Interaction with damaged vessel wall in vivo in humans induces platelets to express CD40L resulting in endothelial activation with no effect of aspirin intake

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Giannini S, Falcinelli E, Bury L, Guglielmini G, Rossi R, Momi S, Gresele P: Interaction with damaged vessel wall in vivo in humans induces platelets to express CD40L, resulting in endothelial activation with no effect of aspirin intake. Am J Physiol Heart Circ Physiol 300: H2072–H2079, 2011. First published March 4, 2011; doi:10.1152/ajpheart.00467.2010.—Activated platelets express CD40L on their plasma membrane and release the soluble fragment sCD40L. The interaction between platelet surface CD40L and endothelial cell CD40 leads to the activation of endothelium contributing to atherothrombosis. Few studies have directly demonstrated an increased expression of platelet CD40L in conditions of in vivo platelet activation in humans, and no data are available on its relevance for endothelial activation. We aimed to assess whether platelets activated in vivo at a localized site of vascular injury in humans express CD40L and release sCD40L, whether the level of platelet CD40L expression attained in vivo is sufficient to induce endothelial activation, and whether platelet CD40L expression is inhibited by aspirin intake. We used the skin-bleeding-time test as a model to study the interaction between platelets and a damaged vessel wall by measuring CD40L in the blood emerging from a skin wound in vivo in healthy volunteers. In some experiments, shed blood was analyzed before and 1 h after the intake of 500 mg of aspirin. Platelets from the bleeding-time blood express CD40L and release soluble sCD40L, in a time-dependent way. In vivo platelet CD40L expression was mild but sufficient to induce VCAM-1 expression and IL-8 secretion in coinabulation experiments with cultured human endothelial cells. Moreover, platelets recovered from the bleeding-time blood activated endothelial cells; an anti-CD40L antibody inhibited this mechanism. Platelets recovered from the bleeding-time blood express CD40L and release soluble sCD40L, in a time-dependent way. In vivo platelet CD40L expression was mild but sufficient to induce VCAM-1 expression and IL-8 secretion in coinabulation experiments with cultured human endothelial cells. Moreover, platelets recovered from the bleeding-time blood activated endothelial cells; an anti-CD40L antibody blocked this effect. On the contrary, the amount of sCD40L released by activated platelets at a localized site of vascular injury did not reach the concentrations required to induce endothelial cell activation. Soluble monocyte chemoattractant protein-1, a marker of endothelium activation, was increased in shed blood and correlated with platelet CD40L expression. Aspirin intake did not inhibit CD40L expression by platelets in vivo. We concluded that CD40L expressed by platelets in vivo in humans upon contact with a damaged vessel wall activates endothelium; aspirin treatment does not inhibit this mechanism.

bleeding time; flow cytometry; atherosclerosis; platelet activation

CD40L (CD154) is a TRIMERIC TRANSMEMBRANE PROTEIN of the tumor necrosis factor family expressed on several cell types, including activated CD4+ T cells, mast cells, basophils, eosinophils, smooth muscle cells, and activated platelets. CD40L and its membrane receptor, CD40, represent a system involved in cell communication, with CD40L inducing activation of CD40-expressing cells. This interaction mediates signals leading to responses that have a key role in immune activation, inflammation, atherosclerosis, and thrombosis.

Platelets constitutively express CD40 on their plasma membrane, whereas CD40L, cryptic in resting platelets, is exposed at their surface upon activation (1) from where it is then cleaved, producing a soluble fragment released in the circulation, sCD40L (20).

Platelet surface CD40L is proinflammatory and procoagulant and can induce the activation of normal endothelium by ligation of CD40 on endothelial cells, leading to the upregulation of adhesive and procoagulant molecules and to the release of cytokines, chemokines, and matrix metalloproteinases (MMPs), thus turning the normally antiatherothrombogenic internal lining of blood vessels into a prothrombotic surface (14, 21, 22, 28, 40).

Not only CD40L expressed on activated platelets but also sCD40L may exert a procoagulant, prothrombotic, and proinflammatory activity because of its ligation to platelet αIIBβ3 and to endothelial CD40, respectively (1). However, the inflammatory action of sCD40L is debated because some studies have questioned the ability of sCD40L to induce endothelial activation (22).

