

## Platelets and Blood Cells

# Clopidogrel inhibits platelet-leukocyte adhesion and platelet-dependent leukocyte activation

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### Summary

Clopidogrel is considered to be an important therapeutic advance in anti-platelet therapy. We investigated whether inhibition by clopidogrel results in a reduced capacity of platelets to adhere and stimulate pro-atherothrombotic and inflammatory functions in polymorphonuclear leukocytes (PMN) and in monocytes (MN). An eventual effect on these processes could further substantiate anti-atherothrombotic properties of this drug. The effects of clopidogrel or of its active metabolite were investigated on ADP or thrombin receptor-induced platelet activation and on platelet-leukocyte interactions *ex vivo* in the mouse or *in vitro* in isolated human cells or whole blood, respectively. Clopidogrel inhibited platelet aggregation, expression of P-selectin, platelet-PMN adhesion and platelet-dependent

ROS production in mouse PMN. Similarly pretreatment of human platelets with the active metabolite of clopidogrel *in vitro* resulted in a profound inhibition of platelet P-selectin expression, platelet-PMN adhesion and production of ROS by PMN. Pretreatment with the active metabolite of clopidogrel significantly impaired the ability of platelets to up-regulate the expression of TF procoagulant activity in MN, in a washed cell system. Moreover, the active metabolite of clopidogrel inhibited rapid TF exposure on platelet as well as on leukocyte surfaces in whole blood. By reducing platelet-dependent up-regulation of inflammatory and pro-atherothrombotic functions in leukocytes, clopidogrel may reduce inflammation that underlies the chronic process of atherosclerosis and its acute complications.

### Keywords

Clopidogrel, P-selectin, platelet-leukocyte interaction, ROS, tissue factor

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### Introduction

ADP plays a fundamental role in the amplification and propagation of platelet activation at the site of vascular injury. Two G-protein coupled receptors largely responsible for platelet responses to ADP have been identified: P2Y<sub>1</sub> and P2Y<sub>12</sub>. These receptors mediate different activation pathways including cytosolic calcium increase / phospholipase C activation and inhibition of adenylate cyclase / PI3K activation, respectively (1, 2). The clinical relevance of ADP is demonstrated by the clinical efficacy of the antithrombotic drugs ticlopidine and clopidogrel, which specifically target P2Y<sub>12</sub> (3–5).

As soon as platelets become activated, at the site of vascular injury, platelet-leukocyte interactions are initiated (6) and leuko-

cytes involved in the process contribute in different ways to the initiation and progression of atherosclerosis (7, 8). Thus, it is reasonable to speculate that inhibition of platelet-leukocyte interaction may reduce the inflammatory reaction at the site of atherosclerotic lesion. Previous studies investigated the possible effect of current anti-platelet therapies on platelet-leukocyte interactions.

Aspirin treatment does not affect platelet-leukocyte interaction (9, 10), while peptide and non peptide IIb/IIIa inhibitors, may increase heterotypic platelet-leukocyte interaction. Moreover the anti-IIb/IIIa antibody Abciximab was shown to reduce (11) or increase platelet-leukocyte interaction (12).

In contrast, a number of recent studies consistently showed that treatment of cardiovascular patients with clopidogrel re-

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duces circulating platelet-leukocyte conjugates and inhibits the formation of these mixed aggregates after platelet stimulation in whole blood *ex vivo* (10, 12–14).

Clopidogrel is a pro-drug that is inactive *in vitro*, and which requires hepatic metabolism to exhibit platelet inhibitory activities (15). Recent studies identified the chemical structure of an active metabolite generated from human liver microsomes incubated with clopidogrel (15, 16). *In vitro* studies demonstrated that this compound exhibited the biological activities of clopidogrel observed *ex vivo*, i.e. irreversible inhibition of  $^{32}\text{P}$ -2Mes-ADP binding, selective inhibition of ADP-induced platelet aggregation and adenylyl cyclase down-regulation (15).

In the present study we investigated the ability of clopidogrel or its active metabolite, respectively, to inhibit platelet-PMN adhesion and platelet-dependent generation of reactive oxygen species *ex vivo*, in the mouse, and *in vitro*, in human cells. Moreover, in human cells, we investigated the effect of the active metabolite of clopidogrel on platelet-dependent increase of TF procoagulant activity in platelet-MN washed cell suspensions, and on rapid exposure of immunoreactive TF molecules on platelets and leukocytes in whole blood.

## Materials and methods

### Reagents

Clopidogrel was provided by Sanofi-Synthelabo Recherche (Toulouse, France). The active metabolite, a stereoisomer of the primary chemical structure 2-{1-[1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-4-sulfanyl-3-piperidinylidene} acetic acid, was purified from supernatant of human liver microsomes incubated with the 2-oxo-precursors as previously described (16).

### Ex vivo experiments in the mouse

Animal studies were performed using protocols that were approved by the institutional Animal Use and Care Committee. Swiss CD-1 male mice (Charles Rivers Laboratories, Calco, Lecco, Italy) were used for the study. 15–20 controls and clopidogrel treated mice were used for each single experiment. Clopidogrel or vehicle (water) was administered by oral gavage. Animals were either untreated or treated by with 25 mg/kg twice a day for two days plus a 25 mg/kg dose 1–2 hours before obtaining blood for the experiment on day 3.

### Preparation of mouse platelets and PMN

Mice were anaesthetized with a mixture of ketamine and xylazine (100 mg/kg and 20 mg/kg respectively) administered intraperitoneally and blood was collected in 0.38% trisodium citrate (final concentration) by cardiac puncture (17). Blood from 15–20 control or clopidogrel treated mice was pooled and centrifuged at 80 g for 15 minutes at room temperature to obtain platelet-rich plasma (PRP). Mouse platelets and PMN were isolated as previously reported (17).

Briefly, platelets were pelleted by centrifugation in the presence of 1  $\mu\text{M}$  PGE<sub>1</sub> and resuspended in HEPES-Tyrode buffer (pH 7.4) containing 1 mM CaCl<sub>2</sub> and MgCl<sub>2</sub>.

Following the removal of PRP, a buffy coat was prepared from the remaining blood by gravity sedimentation of the red blood cells through Dextran T500. The buffy coat was removed,

and the PMNs were isolated from the buffy coat by centrifugation on a Ficoll-Hypaque gradient. The contaminating erythrocytes were removed by hypotonic lysis. Following this procedure, the leukocyte population consisted of ~70% PMNs and ~30% lymphocytes. The leukocytes were washed, resuspended in Tyrode's buffer at  $5 \times 10^6$  cells/ml and kept on ice until the experiment.

