Animal Models

Effect of chronic treatment with acetylsalicylic acid and clopidogrel on atheroprogression and atherothrombosis in ApoE-deficient mice *in vivo*

Christian Schulz[1], Ildiko Konrad[1], Susanne Sauer[1], Lena Orschiedt[1], Maria Koellnberger[1], Reinhard Lorenz[2], Ulrich Walter[3], Steffen Massberg[1,4]

[1]Deutsches Herzzentrum Muenchen and 1. Medizinische Klinik, Klinikum rechts der Isar, Technische Universitaet, Muenchen, Germany; [2]Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Universitaet Muenchen, Germany; [3]Institut für Klinische Biochemie und Pathobiochemie, Julius-Maximilians-Universität, Würzburg, Germany; [4]The CBR Institute and Department of Pathology, Harvard Medical School, Boston, Massachussetts, USA

Summary

Acetylsalicylic acid (ASA) and the thienopyridine clopidogrel are established anti-platelet drugs that significantly reduce secondary cardiovascular events in patients with manifest atherosclerosis. However, their impact on atherosclerotic lesion development remains controversial. Four-week-old ApoE-deficient mice were randomly assigned to four groups receiving a cholesterol diet together with either ASA (5 mg/kg), or clopidogrel (25 mg/kg), or a combination of both ASA and clopidogrel, or vehicle for 8–12 weeks. Using intravital microscopy we found that daily administration of ASA in combination with clopidogrel reduces platelet thrombus formation following rupture of atherosclerotic plaque *in vivo* by ~50%. However, therapy with ASA or clopidogrel alone, or in combination for a period of 8–12 weeks had no significant effect on adhesion of platelets to dysfunctional endothelial cells or on atherosclerotic lesion formation in the aortic root or the carotid artery. In conclusion, anti-platelet therapy is effective in reducing platelet adhesion and subsequent thrombus formation following rupture of atherosclerotic plaque *in vivo*. However, our data do not support a role of either drug in the primary prevention of atherosclerosis in ApoE-deficient mice.

Keywords

Atherosclerosis, platelet pharmacology, antiplatelet agents, atherothrombosis, animal models

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Introduction

Adhesion of platelets to the dysfunctional endothelium induces inflammation within the arterial wall, an essential step in the initiation of atherosclerotic lesion development (1, 2). Specific inhibition of platelet-endothelial cell interactions, e.g. by blockade of platelet glycoproteins (GP)Ibα and GPIIb-IIIa, reduces leukocyte accumulation in the arterial intima and attenuates atherosclerotic lesion formation (1, 2). In more advanced atherosclerosis, disruption of atherosclerotic plaque triggers rapid adhesion of large numbers of platelets to the exposed subendothelial matrix with subsequent platelet aggregation leading to arterial occlusion (2–4). Hence, platelet-vessel wall interactions play an important causative role both in the onset of the atherosclerotic process itself and for the complications of advanced atherosclerotic vascular disease.

The COX-1 inhibitor acetylsalicylic acid (ASA) and the specific P2Y12 antagonist clopidogrel are anti-platelet agents with well-established efficacy in the *secondary prevention* of cardiovascular events in patients with advanced atherosclerosis (5). Both ASA and clopidogrel per se prevent platelet aggregation at sites of endothelial disruption occurring either spontaneously or following coronary angioplasty. Notably, subjects respond individually to anti-platelet therapy, which is due to either pharmacokinetic or pharmacodynamic mechanisms. For example, the efficacy of low-dose ASA is substantially lower in diabetic subjects compared to non-diabetic individuals (6). Thus, combined treatment with ASA and clopidogrel is currently thought to

Correspondence to:
Christian Schulz
Deutsches Herzzentrum – Cardiology
Lazarettstrasse 36
D-80636 Munich, Germany
Tel.: +49 89 1218 4011, Fax: +49 89 1218 4013
E-mail: schulz@dhm.mhn.de

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represent a more effective anti-platelet strategy than treatment with either drug alone (7). In fact, recent data derived from experimental studies suggest a superior efficacy of the combined anti-platelet therapy with ASA and clopidogrel on platelet thrombus formation *in vivo* (7–9). However, the potential role of ASA and, in particular, of clopidogrel in *primary prevention* of the atherosclerotic process itself remains controversial (10–12).

