ageing. The goal of our study was to inves tigate the *in-vitro* effect of atorvastatin on telomerase and the mechanistic relation between proliferation and telomerase in this context.

2. Methods

2.1. T-cell receptor ligation and culture of human PBMCs

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-hypaque density gradient (using Biochrom AG Biocoll L 6113/5) and prepared at 5×10^6/ml in supplemented RPMI 1640. For T-cell activation, 24 well plates (VQR 734-2325) were coated overnight with anti-CD3 (BD 550367) and anti-CD28 (BD 555725) antibodies prepared at 1 μl/ml PBS at 4 °C, except for unstimulated controls. Cells were then cultured in RPMI 1640 (Gibco 21875-034) supplemented by 3 mM L-glutamine, 10% foetal bovine serum (PAA A15-151) and 30 μg/ml of pen/strep (Gibco 15070-063). Buffy coats were purchased from the National Blood Service, Newcastle Upon Tyne. Ethical approval was granted by the Newcastle University faculty of medical sciences ethics committee, 000205/2009.

2.2. Animals

TERT (Jax strain B6.129S-Tert tm1Yjc/J) [31] and TERC (Jax strain B6.Cg-Terc tm1Rdp/J) [32] animals were purchased from Jackson Laboratory, Maine, USA. *mTert*-GFP transgene reporter mice were previously described [33,34]. To confirm the phenotype of GFP transgenic mice, blood from the mouse tail was taken and mixed with 1% of PBS/EDTA solution before lysis of red blood cells in Red Blood Cell Lysing Buffer (Life Technologies uk) for 20 min at 4 °C. Cells were then washed and resuspended in PBS solution and DAPI to confirm cell viability. All the samples were processed using FACS Calibur (BD Biosciences, UK) and then analysed with BD FACSDiva software. All work complied with the guiding principles for the care and use of laboratory animals in the UK. Mice were provided with sawdust and paper bedding and had ad libitum access to food and water. Mice were housed at 20 ± 2 °C under a 12 h light/12 h dark photoperiod. All mice were held under the UK Home office animal licence PPL 60/3864.

2.3. Mouse PBMC cell culture

Cells were grown in RPMI 1640 (Gibco 21875-034) supplemented with 0.5 mM 2-mercaptoethanol (Sigma M7522), 25 mM Hepes Buffer (Gibco 15630-080), 10% FBS (PAA A15 151). 24 well plates were coated overnight with anti CD3 (BD 553238) and CD28 (BD 553295) antibodies prepared at 1 μl/ml PBS at 4 °C. PBMCs were isolated by Ficoll-hypaque density gradient and prepared at 2×10^6/ml in supplemented RPMI 1640.

2.4. Long-term culture and growth curves

Long-term culture of human PBMCs was prepared at 5×10^6 cells per 2 ml RPMI 1640, and supplemented with MACSibead human T cell activation beads at a ratio of 1 bead to every 4 cells (Militenyi 130-091-441) in flat bottom 24 well plates. Medium was changed as cells were counted by Neubauer chamber, and re-stimulated at day 14 with additional beads as per manufacturer's instructions. Mouse cultures were established from 2×10^6 cells per ml RPMI 1640 supplemented by MACSibead mouse T cell activation beads (Militenyi 130-093-627) at a ratio of 1 bead to every 2 cells. Medium was changed every 2–3 days with addition of IL-2 and atorvastatin (cells split 1:2 if necessary) and cells re-stimulated every 7 days as per manufacturer's instructions. Individual wells were compiled after a total cell number of 25×10^6 was reached, the resultant aspirated pellet resuspended in 50 ml complete mouse medium to achieve the required 2×10^6/ml in 50 ml flasks.

2.5. Hypoxia and normoxia culture conditions

Hypoxia (3%) and normoxia (atmospheric 20%) culture conditions were maintained throughout experiments in Heraeus Hera Cell 150 incubators. Conditions of 5% CO_2 and 37 °C were constant.

2.6. Pharmacological activators and inhibitors

Atorvastatin was kindly donated by Pfizer and prepared in dimethyl sulfoxide (DMSO, Sigma 472301) to working dilutions of 5 μM, 2 μM, 0.3 μM, 0.1 μM and 0.02 μM. Interleukin-2 (IL-2, R&D 202-IL) was prepared from frozen stocks (−20 °C) of 100 μg/ml and added in a working concentration of 1.5 μg/ml. Ly294002 (Cell Signaling 9901) was prepared at working doses of 10 μM, 2 μM and 0.4 μM in DMSO. Akt kinase 1/2 inhibitor (Sigma A6730) was prepared at working doses of 10 μM, 2 μM and 0.4 μM in DMSO. For all experiments and conditions, DMSO concentrations in culture medium never exceeded 0.1% and 0.1% DMSO was added to control wells.

2.7. Addition of LDL cholesterol in cholesterol-free media

Human plasma LDL cholesterol (Sigma L7914) was diluted in 150 mM NaCl and 0.01% EDTA to working stocks of 200, 100 and 50 mg/dl added to RPMI supplemented by 10% lipoprotein deficient serum (Sigma S5394) and 30 μg/ml of pen/strep (Gibco 15070-063). Cholesterol was added serially at each media change every 2–3 days.

2.8. Immunomagnetic sorting of human CD4 and CD8 T-cells

Single cell suspensions of PBMCs were centrifuged in a Ficoll-Hypaque gradient, and re-suspended in 90 μl ice-cold MACS buffer (2 mM EDTA, 0.5 ml BSA per 100 ml PBS) per 10^7 cells. PBMC suspensions were then incubated with 10 μl per 10^7 cells anti-CD8 mAb labelled magnetic beads (Militenyi Biotec 130-045-201) under refrigerated conditions to prevent antibody capping and non-specific binding. Cells were then passed through a pre-cooled magnetic LS column (Militenyi 130-042-401) apparatus and the column was repeatedly washed with buffer. Immediately collected cells constituted a fraction depleted of CD8 T cells, which were retained in the column and forced into a separate collection tube by replacement of the column plunger. This fraction was passed down the column a second time to improve its purity. The depleted fraction was identically treated with anti-CD14 beads (Militenyi 71-5775-40), and the depleted fraction with anti CD4 beads (Militenyi 71-5775-40). A multi conjugated anti CD3 (PE), CD8 (FITC), CD4

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