### Mouse platelet-PMN adhesion in suspension

Mouse platelet-PMN adhesion in suspension was investigated by flow cytometry as previously described (17). Briefly, leukocytes were stained with the vital red fluorescent, nuclear dye hydroethidine (HE) (Molecular Probes, Eugene, OR, USA) (20  $\mu\text{g}/\text{ml}$  per  $5 \times 10^7$  PMN/ml for 30 minutes at 4°C, and washed platelets with the green fluorescent probe 2', 7'-Bis-(2-carboxyethyl)-5(6) carboxy-fluoresceine triacetoxymethyl ester (BCECF-AM) (Sigma-Aldrich, St Louis, MO, USA) (2  $\mu\text{g}/\text{ml}$  per  $5 \times 10^8/\text{ml}$  for 30 minutes at 37°C).

Platelets were stimulated with different concentrations of ADP (Mascia Brunelli, Milan, Italy) or thrombin (Sigma-Aldrich) in the presence of 800  $\mu\text{moles}/\text{l}$  of peptide RGDS (Sigma-Aldrich). After 1 minute of stimulation PMN were added at a ratio PMN:platelets of 1:10 and mixed cell suspensions were further incubated in a final volume of 250  $\mu\text{l}$  in siliconized glass cuvettes (internal diameter 6 mm; ChronoLog, Mascia Brunelli, Milan, Italy) placed in an aggregometer (Platelet Ionized Calcium Aggregometer, PICA, ChronoLog) at 37°C and stirred at 1,000 rpm using a magnetic stirrer and a 4 mm iron stir bar. The interaction was stopped at 1 minute by the addition of one volume of 2% paraformaldehyde. A negative control was included in each experiment and consisted of the same sample prepared in the presence of 5 mM EGTA to prevent divalent cation-dependent platelet-PMN interactions.

### Human platelet-PMN adhesion in suspension

Human platelets and PMN were isolated from whole blood of healthy donors as previously described (18).

Human platelet-PMN adhesion in suspension was investigated by flow cytometry as described above for the mouse cells with minor modifications. Human platelets were loaded with the green fluorescent probe by incubating PRP, with BCECF-AM for 30 minutes (18). BCECF-loaded platelets were stimulated in PRP with different concentrations of ADP or PAR-1 agonist peptide, SFLLRN (Bachem, Bubendorf, Switzerland) in the presence of 800  $\mu\text{moles}/\text{l}$  of peptide RGDS, after 1 minute of stimulation PMN were added at a ratio PMN: platelets of 1:10 and mixed cell suspensions were further incubated and processed exactly as described for the mouse cells. Clopidogrel active metabolite or its vehicle (NaCl 0.9%) was incubated in the samples for 30 min at room temperature, before stimulation.

### Two-color cytofluorimetric assay of PMN adhesion to platelets in suspension

The formation of platelet-PMN conjugates was evaluated with a FACStar flow cytometer (Becton Dickinson, Milan, Italy) and platelet-PMN adhesion quantified exactly as previously reported (17, 18). Briefly, PMNs were distinguished from contaminating lymphocytes on the basis of forward and side scatter (17). The

threshold for BCECF (platelet) fluorescence (FL1) in the PMN gate was set on a negative control sample containing 5 mM EGTA (90% of events below the threshold). The percentage of PMN with BCECF fluorescence above the threshold reflects PMN with bound platelets ("positive" PMN). The mean number of adherent platelets per positive PMN was determined by dividing the mean BCECF (FL1) fluorescent intensity of the positive PMN population by the mean BCECF (FL1) fluorescent intensity of a sample of BCECF-labeled platelets alone. This value multiplied by the percentage of PMN binding platelets gives the number of platelets recruited by a standard population of 100 PMN.

### Cytofluorimetric analysis of P-selectin expression on platelets

P-selectin expression on resting or stimulated mouse platelets was monitored by flow cytometry using rat anti-mouse P-selectin monoclonal antibody RB40 (Pharmingen, San Diego, CA, USA). The rat monoclonal antibody M1/70 (Pharmingen), which recognizes mouse Mac-1, was used as a negative control. The binding of the primary antibody was revealed by Alexa Fluor-488 goat anti-rat IgG (Molecular Probes). P-selectin expression by human platelets was analyzed in PRP using the anti-human P-selectin antibody WAPS (19), the anti  $\beta$ 2-integrins IB4 (19), as negative control, and Alexa Fluor-488 goat anti-mouse IgG as secondary antibody (Molecular Probes). Both WAPS and IB4 were purified from mouse ascites or supernatants of hybridoma cells (ATCC, Manassas, VA, USA) using Protein G-Sepharose (Amersham Biosciences (Little Chalfont, Bucks. UK) affinity column.

### Aggregation experiments

Platelet aggregation was monitored as previously described (15).

### Measurement of oxygen burst in PMN

As an index of platelet-induced PMN activation, we evaluated oxygen radical production in PMN by flow cytometry. For this purpose, PMN were loaded with 2',7-dichlorofluorescein diacetate (DCFDA) (Sigma-Aldrich), 40  $\mu$ g/ml of DCFDA for 30 minutes in ice, washed and resuspended in Hepes Tyrode buffer. DCFDA is a cell permeable non fluorescent probe which is de-esterified and trapped in the cytoplasm where it turns to highly fluorescent 2',7-dichlorofluorescein upon oxidation. It is widely used for sensitive and rapid measurement of oxygen reactive species production in myeloid cells (20).

DCFCA-loaded PMN were incubated alone or mixed with unstimulated or with thrombin-activated washed platelets. After 10 minutes of stimulation cells were fixed with 1% PFA and analyzed by flow cytometry. A sample of unstimulated PMN maintained in ice was used to evaluate the basal fluorescence signal of DCFDA-loaded PMN. This sample was used to set a threshold to identify PMN showing increased ROS production. Values are reported as percentage of PMN showing fluorescence values higher than this threshold.

### Tissue factor assay

Enriched MN cell suspension was obtained from whole blood collected from healthy donors by Lymphoprep (Nycomed Phar-

