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Brief communication

Ezetimibe and atorvastatin both immunoregulate CD4+ T cells from cardiac transplant recipients *in vitro*

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ABSTRACT

Background: Statins are LDL lowering agents that reduce cardiac allograft vasculopathy (CAV) incidence after cardiac transplantation. Furthermore, 'pleiotropic effects' including immunomodulation have been demonstrated by statins following transplantation. It has also been previously suggested that ezetimibe may exert specific effects on the innate immune system *in vitro*. We compared the effects of ezetimibe and atorvastatin on T lymphocytes *in vitro* on the justification that these cells are implicated in the pathogenesis of atherosclerosis, allograft rejection and CAV.

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from 30 cardiac transplant recipients and co-cultured with the study drug (or placebo) over 48 h. In total, 150 cultures were performed (5 per patient). Drug concentrations were calculated to simulate 10 mg or 100 mg daily in a 70 kg adult. Flow cytometry was performed to analyse T lymphocyte counts and functional characteristics.

Results: Ezetimibe reduced the standard CD3+CD4+ T cell count and CD3+CD4+CD45ro T memory count by dose linear effect ($p<0.001$). Atorvastatin also reduced the CD3+CD4+ T cell count and CD3+CD4+CD45ro T memory count by dose linear effect ($p=0.005$). Neither drug affected CD3+CD8+ cytotoxic T cells.

Discussion: Both atorvastatin and ezetimibe may have selective immunomodulatory properties independent of their mechanisms of LDL lowering, given that both drugs affect CD4 T helper cells but have no effect on CD8 cytotoxic lymphocytes *in vitro*. Although speculative, both of these agents could potentially offer benefits to the transplant patient by modulating important components of the adaptive immune system. CD4+ cells in particular are implicated in both CAV and rejection processes.

1. Introduction

Hyperlipidaemia is a crucial factor in the development of cardiac allograft vasculopathy (CAV) [1] and resultantly 60–80% of all cardiac transplant recipients receive lipid lowering agents [2]. HMG–CoA reductase inhibitors (statins) remain the first choice for such agents. They have been observed to both reduce mortality [3,4] and reduce the incidence of CAV in this setting [4–6]. Furthermore, statins have been demonstrated to possess many other properties in addition to their lipid lowering potential. An example particularly relevant to the transplant setting is the potential for immunomodulation [7–9]. Indeed a previous study has shown that statins reduce the incidence of acute rejection episodes causing haemodynamic compromise [5]. However despite the use of statins, many patients continue to have high circulating lipid levels which lead to the consideration of additional lipid lowering agents. Ezetimibe is a cholesterol absorption inhibitor with good low density lipoprotein (LDL) lowering efficacy and is often considered as additional treatment for such patients. Yet whether this non-statin lipid lowering agent also possesses pleiotropic effects independent of its cholesterol lowering activity is largely unknown and is certainly a subject of current debate [10–12]. Interestingly, ezetimibe was previously shown *in vitro* to modify the expression of several key surface molecules on monocyte derived macrophages [13]. In particular, CD13 expression was significantly reduced suggesting that ezetimibe has an effect on the lipid raft assembly of these cells. In response to these results, we hypothesized that ezetimibe may have other immunomodulatory effects on alternative elements of the immune system that hold importance after transplantation. Specifically, we investigated the differences between atorvastatin, ezetimibe and placebo on their potential to modulate T lymphocytes during PBMC culture *in vitro*. Following cardiac transplantation, premature atherosclerosis of the coronary vasculature (CAV) occurs with high prevalence and remains the leading cause of mortality for cardiac transplant patients ≥5 years after transplant [14]. Importantly, CAV appears to be dependent on T lymphocyte activity [15,16]. In particular, it has been shown that CD4+ T cell subsets with memory function significantly infiltrate the walls of allograft coronary arteries [17] and the myocardium [18]. Outside the

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Table 1
Culture well compositions.

Culture well	1	2	3	4	5
Drug	Placebo (purified water)	Ezetimibe	Ezetimibe	Atorvastatin	Atorvastatin
Dose simulation (in 70 kg adult)	n/a	10 mg	100 mg	10 mg	100 mg
Actual culture well concentration	n/a	142 ng/ml	1420 ng/ml	142 ng/ml	1420 ng/ml

Five separate culture wells were made from each patient, to allow for analysis of two concentrations of two agents, in addition to placebo. Doses were simulated by estimating the weight per volume of distribution in a 70 kg adult (assuming 1 kg body weight = 1 l volume).

transplant setting, T lymphocytes have also been identified to play a pivotal role in the development of atherosclerosis and acute coronary syndromes [19–21].

2. Methods

Stable heart transplant recipients were recruited from the outpatient setting. Patients were considered clinically stable if they had no previous hospital admissions in the preceding 3 months and also no history of graft rejection (≥2A ISHLT criteria) during the same time period.