Therefore, the aim of the present study was to address the impact of repeated administration of the anti-platelet drugs ASA and clopidogrel (alone or in combination) on the primary prevention of atherosclerotic lesion development in ApoE-deficient mice. Furthermore, we determined the effect of both anti-platelet strategies on platelet adhesion to the dysfunctional endothelium during atherogenesis and on platelet thrombus formation following rupture of atherosclerotic plaque using intravital videofluorescence microscopy.

Material and methods

Animals

Four-week-old male ApoE$^{-/-}$ (C57BL/6J-ApoE^{tm1Unc}) mice (The Jackson Laboratory) were fed a 0.25% cholesterol diet (Harlan research diets, 0% cholate) for another eight or 12 weeks, resulting in serum cholesterol levels between 830 and 990 mg/dl and triggering an inflammatory endothelial phenotype (1, 2). Mice were randomly assigned to four groups receiving either acetylsalicylic acid (ASA) (5 mg/kg/day; Bayer), clopidogrel (25 mg/kg/day; Bristol-Myers Squibb), ASA (5 mg/kg/day) plus clopidogrel (25 mg/kg/day), or vehicle. We applied ASA at a dose of 5 mg/kg per day, which resembles the doses used clinically in humans for primary and secondary prevention of cardiovascular events (13–15). This specific clopidogrel dose was chosen, since similar doses have previously been reported to inhibit platelet function in mice (16, 17). The medication was administered via a gastric tube to ensure proper uptake of exact amounts of the study medication. All mice received the assigned study medication daily over a period of either eight or 12 weeks starting at the age of four weeks parallel to cholesterol feeding. Consequently, experiments were performed with 12-week-old and 16-week-old mice. All experimental procedures were approved by the German legislation on protection of animals.

Measurement of serum thromboxane

Serum thromboxane (TxB2) was measured as previously described (8, 18). Briefly, blood was collected by retroorbital puncture from anesthetized mice into non-anticoagulated glass tubes and placed in a water bath at 37°C for 1 h for serum preparation. Immunoreactive TxB2 was measured in serum samples by a highly specific radioimmunoassay (18).

Platelet aggregation studies

Platelet aggregation was performed *ex vivo* as previously described (8, 19). Whole blood was collected by cardiac puncture from anesthetized mice using citrate as an anticoagulant. Platelet rich plasma (PRP) was prepared by 10 min centrifugation at 250 *g*. PRP was gently transferred to a fresh tube, and centrifugation was repeated at 2,000 *g* for 10 minutes. The platelet pellet was resuspended in HEPES-modified Tyrode solution (20) and platelet count was adjusted to 250,000/µl. Aggregation was started by addition of 20 µM adenosine 5´-diposphate (ADP) and followed in an aggregometer (Chronolog) by recording the light transmission (20).