In humans elevated plasma sCD40L has been associated with several atherosclerotic risk factors, like hypercholesterolemia (9), diabetes (26) or the metabolic syndrome (37), and with acute cardiovascular diseases, like acute coronary syndromes (20), percutaneous coronary interventions (45), and cardiopulmonary bypass (34).

On the contrary, only a few studies have demonstrated an increased expression of membrane-bound CD40L on platelets in conditions associated with in vivo platelet activation (13, 41). Although the proinflammatory activity of platelet-expressed CD40L on the endothelium has been characterized in vitro (21), it is not yet clear whether the amounts of membrane-bound or of soluble CD40L expressed by platelets in vivo in pathological conditions are within the range found to be able to induce endothelial activation in vitro (21). Moreover, considering the short half-life of platelet-expressed CD40L, CD40L-driven atherosclerosis progression has been claimed to be sustained mainly by activated CD4+ T cells (4).

The template skin-bleeding time is a model of microvascular injury in vivo, which allows the study of the kinetics of the localized activation of the cellular and plasmatic components of hemostasis in humans by the analysis of the blood emerging from the skin wound. Indeed, increased levels of plasmatic markers of platelet activation, such as thromboxane B2 (TxB2), β-thromboglobulin, platelet factor 4, are detected in blood emerging from the bleeding-time wound and increase progres-
sively, compatible with the ongoing formation of an in vivo platelet plug (6, 18, 44). Moreover, platelets in skin-wound-shed blood exhibit higher levels of surface activation markers, such as lysosomal membrane glycoproteins (LIMP and LAMP-2) and P-selectin (P-sel), compared with circulating platelets (6), increased activation of GPIIb/IIIa and enhanced fibrinogen binding, and a downregulation of the GPIb/IX complex (30). Using this model, it has been possible to demonstrate that activated platelets release several biologically active proteins in vivo in humans, such as lysosomal enzymes (β-Hex) (6), MMP-2 (11) or vascular endothelial growth factor (VEGF), a potent angiogenic factor (46).

This model proved also useful to assess the antiplatelet or anticoagulant activity of several drugs in vivo, including aspirin (24) and nitric oxide-donating agents (19) or statins (42).

Using this model, the aim of our study was to evaluate whether platelets activated in vivo in humans by the exposure to a damaged vessel wall express CD40L on their surface in amounts sufficient to initiate an inflammatory response of the endothelium. To this purpose, we measured the expression of CD40L on platelets and the release of sCD40L at a localized site of vascular injury in healthy volunteers and then investigated whether the degree of platelet CD40L expression attained in vivo is sufficient to activate endothelial cells. Finally, we evaluated the effect of aspirin on the expression of CD40L by locally activated platelets in vivo.

MATERIALS AND METHODS

Subjects. Twenty-two healthy adult volunteers (12 males, 10 females, mean age 41.3 ± 9.1 yr, range 27 to 59 yr) who had not ingested any drug within the previous 10 days were studied. All tests were conducted in the morning between 8:00 and 10:00 A.M., under fasting conditions. Mean platelet count was 204 × 10^6/ml (range: 121–300 × 10^6/ml). In females all studies were performed in the luteal phase of the ovarian cycle. In a selected subgroup (n = 6) (all males, mean age 31.2 ± 4.4 yr, range 26 to 38 yr) studies were performed before and 1 h after the oral intake of 500 mg of aspirin.

Written informed consent was obtained from all volunteers before inclusion in the study; all studies were carried out in conformity to the declaration of Helsinki and approved by Comitato Etico delle Aziende Sanitarie dell’Umbria (Ethics Committee) protocol number 1497/09.

Bleeding time. A standardized template bleeding time was performed as previously described (11) by the same operator throughout the study, using a Simplate II device (Organon Teknika, Jessup, MD). The boundaries between stained and unstained platelet-rich plasma (PRP) was obtained from shed blood as per definition (24).

