Microvascular Research 77 (2009) 364–369

Contents lists available at ScienceDirect

Microvascular Research

journal homepage: www.elsevier.com/locate/ymvre

Regular Article

Clopidogrel attenuates atheroma formation and induces a stable plaque phenotype in apolipoprotein E knockout mice

Arnon Afek, Evgeny Kogan, Sofia Maysel-Auslender, Adi Mor, Ehud Regev, Ardon Rubinstein, Gad Keren, Jacob George *

The Department of Cardiology, Tel Aviv Sourasky Medical Center, Tel Aviv University, Sackler School of Medicine, Tel Aviv, Israel

ARTICLE INFO

Article history:
Received 23 May 2008
Revised 29 December 2008
Accepted 8 January 2009
Available online 31 January 2009

Keywords:
Atherosclerosis
T cells
Immune system clopidogrel
Endothelial progenitors

ABSTRACT

Aim: Clopidogrel is a widely used anti-thrombotic for the prevention of stent thrombosis and cardiovascular events in patients with coronary atherosclerosis. Clopidogrel has been shown to exhibit anti-inflammatory effects that are related to the attenuated activation of platelets. Atherosclerosis is a complex process in which the immune system and the endothelium appear to play a prominent role. Herein, we tested the hypothesis that clopidogrel will influence plaque size and composition in the atherosclerosis prone apolipoprotein E knockout (apoE KO) mouse model.

Methods and results: Eight week old mice were fed daily with either PBS, 1 mg or 2 mg of clopidogrel for 10 weeks. Plaque size was evaluated in the aortic sinus and cellular and humoral responses were studied as well as splenic and bone marrow endothelial progenitors by FACS.

Treatment with either 1 mg and 2 mg of clopidogrel significantly reduced plaque size and augmented its stability by increasing atheromatous fibrous area. Whereas antigen specific oxLDL immune response was not influenced by clopidogrel feeding, the number of atheroprotective regulatory CD4+CD25+ T cells was significantly increased. Moreover, clopidogrel treatment resulted in a prominent rise in splenic but not bone marrow derived Sca-1+/flk-1+ endothelial progenitors.

Conclusion: Clopidogrel significantly reduces atheroma burden and stabilizes aortic sinus plaques in apoE KO mice. These effects may partially be mediated by upregulation of the regulatory T cell pool and splenic endothelial progenitor cells. These findings may expand the potential applications of clopidogrel in human subjects.

Introduction

The role of the immune system in the pathogenesis of the atheroma is well established (Hansson, 2005; Libby, 2002; Binder et al., 2002). Among the studies lending support to this contention, are reports showing that immune modulating strategies influence the size and the composition of the plaque. T cells are present in human (Hansson et al., 1989) and murine (Roselaar et al., 1996) atherosclerotic plaques and adoptive transfer studies confirm the role of cellular immunity in the pathogenesis of atherosclerosis (Zhou et al., 2000; George et al., 2000, 2001).

Clopidogrel is a platelet $P2Y_{12}$ receptor inhibitor that was approved for the prevention of ischemic stroke, myocardial infarction, and vascular death in patients with symptomatic atherosclerosis (CAPRIE Steering Committee, 1996). Following publication of the CURE trial (Yusuf et al., 2001), the use of clopidogrel added to standard therapy was approved for the reduction of atherothrombotic events in patients with acute coronary syndromes. Recently, an important beneficial role has also been established for clopidogrel in patients with ST elevation acute myocardial infarction (Sabitine et al., 2005).