Determination of platelet adhesion *in vivo*

Platelet adhesion dynamics in the process of atherosclerosis were monitored *in vivo* with the use of video fluorescence microscopy (1, 2). Mice were anesthetized by intraperitoneal injection of a solution of midazolame (5 mg/kg body weight; Ratiopharm), medetomidine (0.5 mg/kg body weight; Pfizer), and fentanyl (0.05 mg/kg body weight; CuraMed Pharma GmbH). The common carotid artery was carefully exposed at a distance of ~3 mm distal and 7 mm proximal to the carotid bifurcation. The exposed tissue was continuously superfused with a thermostated bicarbonate-buffered saline solution equilibrated with 5% CO_2 in nitrogen to maintain physiological pH (1, 2). Polyethylene catheters were implanted into the right jugular vein for injection of fluorescent dyes. Platelets were labeled with 5-carboxyfluorescein diacetate succinimidyl ester (DCF) and 50 x 10^6 platelets per 250 µl were injected intravenously into the jugular vein of ApoE$^{-/-}$ mice. In each group, donor and recipient were age-matched ApoE-deficient subjected to the same protocol with respect to a) age, b) duration of cholesterol-enriched diet, and c) duration and type of anti-platelet treatment. Interaction of platelets with the uninjured vascular wall of the atherosclerotic common carotid artery was visualized using a BX51W1 microscope (20x water immersion objective, Olympus) equipped with a MT20 monochromator for epi-illumination. All images were evaluated off-line using a computer-assisted image analysis program (Cap Image 7.4; Dr. H. Zeintl, Ingenieurbuero Dr. Zeintl, Heidelberg, Germany). The number of adherent platelets was quantified 15 minutes after injection of labeled platelets and calculated as n/mm^2 surface area (n=6–8 carotid arteries per group). To study platelet-vessel wall interaction during atherothrombosis, the common carotid artery was dissected free and ligated vigorously near the carotid bifurcation for 5 min to induce plaque rupture. Before and after vascular injury, fluorescent platelets were visualized in situ by in-vivo video fluorescence microscopy. The number of firmly adherent platelets is given as n/mm^2 surface area (n=6 per group).

Determination of atherosclerotic lesion development

To assess the impact of chronic treatment with ASA and/or clopidogrel on atherosclerotic lesion development, ApoE$^{-/-}$ mice were sacrificed at the age of 12 and 16 weeks after eight weeks and 12 weeks on cholesterol diet and daily administration of study drugs or vehicle. Carotid arteries and aortic root of 12-week-old and 16-week-old mice were processed for histomorphometry as described previously (1). In brief, vessels were stained with Sudan III and plaque extension was quantified by an image analysis program (Cap Image 7.4). Plaque area is given as µm^2 Sudan III-stained surface area in % of vessel surface area.

Statistics

Comparisons between groups were performed using a one-way ANOVA with a Tukey post-hoc test. Data are presented as mean +/- standard deviation (SD) or single measurements with median. A value of $p < 0.05$ was regarded as significant.