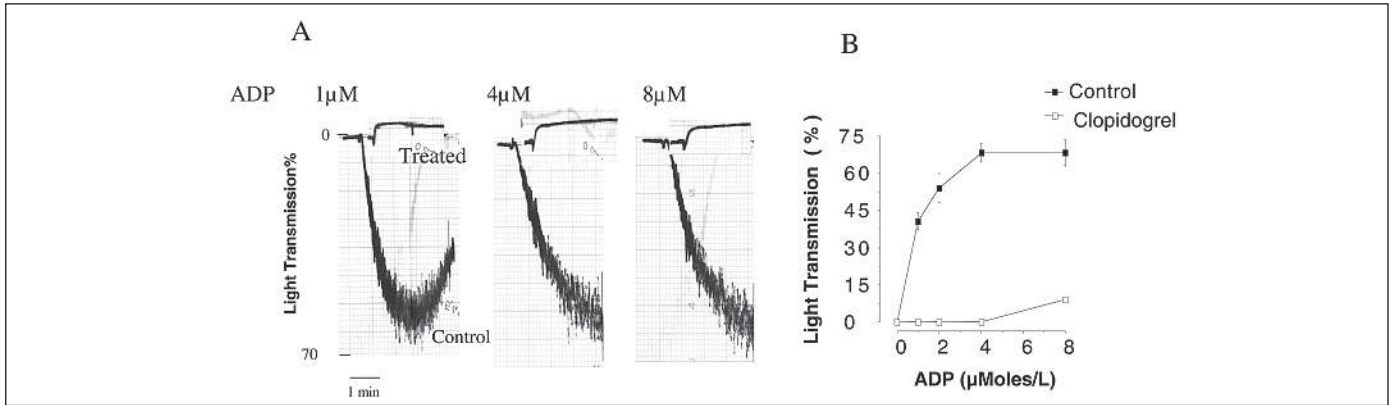
ma AS, Oslo, Norway) sedimentation as previously described (21). The MN in this population were 25–30%, as assessed by non-specific esterase staining, and less than 2 platelets per MN were counted by microscopy. MN cells were mixed with washed platelets (1/40 ratio) which had been pretreated or not with the active metabolite of clopidogrel (5  $\mu$ moles/l) for 30 min at room temperature. Mixed cell suspensions were stimulated with bacterial endotoxin (LPS, (*Escherichia coli* 055:B5, Sigma-Aldrich, Milan, Italy), 100 ng/ml, in combination with the PAR-1 activating peptide SFLLRN for 6 hours at 37°C. In each experiment a sample of MN was stimulated in the absence of platelets.

After incubation, cells were disrupted by 3 freeze-thaw cycles, and procoagulant activity was assessed by a one stage clotting time (21). Results were expressed in arbitrary units (U) by comparison with a standard curve obtained using a human brain thromboplastin standard kindly donated by Dr. L. Poller, (University of Manchester, Manchester, U.K.) This preparation was assigned a value of 1000 U for a clotting time of 20 sec. The procoagulant activity generated in this model is completely inhibited by an anti-TF antibody (kindly donated by Dr Y. Nemer-son, Mt. Sinai Medical Center, New York, USA) indicating that the activity is solely attributable to TF [not shown].

### Flow cytometric analysis of platelet P-selectin, mixed platelet-leukocyte conjugates, and immunoreactive TF in whole blood

Whole blood was incubated in the presence of the active metabolite (5  $\mu$ moles/l) or vehicle at room temperature for 30 min. The samples were then stimulated with ADP (10  $\mu$ moles/l) or with the thrombin receptor agonist peptide SFLLRN (10  $\mu$ moles/l) for 15 min in the presence of 800  $\mu$ moles/l RGDS to prevent platelet-platelet aggregation. A ten  $\mu$ l aliquot of non stimulated or ADP or SFLLRN-activated blood were then incubated with Tyrode's buffer (90  $\mu$ l) containing primary mouse monoclonal antibodies against P-selectin (WAPS), IIB/IIIa (clone 10E5, IgG1, kindly provided by Dr B. Coller, Rockefeller University, NY), TF (Clone 4508, IgG1, American Diagnostica, Ortho Clinical Diagnostics, Milan, Italy) or an irrelevant mouse IgG1 (10  $\mu$ g/ml final dilution) for 15 min. Samples were incubated with Alexa Fluor-488 goat anti-mouse IgG as secondary antibody (Molecular Probes) for a further 15 min. After immunofluorescent staining, erythrocytes were lysed by FACS™ lysing solution (Becton Dickinson) according to the manufacturer's instruction and samples immediately analyzed by FACS. Cytometric parameters were acquired on a logarithmic scale. Leukocyte and platelet populations were identified on the basis of forward and side scatter parameters. P-selectin and TF exposure on platelets was measured by recording a total of 4000 events in the platelet population. P-selectin expressing platelets were analysed as percentage of events showing P-selectin specific staining above a threshold set using an irrelevant mouse IgG1.

Expression of immunoreactive-TF was analysed as geometric mean of fluorescence. TF-specific immunofluorescence was calculated, for each sample, as difference between the values of geometric mean fluorescence obtained with the anti-TF antibodies and that obtained with the irrelevant immunofluorescent staining.



**Figure 1: Effect of clopidogrel on ADP-induced platelet aggregation in mouse PRP.** A) The figure shows representative tracings of platelet aggregation in PRP from controls or clopidogrel-treated mice, challenged by ADP in increasing concentrations. B) The graph reports the means±SEM of data from 6 different experiments measuring platelet aggregation in PRP triggered by increasing concentrations of ADP. Each experiment was performed using pools of PRP from 5–10 control or treated mice.

Platelet-leukocyte aggregates were detected as IIB/IIIa positive events in the leukocyte population. Leukocytes positive for anti-IIB/IIIa were assessed as the percentage of total events identified as PMN and monocytes (4000 events recorded), reflecting the percentage of PMN-monocytes with bound platelets, and as the mean intensity of IIB/IIIa specific fluorescence per particle. The latter value divided by the value of IIB/IIIa specific mean fluorescence of the platelet population represents an estimate of the number of platelets associated with 1 leukocyte. This value multiplied by the percentage of PMN binding platelets gives the number of platelets recruited by a standard population of 100 PMN.

TF exposure on leukocytes was measured by recording a total of 2500 events in the leukocyte population.

**Statistical analysis**

Data are presented as Mean ± SEM, and were analyzed by repeated measurement ANOVA or by paired t-test. Differences be-

tween treated and controls have been assessed at each dose level. P<0.05 was considered statistically significant.