Subjects were studied using a standardized template bleeding time assay according to the protocol described (11) and immediately added to tubes containing CTAD in a volume giving the same blood/anticoagulant ratio as for the bleeding-time blood, was collected. Platelet-rich plasma (PRP) was obtained from shed blood as well as from peripheral blood by centrifugation at 150 g for 20 min. The resulting PRP was used for coincubation experiments with human umbilical vein endothelial cells (HUVEC).

Flow cytometric analysis of platelets. Samples were analyzed in an EPICS XL-MCL flow cytometer (Beckman Coulter, Miami, FL). The instrument was equipped with an argon laser operating at 488 nm. FITC and PE fluorescence were detected using 525-nm and 575-nm band-pass filters. Platelets were identified by their light-scatter characteristics and their positivity for platelet-specific mAb (CD41 PE). A total of 5,000 platelets was analyzed for each sample. A negative control was obtained running a sample labeled with an isotypic control antibody. The boundaries between stained and unstained populations were set such that ≥2% of the events in the control tube were scored as positive.

Preparation of washed platelets for coincubation experiments. Citrated-venous whole blood was centrifuged at 150 g for 10 min to obtain PRP. Washed platelets were obtained by gel filtration of PRP onto Sepharose 2B (Sigma Chemical, St Louis, MO) columns equilibrated with a calcium-free Tyrode’s buffer (3.1 mM HEPES, 4 mM NaH2PO4, 137 mM NaCl, 2.6 mM KCl, 1 mM MgCl2, 5.6 mM dextrose, and 0.1% BSA, pH 7.4) as described (38).

To concentrate platelets, gel-filtered platelets were centrifuged at 1,000 g for 10 min, after the addition of 0.02 μM prostacyclin (ICN Biomedicals, Aurora, OH), and the pellet was resuspended at a concentration of 1.5 × 10^6/ml in Tyrode’s buffer and M199 1:1.

Isolation and culture of HUVEC. HUVEC were isolated from umbilical cords from healthy women after delivery, as previously described (16) using 0.1% collagenase type IA (Sigma Chemical) and were cultured in M199 medium supplemented with 20% FBS (GIBCO, Gaithersburg, MD), 50 μg/ml endothelial cell growth supplement (Sigma Chemical), 100 μg/ml streptomycin, 100 U/ml penicillin, 100 μg/ml heparin, and 2 mM L-glutamine. HUVEC were used at their second passage.
Culture of human aortic endothelial cells. Human aortic endothelial cells (HAEC) (10) were purchased from Cascade Biologics (Gibco-Invitrogen) and cultured in Medium 200 (Gibco-Invitrogen) supplemented with Low Serum Grow Supplement (Gibco-Invitrogen) medium containing 2% fetal bovine serum, 1 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, and 10 µg/ml heparin. HAEC were used from passage III to passage VI. These cells where confirmed to be HAEC by morphological analysis and by the expression of Factor VIII-related antigen and CD31.

Stimulation of endothelial cells. Washed human platelets were stimulated with bovine α-thrombin (Sigma Chemical) (0, 0.01, 0.03, 0.06, 0.1 U/ml) for 10 min. Confirmatory experiments stimulating platelets with human α-thrombin (a kind gift of Prof. De Cristofaro, Catholic University of Rome, Rome, Italy) were performed. In some experiments platelets were preincubated for 20 min with 20 µg/ml of the blocking anti-CD40L mAb TRAP1 (Immunotech) before stimulation.

Thrombin was then neutralized with 10 U/ml of hirudin (Knoll, Ludwigshafen, Germany), and the platelet suspension was used in part to measure P-sel and CD40L expression, by flow cytometry, and in part added to 24-well plates covered with a monolayer of confluent endothelial cells (HUVEC or HAEC) and incubated for 4 h without agitation at 37°C and a 0.5% CO2 atmosphere. In some experiments, HUVEC or HAEC were incubated with recombinant sCD40L (Biosource, Camarillo, CA) (1 to 1.000 ng/ml).

After incubation, endothelial cell supernatants were collected, centrifuged at 12,000 g, and stored at −80°C for later IL-8 assay by ELISA (Bender MedSystem).