2.1. Culture protocol

Whole blood was collected into EDTA vacutainers after routine venepuncture. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood using Ficoll–Paque density gradient centrifugation. Culture medium consisted of standard RPMI, 10% fetal bovine serum, 100 U penicillin–streptomycin and 2 mM/l L-glutamine. Each PBMC sample was reconstituted with culture medium and equally aliquotted in accurate volumes into 5 culture wells.

10 mg of atorvastatin or ezetimibe were each mixed with 100 ml of purified water to form a colloidal suspension. Specific amounts of the resulting suspension were then diluted further depending on the final concentration required and then carefully added to the culture wells. Final culture well concentrations were made to approximately simulate 10 mg or 100 mg doses, per standard adult weight (70 kg). For this, we assumed that 1 kg of body weight was equivalent to 1 litre volume of distribution. Ezetimibe and atorvastatin at the dose of 10 mg daily are typically considered the standard for initiating cholesterol reduction treatment [22,23]. We included the simulation of supratherapeutic doses to observe for dose effect.

A placebo well was also included and consisted of the addition of purified water only. This resulted in 5 PBMC cultures per patient (see Table 1). All cultures were incubated in humidified conditions at 37 °C for 48 h. Samples were then immunophenotyped using flow cytometry.

Table 2
Antibody combinations for cell phenotyping of T lymphocytes.

FITC	PE–Cy5	PE
T helper/memory conjugate		
CD3	CD4	CD45ro
Cytotoxic T cell conjugate		
CD3	CD8	CD107a

FITC = fluorescein isothiocyanate; PE–Cy5 = phycoerythrin–Cy5; PE= phycoerythrin. The flow cytometer was able to process combinations of three antibodies, enabling the separation of T helper and memory T cells, plus cytotoxic T cells with or without the expression of an activation marker.

Table 3
Breakdown of statin therapy in the cardiac transplant cohort ($n = 30$).

Statin type	Number of patients (%)	Average dose
Simvastatin	4 (13)	30.0 mg
Pravastatin	13 (43)	25.7 mg
Atorvastatin	8 (27)	32.9 mg
Fluvastatin	3 (10)	26.7 mg
None	2 (7)	
Total	30 (100)	

2.2. Flow cytometry

Sample preparation: cultured cells were immunophenotyped for CD4 and CD8 T cells via combinations of conjugated antibodies (Becton Dickinson, Oxford, UK) See Table 2. Cells were analysed in a final volume of 500 μl PBS. Flow cytometric analysis was performed using an EPICS XL (Beckman Coulter) flow cytometer using previously published methods [24]. Gating strategies and data analysis were created using EXPO32 ADC Analysis software (Beckman Coulter). For each individual sample, cell counts were performed using a standard machine protocol set at a 'medium' event intensity over a 180 s period.

2.3. Statistics

Statistical Package for the Social Sciences (SPSS) version 14.0 was used to analyse data. Data was assessed for its distribution using skewness, kurtosis and Kolmogorov–Smirnov test prior to significance analysis. Assessment of individual concentrations against placebo (or each other) was performed using Wilcoxon Signed Ranks Test. Assessment for dose linear effects of each drug used ANOVA general linear model (GLM). The data underwent logarithmic conversion prior to analysis (given its non parametric distribution). For all analyses, p values ≤ 0.05 were considered as statistically significant.

3. Results

3.1. Demographics

30 cardiac transplant patients were recruited, resulting in a total of 150 cultures. Mean age was 57 years (range 35–75 years) and 78% of patients were male. The mean time since transplant was 2433 days (range 346–6380 days). 90% of patients were administered cyclosporin as their primary immunosuppressive agent, with an average

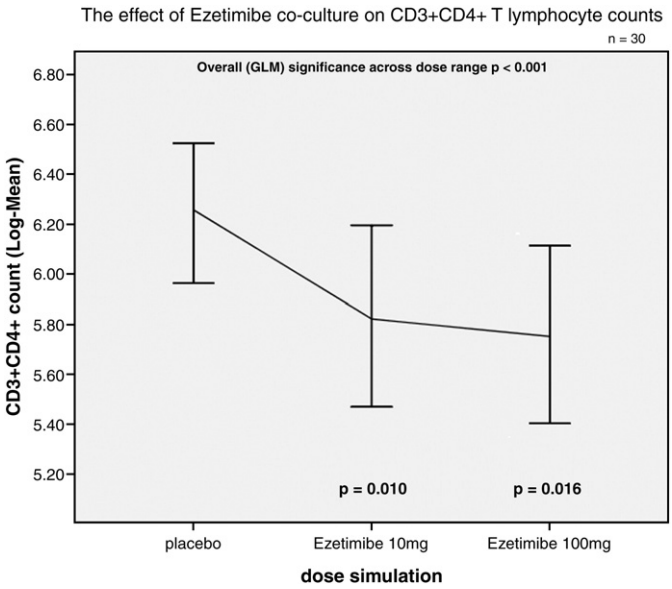

Fig. 1. This graph shows the reduction in logarithmic means (bars represent standard errors) of the CD3+CD4+ T cell count when ezetimibe was co-cultured with PBMCs.