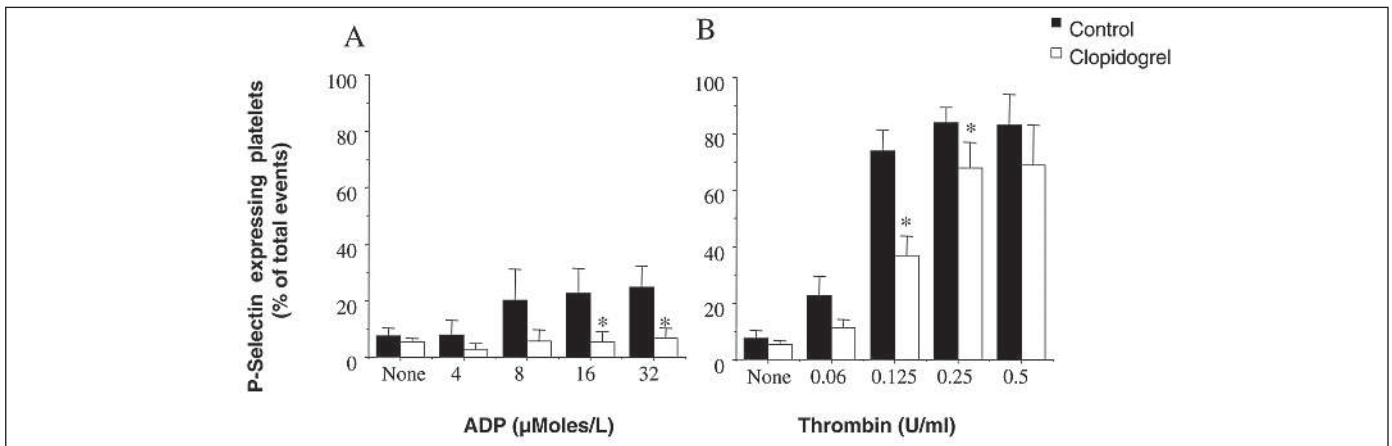
**Results**

**Effect of clopidogrel on platelet aggregation**

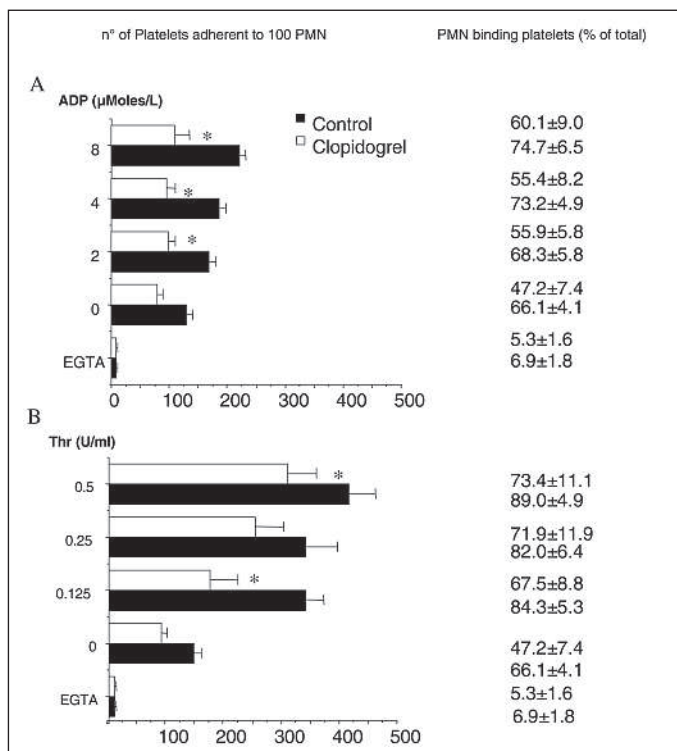
Initial experiments were performed to confirm that treatment of mice with clopidogrel inhibits platelet aggregation triggered by ADP *ex vivo*. As shown in Figure 1, clopidogrel abolished platelet aggregation, in PRP, induced by increasing concentrations of ADP.

**Effect of clopidogrel on P-selectin expression**

As reported in Figure 2, 7.7±2.5% (Mean± SEM, n=8) of unstimulated mouse washed platelets expressed P-selectin. This value was reduced to 5.4±1.2% (n=8) in platelets from clopidogrel treated mice.



**Figure 2: Effect of clopidogrel on ADP and thrombin induced P-selectin expression in mouse platelets.** Platelets from control (filled bars) or clopidogrel treated mice (empty bars) were stimulated with agonists in increasing concentrations. P-selectin expression was quantified by cytometry and expressed as the percentage of positive platelets. The graphs report the means±SEM of data from 4 and 8 different experiments for ADP (A) or thrombin-induced (B) P-selectin expression by washed platelets, respectively. Each experiment was performed using pools of PRP from 5–10 control or treated mice. \*denotes P<0.05 levels of significance.

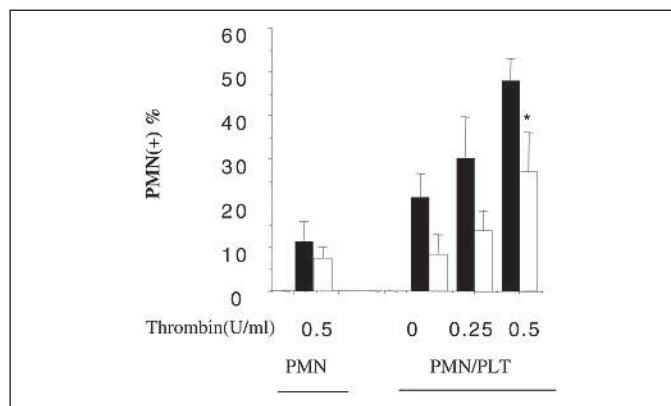


**Figure 3: Effect of clopidogrel on mouse platelet-PMN adhesion.** Platelet-PMN adhesion was evaluated by flow cytometry. PMN and platelets were obtained from mice treated (empty bars) or not (filled bars) with clopidogrel. The bars indicate the number of platelets recruited by 100 PMN. This was determined as follows: the mean fluorescent intensity (FLI) of the PMN population showing the platelet marker (BCECF) was divided by the mean fluorescent intensity (FLI) of the BCECF-labeled platelets. This value, which represents an estimate of the number of platelets per leukocyte conjugate, multiplied by the percentage of PMN showing the platelet marker, gives the number of platelets recruited by a standard population of 100 PMN. The numbers indicate the percentage of PMN binding platelets. Data are Means±SEM of 4 and 8 different experiments performed for ADP (A) or thrombin (B), respectively. Each experiment was performed using a pool of blood from 15–20 controls or treated mice. \*denotes  $P < 0.05$  levels of significance.

Stimulation with ADP slightly increased the percentage of platelets expressing detectable amounts of P-selectin reaching a plateau of  $20.1 \pm 11\%$  at  $8 \mu\text{moles/l}$  of ADP (Mean±SEM,  $n=4$ ); this effect was abolished in platelets from clopidogrel-treated mice (Fig. 2A). Similarly, the dose-dependent increase of P-selectin expression induced by thrombin was significantly lower in platelets from clopidogrel-treated mice than in controls (Fig. 2B).

Stimulation of platelets in mouse PRP with  $10 \mu\text{moles/l}$  ADP or  $0.5 \text{ mmoles/l}$  of arachidonic acid increased the percentage of P-selectin expressing platelets from 1.3 in unstimulated PRP to 16 and 77.8%, respectively (Mean,  $n=2$ ). Treatment with clopidogrel abolished P-selectin expression by ADP and reduced that triggered by arachidonic acid (1.2 and 15.3%, respectively, data not shown).

Thus treatment with clopidogrel *in vivo* significantly reduced the capacity of platelets to express P-selectin following activation with strong stimuli *ex vivo*.