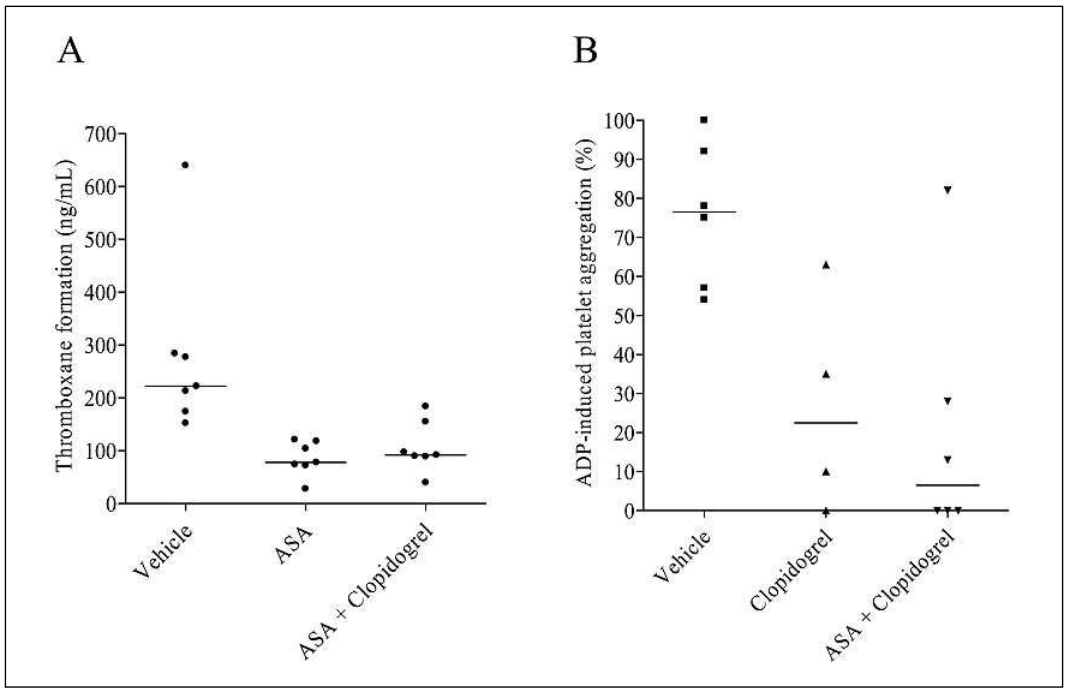

Figure 1: Effect of ASA and clopidogrel on platelet function. A) Effect of ASA (5 mg/kg) alone or in combination with clopidogrel (25 mg/kg) on thromboxane B2 formation during standardized clotting of murine whole blood (n=6, P<0.01 for ASA vs. vehicle and P<0.05 for ASA + clopidogrel vs. vehicle). B) Effect of clopidogrel (25 mg/kg) alone or in combination with ASA (5 mg/kg) on ADP (20 μM)-induced platelet aggregation of isolated murine platelets (n=4–6, P<0.05 for clopidogrel vs. vehicle and P<0.01 for ASA + clopidogrel vs. vehicle). Dots represent single measurements; bars denote median.

Results

Effect of ASA and clopidogrel on platelet function

First we defined whether doses of ASA recommended clinically for primary and secondary prevention of cardiovascular diseases in humans (13–15) are sufficient to inhibit platelet function in mice. To do this, we treated mice with ASA (5 mg/kg daily) for one week and then measured serum thromboxane B2 formation with a highly specific radioimmunoassay after spontaneous aggregation of (non-anticoagulated) whole blood (18). Importantly, 5 mg/kg ASA strongly reduced serum thromboxane B2 formation (Fig. 1A). Notably, additive treatment with clopidogrel had no additional effects on the inhibitory action of ASA.

To investigate the effect of P2Y12 antagonism on platelet adhesion and atheroprogression, a dose of 25 mg/kg clopidogrel (daily) was chosen which had been previously shown to inhibit platelet function in mice (16, 17). In fact, when we treated mice with 25 mg/kg clopidogrel alone mean platelet aggregation induced by 20 μM ADP was reduced by ~64% (Fig. 1B). The extent of inhibition of platelet function by clopidogrel was similar to that which had been previously reported by us and others in humans (19, 21, 22).

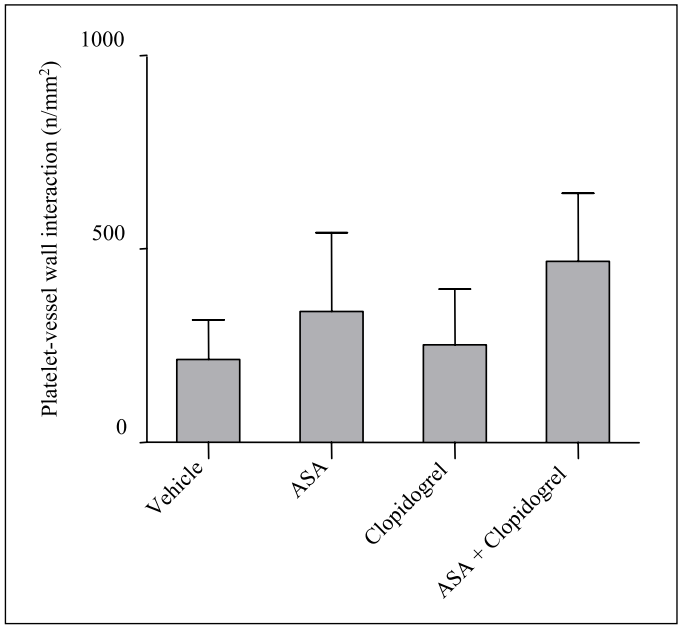

Figure 2: Effect of ASA and clopidogrel on platelet adhesion to atherosclerotic endothelium *in vivo*. Platelet interaction with the atherosclerotic endothelium was assessed by intravital microscopy in 16-week-old ApoE$^{-/-}$ mice. Adhesion of DCF-labeled platelets was quantified in the common carotid artery and is given as n/mm^2 surface area (n=6–8; mean ± SD; P=n.s.).