Platelet $P2Y_{12}$ receptor is of critical importance in attenuation of platelet activation and thus represents an effective pharmacological target for the inhibition of platelet aggregation and prevention of atherothrombotic events (Herbert, 2004). However, immune mediated responses that are implicated in the pathogenesis of atherosclerosis and atherothrombosis and depend on platelets, are also reduced by ADP-receptor antagonism (Hermann et al., 2001). Thus, the release of CD40 ligand (CD154) from platelets is inhibited by treatment with clopidogrel (Klinkhardt et al., 2003). Additionally, the expression of P-selectin (an adhesion protein involved in platelet–leukocyte interactions) on stimulated platelets is also reduced by clopidogrel treatment (Klinkhardt et al., 2003, 2002). In human trials, there is also evidence that supports the role of clopidogrel as an anti inflammatory agent other than its well known anti-thrombotic properties (Solheim et al.; 2006, Azar et al., 2006; Graff et al., 2005).

In view of the potential influence of clopidogrel on the immune system, we tested the hypothesis that clopidogrel could attenuate plaque formation and stability in the atherosclerosis prone apolipoprotein E knockout mouse.

* Corresponding author. Fax: +972 3 5469832.
E-mail address: jacobg@post.tau.ac.il (J. George).

0026-2862/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.mvr.2009.01.009

Materials and methods

Animals

ApoE-KO mice on a C57BL/6 background (Plump et al.; 1992) and their wild-type litters were purchased from Jackson Laboratories and grown at the local animal house. Mice were fed a normal chow diet containing 4.5% fat by weight (0.02% cholesterol).

Experimental groups

Eight week old female, apoE KO mice (10/group) were fed once daily with clopidogrel 1 mg, 2 mg (both, dissolved in PBS) or PBS as control. Animals were fed for 10 weeks until sacrifice, when their hearts and aortas were obtained for measurement of atherosclerotic plaque size.

Lipid profile

Total plasma cholesterol and triglyceride levels were determined using an automated enzymatic technique (Boehringer Mannheim, Germany).

Splenocyte proliferation assays

Splenocytes from clopidogrel 1 mg, 2 mg and PBS treated mice (1×10^6 cells/ml) were incubated in triplicates in 0.2 ml of culture medium in microtiter wells in the presence or in the absence of 1 μg/ml oxLDL or MDA-LDL for 72 h. In brief, after 72 h of proliferation, XTT reagent was added to wells according to manufacturer's instructions. After 2 h of incubation orange color that developed is measured on ELISA reader and it is proportional to amount of cells in the well.

For in vitro proliferation splenocytes from apoE KO mice (1×10^6 cells/ml) were incubated for 72 h in presence of clopidogrel (0.5–10 μg/ml) in 0.2 ml of culture medium. Detection of proliferation was done as described above.

Detection of anti-oxidized-LDL and anti-MDA LDL antibodies by ELISA

Ninety-six-well polystyrene plates (Nunc) were coated with either copper-oxidized LDL, native LDL (at a concentration of 5 μg/ml in PBS), or PBS alone overnight at 4 °C. After 4 washes with PBS containing 0.05% Tween and 0.001% aprotinin (Sigma), the plates were blocked with 2% BSA for 2 h at room temperature. Serum fractions were diluted to 1:50 in PBS–0.05% Tween–0.2% BSA and added to the wells. After additional overnight incubation the plates were washed, and alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch laboratories Inc), diluted 1:10 000 in PBS–0.05% Tween–0.2% BSA, was added for 1 h at room temperature. After extensive washing, 1 mg/ml p-nitrophenyl phosphate (Sigma) in 50 mmol/l carbonate buffer containing 1 mmol/l $MgCl_2$, pH 9.8, was added as a substrate. The reaction was stopped after 30 min by adding 1 mol/l NaOH. The optical density was read at a 405-nm wavelength in a Titertek ELISA reader (SLT Laboratory Instruments). Levels of anti-oxLDL antibodies were calculated as the level of binding to native LDL subtracted from that for the binding to oxLDL (George et al., 1998).