**Figure 4: Effect of clopidogrel on mouse platelet-dependent ROS production in PMN.** PMN alone (PMN) or in suspension with platelets (PMN/PLT) prepared from control (filled bars) or clopidogrel treated mice (empty bars) were stimulated by different concentrations of thrombin. Results are percentage of PMN producing ROS over a basal level. Data are mean±SEM from 3 different experiment using pools of blood from 15–20 untreated or clopidogrel treated mice, \*denotes  $P < 0.05$  levels of significance.

#### Clopidogrel reduces platelet/PMN adhesion

The formation of platelet/PMN conjugates in cell suspensions incubated under stirring (1000 rpm) was investigated by double color flow cytometry. In parallel with the reduction of P-selectin expression, ADP or thrombin-activated platelets from clopidogrel-treated mice showed a reduced capacity to form mixed conjugates with PMN. As shown in figure 3, both the number of platelets recruited by PMN as well as the percentage of PMN binding platelets were reduced in mixed cell suspensions from clopidogrel-treated animals. In selected experiments platelets from untreated animals were mixed with PMN from clopidogrel-treated mice. In this condition, adhesion was similar to that of cells from untreated animals (data not shown) indicating that the inhibitory effect of clopidogrel on platelet/PMN adhesion is mainly due to a reduced platelet response.

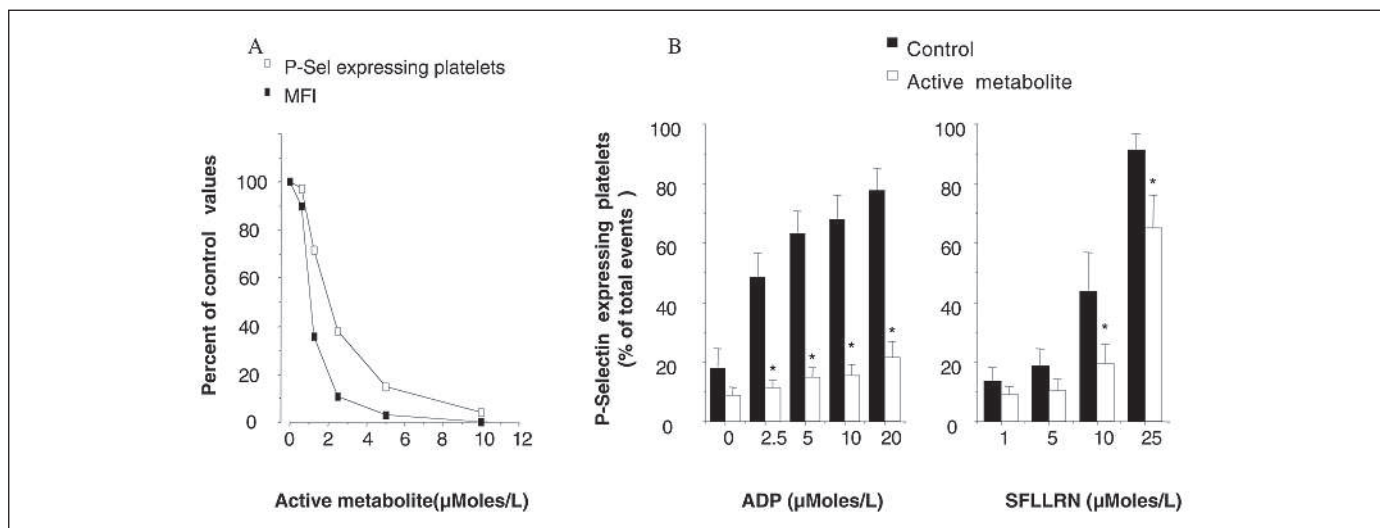
#### Clopidogrel reduces platelet-dependent respiratory burst in PMN

To investigate whether the reduced adhesion resulted in a reduced functional response of leukocytes, we evaluated the production of reactive oxygen species in PMN loaded with the fluorescent probe DCFDA, by flow cytometry.

As shown in figure 4, thrombin did not stimulate respiratory burst in PMN incubated alone, but dose-dependently increased ROS production in the presence of platelets. This increase was significantly lower in mixed cells suspension from clopidogrel-treated mice, than in cells from controls.

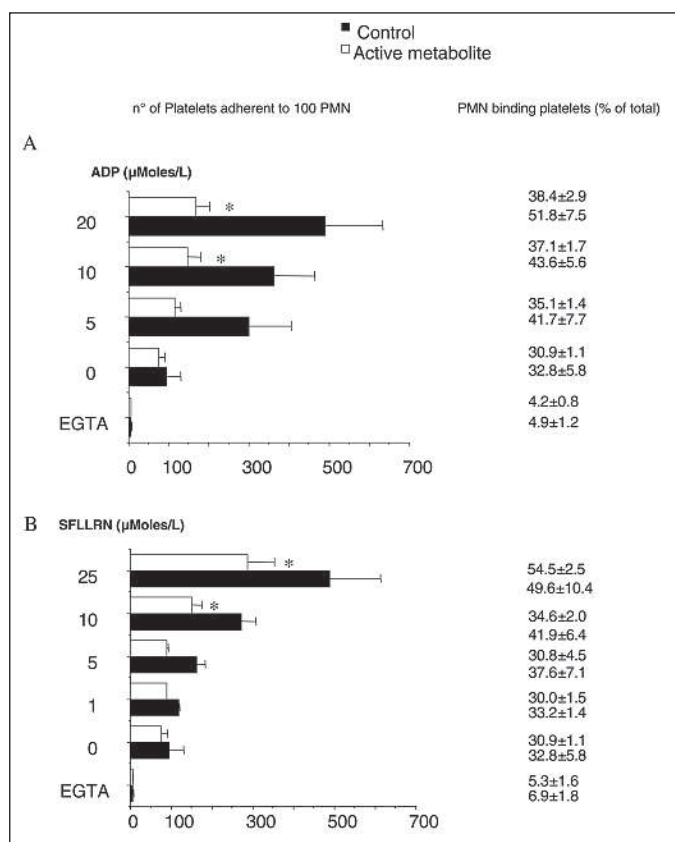
#### Effect of the active metabolite of clopidogrel on P-selectin expression

Preincubation of human PRP for 30 minutes with increasing amounts of the active metabolite dose-dependently inhibited P-selectin expression triggered by ADP ( $10 \mu\text{moles/l}$ ), reaching a maximal effect at a concentration of  $5 \mu\text{moles/l}$  (Fig. 5, panel A). At this concentration the active metabolite totally abolished P-selectin expression triggered by increasing concentrations of



**Figure 5: Effect of clopidogrel active metabolite on ADP or SFLLRN-induced P-selectin expression in human platelets.** A) Human PRP, was incubated for 30 minutes with the active metabolite in increasing concentrations then triggered with ADP (10 μmoles/l). P selectin expression was analyzed by flow cytometry. The results are reported as percentage of the values obtained in the absence of clopidogrel active metabolite of both the percentage of platelets expressing

P-selectin and the mean fluorescence intensity (MFI). B) Platelets in PRP were incubated in the absence (filled bars) or in the presence (empty bars) of the active metabolite (5 μmoles/l) for 30 minutes. Then PRP was activated with ADP or PAR-1 agonist peptide SFLLRN, in increasing concentrations. P-selectin expression was measured by flow cytometry. Data are mean±SEM from 3 different experiments using cells from 3 different donors, \*denotes P<0.05 levels of significance.