Effect of ASA and clopidogrel on platelet adhesion to atherosclerotic endothelium *in vivo*

Platelet adhesion to the vascular wall of 12-week- and 16-week-old ApoE-/- mice was visualized in situ by in-vivo fluorescence microscopy. As previously reported, platelet adhesion to the atherosclerotic endothelium was a prominent phenomenon in vehicle-treated ApoE-/- mice (1, 2). However, platelet-endothelium-interactions in both 12– (not shown) and 16-week-old ApoE$^{-/-}$ mice were unaffected by treatment with ASA and/or clopidogrel (Fig. 2).

Effect of ASA and clopidogrel on atheroprogression

Next, we determined the effect of ASA and clopidogrel on atheroprogression. In brief, we quantified lesion formation in 12– and 16-week-old ApoE$^{-/-}$ mice after eight and 12 weeks of cholesterol feeding, respectively. At this age vehicle-treated ApoE$^{-/-}$ mice exhibited substantial atherosclerotic plaque formation

(Fig. 3A, B). To our surprise, neither ASA, nor clopidogrel, or the combination of ASA and clopidogrel significantly affected atheroprogression in the carotid arteries (not shown) or the aortic root (Fig. 3A, B). Hence, targeting of COX-1 and/or of the ADP receptor P2Y12 does not prevent the development of atherosclerosis in ApoE-deficient mice.

Effect of ASA and clopidogrel on thrombus formation following plaque rupture

Having shown that ASA and clopidogrel do not significantly alter platelet-endothelial cell interactions during atheroprogression or atherosclerotic lesion formation itself, we finally determined the capacity of ASA and clopidogrel to prevent platelet adhesion to the subendothelium following rupture of the atherosclerotic plaque. To test this, we mechanically disrupted the endothelial cell layer covering atherosclerotic lesions in the carotid artery of 16-week-old ApoE$^{-/-}$ mice. Following plaque rupture platelets accumulated rapidly at the site of vascular injury in 16-week-old vehicle-treated ApoE$^{-/-}$ mice (Fig. 4A, B). Clopidogrel alone (by 33%, $P<0.05$ vs. vehicle), but in particular the combined treatment with both ASA plus clopidogrel (by 50%, $P<0.001$ vs. vehicle), substantially reduced platelet adhesion following plaque rupture (Fig. 4A, B). In contrast, ASA monotherapy had no significant effect on platelet adhesion.

Discussion

Platelet adhesion and aggregation are the major triggers of myocardial infarction and stroke in patients with advanced atherosclerosis. ASA and clopidogrel are effective inhibitors of platelet function *in vivo* (23). Consequently, both drugs are part of the standard anti-platelet regimen used for the *secondary prevention* of cardiovascular events (5). However, the efficacy of clopidogrel and ASA for *primary prevention* of atherosclerotic lesion development remains controversial (10, 14, 15).

Here, we utilized intravital videofluorescence microscopy to directly visualize platelet adhesion in a model of plaque rupture in the carotid artery of ApoE$^{-/-}$ mice. We showed that chronic treatment (by gavage) with ASA plus clopidogrel, and to a lesser extent clopidogrel monotherapy, significantly reduces platelet accumulation following rupture of atherosclerotic lesions. These findings are in line with previous studies, showing that clopidogrel, in addition to ASA, significantly reduces thrombotic complications following percutaneous coronary intervention in animal models (7, 24) and humans (25). Notably, platelet thrombus formation has been reported to be inhibited more effectively by combined anti-platelet therapy with ASA and clopidogrel in the presence of a nitric oxide (NO)-releasing compound without increasing prohaemorrhagic side effects (8, 26).

