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

A R T I C L E   I N F O

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

A B S T R A C T

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 apoptotic 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.

© 2009 Elsevier Inc. All rights reserved.

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 P2Y12 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 P2Y12 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.
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 x 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 x 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 MgCl2, 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 IgG2b isotypic control (KLH/G2b-1-2 from SouthernBiotech) and phycoerythrin (PE)-labeled mouse IgM 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
carried out according to Paigen et al. (1987). All animals in the study ($n=9$) were used for analysis.

Staining with Masson’s trichrome was employed to determine fibrous area. The proportional area of the plaque stained positive for collagenous fibrosis was determined by quantitative morphometry.

**Statistical analysis**

All parameters were evaluated by the one way ANOVA test. $P<0.05$ was considered statistically significant. Results are expressed as mean±SEM unless otherwise specified in the text.

**Results**

**The effect of clopidogrel on lipid profile**

We have found that treatment with clopidogrel at either the 1 mg or 2 mg daily doses, did not influence total cholesterol levels. Moreover, triglyceride values also did not change following the two regimens of clopidogrel treatment (data not shown).

**The effect of clopidogrel on cellular immune responses to oxLD**

Next, we tested the effects of oral treatment with clopidogrel, on proliferative capacity of splenocytes to copper oxidized LDL. We have found that treatment with clopidogrel (either 1 mg or 2 mg) did not influence the proliferation of splenocytes to MDA-LDL and copper-oxLDL (Fig. 1A). However, when splenocytes were incubated in vitro with clopidogrel, the 5 and 10 mcg/ml concentrations yielded a significant decrease in spontaneous proliferation (Fig. 1B).

**The effect of treatment with clopidogrel on humoral immune markers**

In the apoE KO mouse model, antibodies to oxidized LDL develop as cholesterol levels rise and atherosclerotic lesions progress. We tested 2 types of IgG antibodies to modified LDL. Treatment with clopidogrel with either 1 mg or 2 mg daily did not alter the levels of IgG antibodies to either copper oxidized LDL or MDA modified LDL measured at sacrifice (Fig. 2).

**The effect of clopidogrel on Treg numbers**

We evaluated the effects of clopidogrel on Tregs by assaying CD4+ CD25high in the spleens from both clopidogrel treatment groups in comparison with controls. We have found that both doses of clopidogrel significantly increased the number of peripheral naturally occurring Tregs as compared with the PBS treated group (Fig. 3).
The effect of clopidogrel on the number of endothelial progenitor cells (EPC)

One of the methods by which EPC in the mouse is by co-expression of Sca-1 and VEGF-receptor 2 (FLK-1) (Werner et al., 2002). We evaluated the numbers of bone marrow and peripheral (splenocyte) EPC by FACS analysis for the respective markers. Treatment with clopidogrel with either 1 mg or 2 mg significantly increased the numbers of EPC in the spleen as compared with PBS (Fig. 4). Clopidogrel treatment did not influence the numbers of bone marrow derived EPC as compared with PBS.

Both clopidogrel regimens resulted in a significant attenuation of aortic sinus atherosclerotic lesion (Fig. 5). Whereas 1 mg of clopidogrel given for 10 week was associated with a reduction of 45% in lesion size, the 2 mg dose resulted in a 48% decrease in atheroma as compared with PBS (P<0.01 for both comparisons).

Aiming to study the effects of clopidogrel on plaque stability we performed Masson’s trichrome staining to detect relative plaque fibrous content. Treatment with a daily dose of 1 mg clopidogrel was associated with a 31% increase in fibrous area, whereas administration of 2 mg daily, increased relative plaque fibrous area by 46% in comparison with PBS (P<0.05 for both comparisons).

Discussion

The pathogenesis of atherosclerosis is complex and, other than deranged lipid metabolism, immune responses and endothelial malfunction have been shown to play a prominent role.

Herein, we tested the effects of the widely employed ADP inhibitor clopidogrel on the size and of stability of the atherosclerotic plaque in the apoE KO mouse model. Despite the well-known role of clopidogrel...
in the prevention of stent thrombosis and adverse myocardial events, there is currently no evidence supporting its effects on plaque size and composition. Such studies are inherently easier to perform in experimental murine atherosclerosis models. Indeed, we have found that treatment with clopidogrel given at 1 mg and 2 mg daily doses equivalent to those given to humans (75 mg and 150 mg, respectively), for a period of 10 weeks significantly reduced the size of atherosclerotic plaques (Fig. 6).