**Figure 6: Effect of clopidogrel active metabolite on human platelet/PMN adhesion.** The bars indicate the relative number of platelets recruited by PMN. The numbers indicate the percentage of PMN binding platelets Data are Means±SEM of 3 different experiments performed for ADP (A) or PAR-1 agonist peptide SFLLRN (B) using cells from 3 different donors, \*denotes P<0.05 levels of significance.

ADP and significantly reduced the dose-dependent stimulation by the PAR-1 agonist peptide SFLLRN (Fig. 5, panel B). Similar results were obtained when control or active metabolite-treated platelets were washed and the expression of P-selectin was measured following platelet stimulation with thrombin (not shown) confirming the already described irreversibility of the inhibitory effect (15).

#### Effect of the clopidogrel active metabolite on platelet-leukocyte adhesion and ROS production

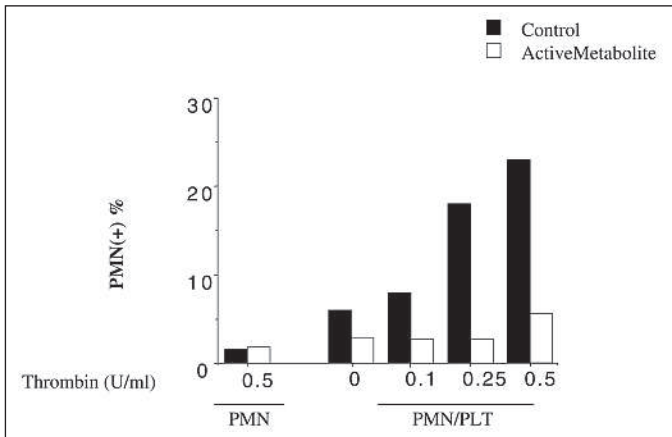
Pretreatment of human platelets with the active metabolite (5 μmoles/l) virtually abolished platelet/PMN adhesion triggered by ADP *in vitro*, and significantly reduced that triggered by PAR-1 agonist SFLLRN (Fig. 6).

Furthermore, production of ROS by PMN coincubated with platelets pretreated with the active metabolite was lower than that observed in the presence of untreated platelets (Fig. 7).

#### Effect of the clopidogrel active metabolite on TF pro-coagulant activity in mixed platelet/MN suspensions

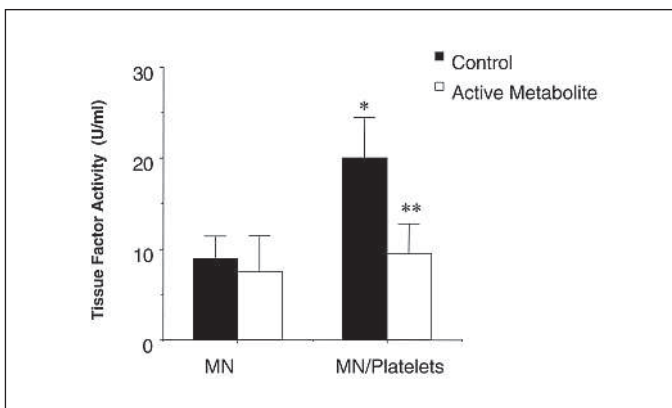
Platelets may up-regulate *de novo* TF synthesis in monocyte via the release of arachidonic acid metabolites and P-selectin (22–24). We investigated the effect of the active metabolite of clopidogrel on platelet-dependent increase of TF activity in human MN cells. In these experiments MN alone or combined with platelets (1:40 ratio) were activated with endotoxin for 6 hours in order to trigger TF gene transcription. SFLLRN was used as a specific platelet agonist. One stage clotting time was used to assess pro-coagulant activity in whole cell lysates.

As shown in figure 8, TF activity triggered by the combination of endotoxin and SFLLRN in platelet-MN mixed cell populations was significantly higher than that expressed by MN alone. Coincubation of MN with control platelets resulted in a



**Figure 7: Effect of clopidogrel active metabolite on human platelet-dependent ROS production in PMN.** PMN alone (PMN) or in suspension (PMN/PLT) with platelets prepared from PRP incubated in the absence (filled bars) or in the presence (empty bars) of active metabolite (5  $\mu$ moles/l) for 30 minutes were stimulated by different concentrations of thrombin. Results are the percentage of PMN producing ROS over a basal level. Data are means of 2 different experiments using different donors.

significant increase of procoagulant activity. This increase was prevented with platelets isolated from PRP previously treated with the active metabolite of clopidogrel. This indicates that the active metabolite inhibits a platelet-dependent function relevant for up-regulation of TF in MN. In agreement with this conclusion the presence of the active metabolite of clopidogrel did not modify TF activity in MN alone.



**Figure 8: Effect of the clopidogrel active metabolite on the platelet dependent TF activity in human MN.** MN cells were isolated and mixed with platelets (1/40 ratio) pretreated in PRP with vehicle (filled bars) or with the active metabolite (5  $\mu$ moles/l) (empty bars) for 30 min at RT and then washed before coinubation. MN alone or mixed cell suspensions were stimulated with LPS (100 ng/ml) in combination with the PAR-1 activating peptide SFLLRN (50  $\mu$ moles/l) for 6 hours at 37°C. After incubation, cells were disrupted by 3 freeze-thaw cycles, and procoagulant activity was assessed by a one stage clotting time. Results were expressed in arbitrary units (U). Data are mean $\pm$ SEM of 8 different experiments using cells from 8 different donors, \*denotes P<0.05 levels of significance (MN/platelets vs MN). \*\*P<0.05 levels of significance (Control vs Active Metabolite).

Short time incubation (15 min) of MN with platelets and SFLLRN did not result in detectable procoagulant activity (not shown), excluding an important pro-coagulant contribution of TF molecules rapidly released by activated cells, in this washed cell system.