However, to our surprise, neither ASA nor clopidogrel were effective in preventing platelet adhesion to the inflamed endothelial surface in the absence of endothelial disruption, an event known to trigger atherosclerotic lesion development (1, 2). At the same time, both inhibition of COX-1 by ASA and blockade of the ADP receptor P2Y12 by clopidogrel were ineffective in preventing atherosclerotic lesion formation in the aortic root.

Previous data obtained in different animal models in which the role of ASA for primary prevention of atherosclerotic vascu-

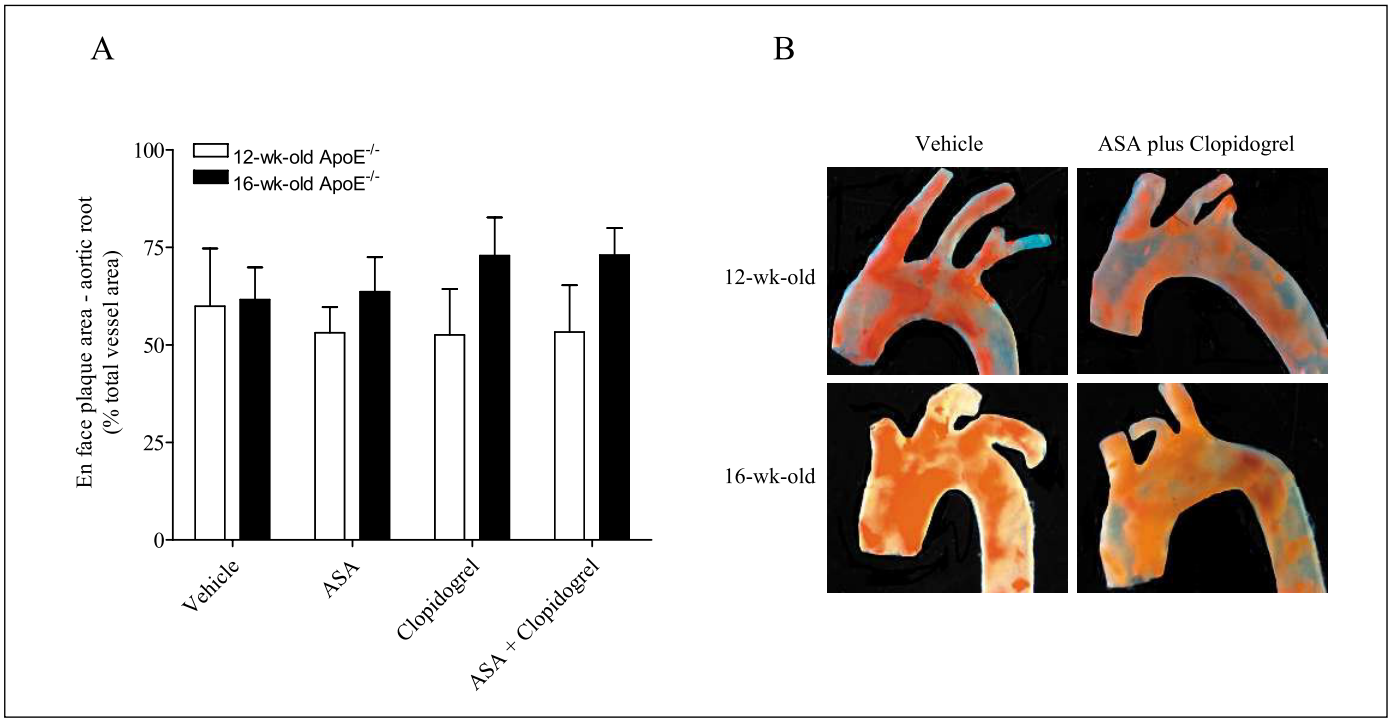

Figure 3: Effect of ASA and clopidogrel on atheroprogression. Four-week-old ApoE$^{-/-}$ mice were randomly assigned to either ASA, clopidogrel, ASA plus clopidogrel, or vehicle for another eight or 12 weeks. While on study medication, mice received a cholesterol-enriched diet. A) Atherosclerotic lesion size was quantified en face in 12– and 16-week-old ApoE$^{-/-}$ mice and indicated as plaque area in percent of total vessel area (n=6–8; mean ± SD; P=n.s.). B) Depicted are representative ex-vivo images from the aortic root of ApoE$^{-/-}$ mice treated with vehicle or ASA plus clopidogrel.