We have studied several mechanisms that could mediate the beneficial effects of clopidogrel. Cellular immune responses are known to influence the size and composition of murine plaques. Indeed, oxLDL reactive lymphocytes have been demonstrated within human plaques (Stemme et al., 1995), and active immunization studies in with different antigens have been shown influence plaque progression (George et al., 1998; Xu et al., 1992; Palinski et al., 1995).

Although no effect of clopidogrel was evident on antibodies to oxLDL, it is nevertheless possible that different timing of clopidogrel with respect to the development of the plaques would have influenced humoral responses.

Additionally, adoptive transfer of antigen-specific and non-specific lymphocytes was demonstrated to promote atherosclerosis (Zhou et al., 2000; George et al., 2000, 2001). In this study we found that treatment with clopidogrel did not influence cellular responses evident by splenocyte proliferation to either oxLDL or the non specific mitogen Conavalin-A, suggesting this mechanisms may not have been involved in the atheroprotective effect of clopidogrel. Interestingly, in vitro proliferation to clopidogrel did produce a suppressive effect on murine splenocytes that was not evident when the treatment was given in vivo. This finding may be explained by the attenuation of clopidogrel’s effect in vivo due to various possible systemic factors such as lipids and cytokines that are not present in the in vitro isolated system.

A relatively new role has recently been ascribed to naturally occurring CD4+CD25+ Tregs in atherosclerosis (reviewed in George, 2008). Consistent with the well documented role of Tregs in suppressing autoimmune responses (Sakaguchi, 2004), others (Ait-Oufella et al., 2006) and we (Mor et al., 2007) have shown that this population of cells exhibit anti-atherosclerotic properties in mice. We have also shown that peripheral population and functional suppressive properties of Tregs are compromised in humans and suggested it may have a role in destabilization of human plaques with consequent development of acute coronary syndromes (Mor et al., 2006). In this study we have found that treatment with clopidogrel resulted in a significant increase in the number of spleen cell derived Treg, a finding that has not yet been associated with the agent. This effect of clopidogrel may be related to a direct effect of clopidogrel on Tregs or their transcriptional activator, or alternatively to a reduction platelet derived cytokine secretion that may influence indirectly, Treg pool. Regardless of the potential mechanism of Treg upregulation, this effect of clopidogrel may be responsible for its anti-atherosclerotic effects.

EPC are reduced in patients with risk factors for atherosclerosis (Hill et al., 2003; Vasa et al., 2001), and mobilized to the periphery in acute coronary syndrome patients (George et al., 2004). Contrasting results have been provided with regard to the role of EPC transfer on atherosclerosis, some of which showed anti-atherogenic (Rauscher et al., 2003) and others showing enhanced atherosclerosis (George et al., 2004).
et al., 2005). In any case, upregulation of the endogenous EPC pool is of possible benefit in atherosclerosis (Vasa et al., 2001) whereas the potential effects of cell transfer remain to be resolved. In the current study, we have found that clopidogrel significantly increased the number of EPC as compared to PBS, thus potentially contributing to the anti-atherogenic properties of the compound. The mechanism responsible for the upregulated EPC pool has not been explored. It could occur due to mobilization from the bone marrow, or alternatively a decrease in the peripheral apoptotic destruction EPC. Indeed, we have recently shown that in humans, a circulating pool of apoptic progenitors is detectable and measurable, and is increased in acute coronary syndrome patients (Shwartzenberg et al., 2007).

In conclusion, we have shown here that clopidogrel has, other than its well known anti-thrombotic properties, a role as an anti-atherogenic and plaque stabilizing compound in apoE KO mice. These findings may be explained by two potentially novel mechanisms of clopidogrel that include upregulation of the peripheral pool of naturally occurring Tregs and EPC, both of which have been shown to possess anti-atherogenic properties. If these findings are to be validated in additional experimental models, the use of clopidogrel could be further expanded to humans with proven atherosclerosis.

Acknowledgments

Supported in part by a grant from the Israeli Science Foundation (JG Grant No. 832/06) and Chief Scientist of Israel (JG).

References


Mor, A., Luboshits, G., Planer, D., Keren, G., George, J., 2006. Support in part by a grant from the Israeli Science Foundation (JG Grant No. 832/06) and Chief Scientist of Israel (JG).