#### Effect of the active metabolite of clopidogrel on P-selectin and TF exposure on platelets, on platelet-leukocyte conjugates and on TF exposure on leukocytes, in whole blood

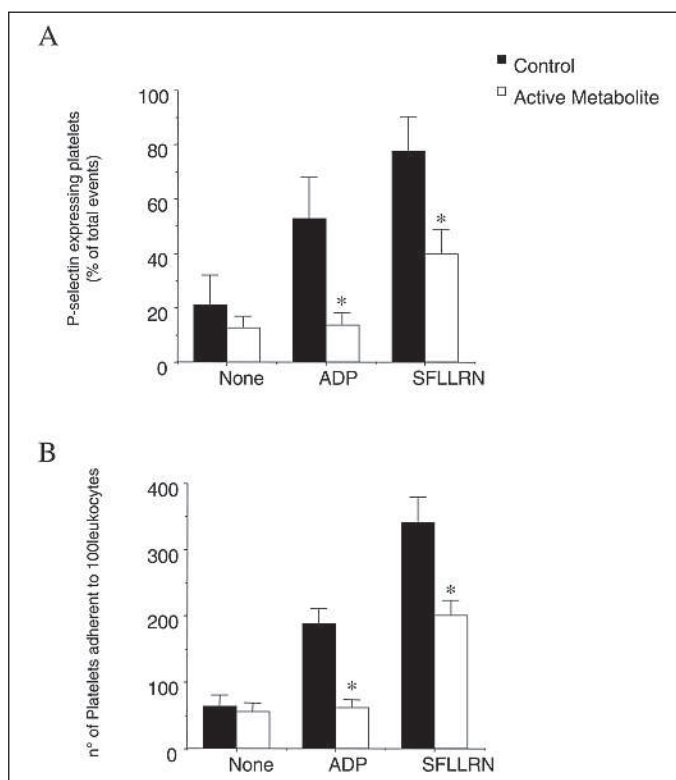
In contrast to the results reported above, Leon et al (25) showed that stimulation of whole blood with ADP or SFLLRN for 15 min resulted in P-selectin-dependent platelet-leukocyte interactions, and rapid immunoreactive-TF exposure at the surface of leukocytes. This process was inhibited by P2Y12 receptor-antagonists. The use of washed cells instead of whole blood as well as detection of TF procoagulant activity instead of immunoreactive TF molecules may account for this apparent discrepancy. Thus, we have further investigated the effect of the active metabolite of clopidogrel on rapid immunoreactive-TF exposure on platelets and leukocyte surface by whole blood flow cytometry. Preincubation of whole blood for 30 minutes with 5  $\mu$ moles/l of the active metabolite of clopidogrel abolished P-selectin expression triggered by ADP (10  $\mu$ moles/l) and significantly reduced stimulation by the PAR-1 agonist peptide SFLLRN (Fig. 9, panel A). In agreement with the effect on P-selectin, the active metabolite of clopidogrel reduced ADP- or SFLLRN-induced platelet-leukocyte conjugates (Fig. 9, panel B).

Flow cytometric analysis of platelets in whole blood showed specific binding of anti-TF antibody (Fig 10, Panel A). Immunoreactive-TF was already detectable, above the signal of an irrelevant mouse IgG1, in resting platelets and was increased after stimulation by ADP or SFLLRN (both 10  $\mu$ moles/l) for 15 min. Interestingly the active metabolite of clopidogrel inhibited rapid exposure of immunoreactive-TF, triggered by ADP or SFLLRN (Fig. 10, panel A). Flow cytometric analysis of events gated in the leukocyte population demonstrated that after 15 min incubation the percentage of leukocytes showing immunoreactive-TF increased in ADP or SFLLRN-stimulated samples and was significantly reduced in samples pretreated with the active metabolite of clopidogrel (Fig 10, panel B).

## Discussion

A number of recent clinical trials demonstrated the efficacy of clopidogrel in reducing major cardiovascular events in patients (26–29). Thus, clopidogrel is now considered an advance in anti-platelet therapy.

The occurrence of platelet-leukocyte interaction in ischemic heart disease is confirmed by increased mixed conjugates in the systemic circulation of patients (30–31). These interactions may play an important role in thrombus formation (32) as well as in the progression of atherosclerosis (7, 8). Thus, their molecular mechanisms may represent novel targets for pharmacological intervention in atherothrombotic disease. However, pharmacological approaches that specifically target these processes are not yet available. Recent studies consistently showed that treatment of cardiovascular patients with clopidogrel reduces circulating pla-

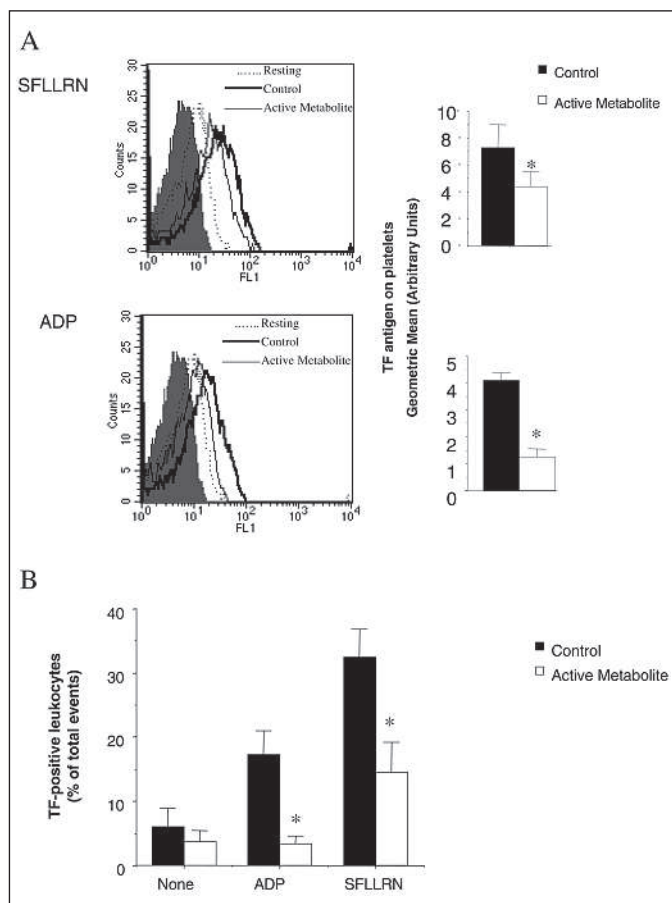


**Figure 9: Effect of clopidogrel active metabolite on P-selectin expression in platelets and on platelet/PMN conjugate formation triggered by ADP or SFLLRN in human whole blood.**