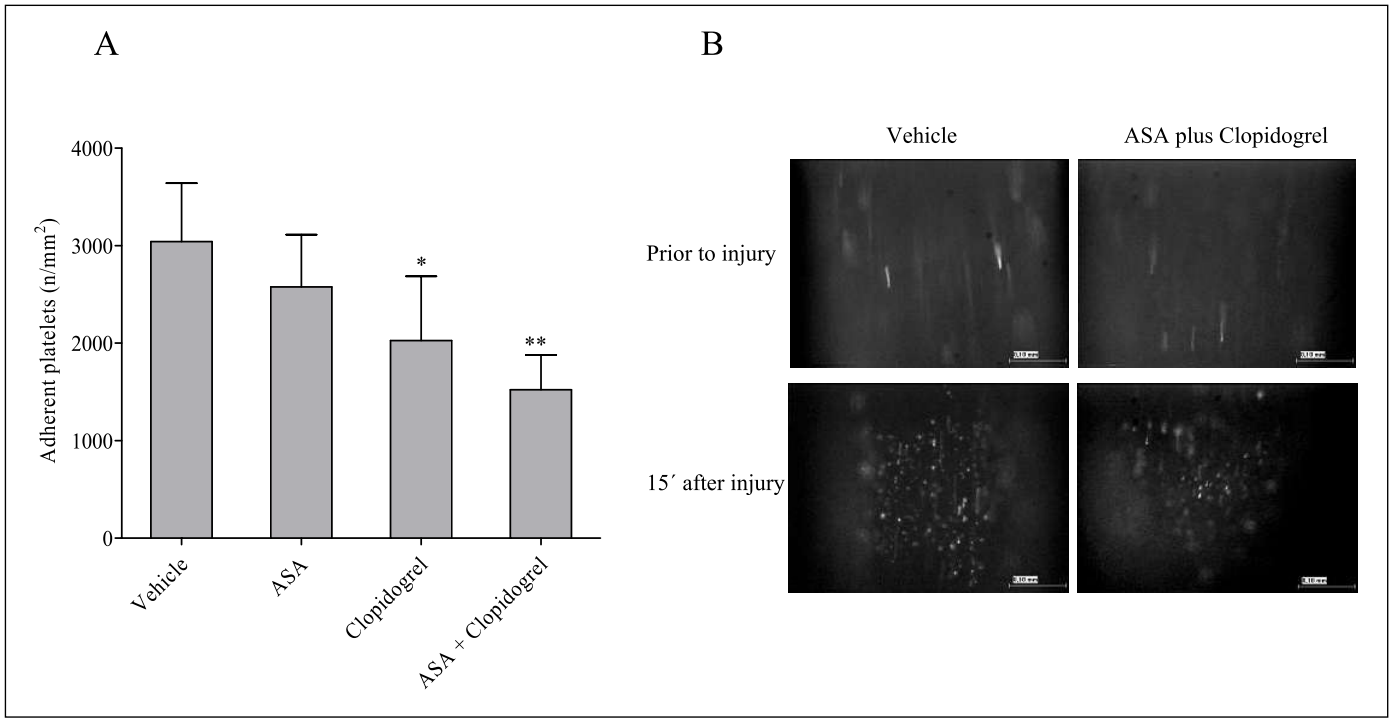

Figure 4: Effect of ASA and clopidogrel on thrombus formation following plaque rupture. Platelet thrombus formation was induced in 16-week-old ApoE$^{-/-}$ mice by ligation of the atherosclerotic carotid artery and assessed by intravital microscopy. A) Adhesion of DCF-labeled platelets was quantified before and after injury and given as n/mm^2 surface area (n=6; mean ± SD; *P<0.05 for clopidogrel vs. vehicle, **P<0.001 for ASA plus clopidogrel vs. vehicle). B) Depicted are representative intravital microscopic images from the carotid artery of ApoE$^{-/-}$ mice treated with vehicle or ASA plus clopidogrel (prior to and 15 minutes after injury).

lar disease has been addressed are controversial. While some studies describe moderate antiatherogenic actions of ASA (11, 15), others report no effects whatsoever (10). We show here that ASA does not significantly attenuate lesion formation. In part, the discrepancy in findings may be explained (1) by the use of different animal models of atherosclerosis, (2) by variations in the composition of the cholesterol-enriched diets, and (3) by the fact that different stages of atherosclerosis were investigated. However, our present findings are in line with a recent randomized clinical trial showing that ASA has no beneficial effect in the primary prevention of cardiovascular disease (14).

The role of clopidogrel for primary prevention of atherosclerosis is even less well understood. Previous experiments performed *in vitro* indicated that clopidogrel exerts inhibitory effects on intimal proliferation of rabbit arteries under culture conditions (27). It was only recently that Li et al. observed that combined anti-platelet therapy with clopidogrel and ASA reduced lesion formation in rabbits (28). However, in their model, the authors induced arterial injury in addition to cholesterol feeding to induce atherosclerotic lesion formation. In our present study, chronic treatment with clopidogrel alone or in combination with ASA did not prevent or attenuate atherosclerotic formation in ApoE-defcient mice exposed to a high cholesterol diet in the absence of any additional mechanical vessel injury (Fig. 2). However, clopidogrel did prevent platelet adhesion when arterial injury was induced (Fig. 3). Hence, in light of the previous findings by Li et al. (28) our present study clearly indicates that clopidogrel