Analysis Tregs by FACS

Splenocytes from sacrificed animals were co-stained with the following monoclonal antibodies: FITC-labeled anti-CD4 (7D4, Miltenyi Biotec), phycoerythrin (PE)-labeled anti-CD25 (GK1.5, Miltenyi Biotec), FITC-labeled mouse IgG2bκ isotypic control (KLH/G2b-1-2 from SouthernBiotech) and phycoerythrin (PE)-labeled mouse IgMκ isotypic control (RTK2118 from BioLegend).

In addition, staining was performed on splenocytes incubated for 72 h with Clopidogrel (0.5–10 μg/ml).

Assessment of bone marrow and spleen cell derived endothelial progenitors

Spleen and bone marrow cells were stained with the following antibodies: PE-anti mouse Flk-1 (Avas12a1; e-Bioscience), FITC-anti mouse-Sca-1 (D7 e-Bioscience) antibodies and corresponding isotype controls.

Assessment of aortic sinus atherosclerosis

Atherosclerotic fatty-streak lesions were quantified by calculating the lesion size in the aortic sinus as previously described (Paigen et al., 1987) with a few modifications. Briefly, the heart and upper section of the aorta were removed from the animals, and the peripheral fat was carefully cleaned. The upper section was embedded in OCT medium and frozen. Every other section (10 μm thick) throughout the aortic sinus (400 μm) was taken for analysis. The distal portion of the aortic sinus is recognized by the 3 valve cusps, which are the junctions of the aorta to the heart.

The extent of atherosclerosis was evaluated blindly by two expert pathologists. Processing and staining of the tissue with oil red O were

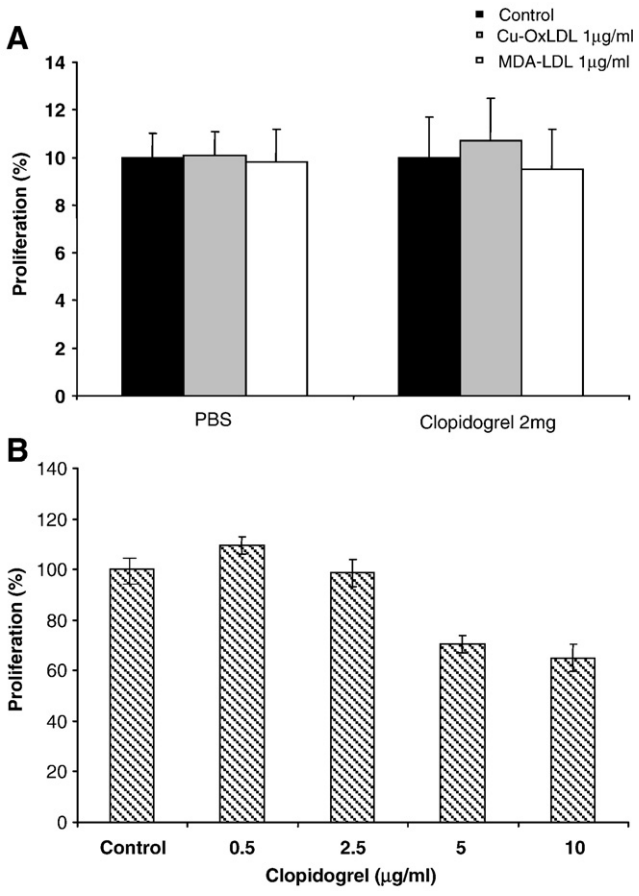

Fig. 1. The effect of clopidogrel on lymphocyte proliferation. Splenocytes were obtained from clopidogrel (2 mg) or PBS treated animals upon sacrifice. Proliferative capacity from 4 animals in each group to MDA-LDL and copper oxLDL was assessed as described in Materials and methods (A). Splenocytes from PBS treated mice were incubated in the presence of different concentrations of clopidogrel for 72 h and proliferation was determined by XTT method as described in Materials and methods (B). Both the 5 and 10 mcg/ml concentrations yielded significant ($P<0.05$) reduction as compared with controls. Results are presented as means ± SD.