Whole blood was incubated in the presence of the active metabolite (5  $\mu$ moles/l) or vehicle at room temperature for 30 min. The samples were then stimulated with ADP (10  $\mu$ moles/l) or with the thrombin receptor agonist peptide SFLLRN (10  $\mu$ moles/l) for 15 min in the presence of 800  $\mu$ moles/l RGDS to prevent platelet-platelet aggregation. A ten  $\mu$ l aliquot of non stimulated or ADP or SFLLRN-activated blood were then incubated with Tyrode's buffer (90  $\mu$ l) containing primary mouse monoclonal antibodies against P-selectin (WAPS), IIb/IIIa (10E5) or an irrelevant mouse IgG1 (all 10  $\mu$ g/ml final dilution) for 15 min. Then samples were incubated with Alexa Fluor-488 goat anti-mouse IgG as secondary antibody for further 15 min. After immunofluorescent staining, erythrocytes were lysed by FACS™ lysing solution (Becton Dickinson) according to manufacturer instructions and samples immediately analyzed by FACS. A) Bars indicate the percentage of platelets expressing P-selectin in vehicle (filled bars) or active metabolite-treated (empty bars) samples. Data are mean  $\pm$  SEM of 3 different experiments using whole blood from 3 different donors, \*denotes P < 0.05 levels of significance. B) The bars indicate the number of platelets recruited by 100 leukocytes (see materials and methods). Data are mean  $\pm$  SEM from 3 different experiments using whole blood from 3 different donors, \*denotes P < 0.05 levels of significance.

telet-leukocyte conjugates and inhibits the formation of these mixed aggregates after platelet stimulation in whole blood *ex vivo* (10, 12–14).

In the present study, using an experimental model in the mouse, we confirm and extend these observations showing that treatment with clopidogrel reduces the capacity of platelets to express P-selectin, to adhere with leukocytes and to cause their activation *ex vivo*. Moreover, our present results show that the pharmacological effect exhibited by clopidogrel *ex vivo* is strikingly similar to the biological effect exhibited by the recently rec-



**Figure 10: Effect of clopidogrel active metabolite on ADP- or SFLLRN-induced expression of immunoreactive TF on platelets and leukocytes in human whole blood.**

Whole blood was treated as in figure 9. A ten  $\mu$ l aliquot of non stimulated or ADP or SFLLRN-activated blood were then incubated with Tyrode's buffer (90  $\mu$ l) containing primary mouse monoclonal antibodies against TF or an irrelevant mouse IgG1 (10  $\mu$ g/ml final dilution) for 15 min. Samples were incubated with Alexa Fluor-488 goat anti-mouse IgG as secondary antibody for a further 15 minutes. After immunofluorescent staining, erythrocytes were lysed by FACS™ lysing solution and samples immediately analyzed by FACS. Cytometric parameters were acquired on a logarithmic scale and immunoreactive TF exposure on platelets or leukocytes was measured by recording a total of 4000 or 2500 events in the platelet or leukocyte population, respectively. A) Representative histograms of flow cytometric analysis of immunoreactive-TF in resting, ADP or SFLLRN-activated platelets in whole blood pre-incubated with or without the active metabolite of clopidogrel. The filled histograms represent the negative staining with an irrelevant mouse IgG. Bars report TF-specific staining as geometric mean of fluorescence. Data are mean  $\pm$  SEM from 3 different experiments using whole blood from 3 different donors, \*denotes P < 0.05 levels of significance. B) Bars report percentages of PMN/monocytes which display TF-specific immunofluorescent staining. Data mean  $\pm$  SEM from 3 different experiments using whole blood from 3 different donors, \*denotes P < 0.05 levels of significance.

ognized active metabolite of the drug, *in vitro*. Previous studies demonstrated that this compound inhibited 2Mes-ADP binding on P2Y<sub>12</sub> (33), but not on P2Y<sub>13</sub>-expressing cells (34). Treatment of platelets with this compound irreversibly inhibited P2Y<sub>12</sub>-mediated effects, i.e., ADP-induced aggregation and down-regulation of adenylyl cyclase, but had no effect on

P2Y1-dependent shape change (15). Moreover the compound did not affect epinephrine-induced platelet aggregation (15). Thus the already reported *in vitro* effects of the compound on platelets reproduced the *ex vivo* activity of clopidogrel, i.e. an irreversible antagonistic activity on the P2Y12 receptor. Our data furthermore support that this compound mediates the effect of the drug *in vivo*.

Although we did not investigate the intracellular mechanism involved in the regulation of P-selectin expression, the results strongly support the concept that intracellular pathways regulated by P2Y12 play an important role in alpha-granule secretion and P-selectin expression. This interpretation is in agreement with previous studies showing that, in patients with genetic defects of P2Y12, granule secretion is severely impaired (35, 36). More recent studies suggested that P2Y12-dependent positive feedback on platelet secretion is mediated by PI-3 kinase (37, 38). Interestingly, P2Y12 plays an important role in PI-3 kinase activation by PAR-1 (39), and this may explain the significant inhibitory effect of clopidogrel on thrombin-induced P-selectin expression. Inhibition of granule secretion by clopidogrel may also result in a reduced availability of platelet-derived soluble agonists, which may contribute to functional PMN activation (40, 41). In fact, clopidogrel substantially impairs the ability of platelets to adhere and promote the production of ROS by PMN.

Recently Leon et al. (25, 42), using a whole blood experimental system, reported that inhibition of ADP receptors in platelets reduces the rapid exposure of TF in mixed platelet-leukocyte conjugates triggered by platelet activation. In agreement with this observation we found that the active metabolite of clopidogrel reduces rapid immunoreactive-TF exposure on both platelet and leukocyte surfaces triggered by ADP or SFLLRN, in whole blood. The source of TF in this model was not investigated, however other studies have shown that platelets contain TF mol-

ecules (43) which may be transferred to leukocytes during platelet-leukocyte interaction (44). Another source of readily available TF are circulating microparticles (45). These microparticles are of myeloid origin, express receptors for P-selectin and rapidly bind activated platelets at the site of vascular injury (46–48). Thus, in addition to endogenous TF molecules, activated platelets may rapidly bind and transfer blood-borne TF to adjacent leukocytes, *via* P-selectin.

In addition to the effect on the rapid immunoreactive-TF exposure in whole blood, we found that the TF-dependent procoagulant activity in MN activated in the presence of platelets in a washed cell system was significantly reduced by the active metabolite of clopidogrel. In the washed cell system TF-dependent procoagulant activity could be not detected after short time incubation (15 min), indicating that the eventual contribution to the overall procoagulant activity of TF molecules rapidly released from washed platelet is negligible. More probably the contribution of platelets in the washed cell system can be explained on the basis of previous studies indicating that P-selectin alone or in combination with released 12-HETE, or other cytokines such as CD40L, up-regulates TF gene transcription (22–24).

In conclusion our data suggest that the pharmacological effect of clopidogrel, targeting platelet functions relevant for their interaction with different classes of leukocyte, may reduce the inflammatory and procoagulant component at the site of vascular lesion, thus slowing the progression and reducing complications of atherosclerosis.

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