plays a minor, if any, role in the primary prevention of atherosclerosis itself, but appears to be beneficial in the prevention of secondary events following disruption of the endothelial cell lining, including arterial thrombosis (shown here) and neointima formation in response to mechanical injury (28). The latter is supported by a recent clinical trial, which demonstrated that sustained dual oral anti-platelet therapy following percutaneous coronary intervention attenuates neointima formation following percutaneous coronary intervention in humans (25). Instead, patients with multiple risk factors do not profit from additional clopidogrel treatment on top of ASA in the primary prevention of cardiovascular events (13).

It is important to emphasize that our present findings do not exclude a role for platelets in the initiation of atherosclerotic lesion formation. In fact, we have previously reported that platelet adhesion to the endothelial surface occurs very early in the process of atherosclerosis. Inhibition of platelet-endothelial cell interactions substantially attenuates atherosclerotic lesion formation (1, 2). Here, we show that treatment with ASA and/or clopidogrel does not affect platelet-endothelial cell interactions in the early stages of atherosclerosis and is ineffective in preventing atherosclerotic lesion formation. Based on our earlier findings this implies that (1) platelet activation through pathways that do not involve COX-1 or P2Y12 signaling, and/or (2) the process of platelet adhesion in itself (e.g. through platelet and/or endothelial integrins) are sufficient to initiate and accelerate atherosclerotic lesion formation. Hence, drugs that specifically target the receptors involved in platelet adhesion to the inflamed endothelial surfaces might provide a more promising strategy in

terms of the primary prevention of atherosclerotic lesion formation.

In summary, we show here that anti-platelet therapy with ASA plus clopidogrel is effective in the inhibition of acute thrombosis following plaque rupture in established atherosclerosis in mice, but does not prevent the development of atherosclerosis *per se*. Thus, further basic investigation using experimental animal and in-vitro models is warranted in order to develop an effective therapeutic intervention during atherosclerotic lesion development.

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