For HUVEC coincubation experiments with platelets activated in vivo, shed-blood-derived PRP was added to 96-well plates covered with a monolayer of confluent HUVEC and incubated for 4 h at 37°C and a 0.5% CO2 atmosphere without agitation.

After incubation with either stimulated platelets or sCD40L, endothelial cells were detached with mild trypsinization (32) (or with 2 mM EDTA, as a control), centrifuged for 5 min at 200 g, resuspended in PBS, and incubated for 30 min in the dark with a saturating concentration of a FITC-conjugated anti-VCAM-1 mAb (CD106, BD Pharmingen). Samples were diluted by adding 0.5 ml of PBS and finally analyzed by one-color flow cytometry.

Measurement of TxB2 production. TxB2 was measured by a specific radioimmunoassay, as previously described (43), in serum obtained from venous blood samples collected in glass tubes containing no anticoagulant and clotted at 37°C for 1 h (35).

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed by using the one-way ANOVA for repeated measures followed by the Tukey’s multiple-comparison posttest or the two-way ANOVA, as appropriate, with the GraphPad Prism version 4.0 for Windows (San Diego, CA). Correlation analyses were carried out with the Pearson’s test. A P value <0.05 was considered as statistically significant.

RESULTS

CD40L expression on platelets in blood emerging from the bleeding-time wound. Bleeding time was 5.8 ± 0.2 min (4.5–7.5, n = 22). CD40L and P-sel expression on platelets in shed blood increased in a time-dependent way, with a significant increase starting from the third and the second minute of bleeding, respectively, and a maximal expression of 15.1 ± 3.5% of positive cells and 31.5 ± 7.5% of positive cells, respectively, at the sixth minute (Fig. 1A), suggesting a progressive activation of platelets passing through the damaged capillary walls. The trend was similar for the two activation antigens, but P-selectin reached a higher platelet surface expression compared with CD40L.

In venous blood platelets expressed 1.8 ± 0.3% of CD40L-positive cells and 2.2 ± 0.3% of P-sel-positive cells. Maximum platelet CD40L expression attained in vivo in shed blood (fifth minute = 9.5 ± 1.1%, range 8.4 to 12.5%) was significantly lower than the expression reached in vitro after platelet stimulation with strong agonists (50 µM TRAP-6, fifth minute = 22.1 ± 4.4%, range 14.2 to 41.1%, P < 0.05).

The efficacy of the blood-collection method in stopping platelet activation from occurring in vitro after sampling was validated by ad hoc experiments. Citrated whole blood was stimulated for 1 min with collagen (20 µg/ml), and aliquots from the sample were transferred to either tubes containing CTAD or tubes containing PBS; afterward, 3- and 5-min samples were analyzed by flow cytometry for CD40L expression, as described above. Results confirmed that platelets from samples collected in CTAD express half the CD40L detected on platelets from samples collected without antplatelet/anticoagulant mixture and that the platelet expression of CD40L was almost unchanged after 1- or 5-min stimulation in samples treated with CTAD, whereas it was strikingly higher at 5 min in samples not treated with the antplatelet/anticoagulant mixture (basal: 2.7 ± 0.6% positive platelets; 1 min: 5.8 ± 2.9%;
PBS = 3 min: 8.4 ± 1.9%, 5 min: 13.3 ± 4.6%, P < 0.05 vs. 1 min; CTAD = 3 min: 5.4 ± 2.6%, 5 min: 6.3 ± 1.8%, P = ns vs. 1 min). These data confirm that the precautions adopted to avoid in vitro platelet activation after blood collection were effective and that the level of expression of CD40L on platelets recovered from shed blood reflects localized in vivo activation.

sCD40L release in shed blood. sCD40L concentration was measured in plasma prepared from the skin-wound-shed blood collected at 1-min intervals. sCD40L levels rose progressively in the bleeding-time blood samples, with a significant rise starting from the second minute, and it reached the maximal concentration after 6 min, indicating progressive shedding from activated platelets during the bleeding-time test. sP-sel release, measured in the same plasma samples, showed a similar pattern although a significant rise was observed already at the first minute (Fig. 1B). sCD40L showed a significant correlation with sP-sel (r² = 0.6, n = 5, P < 0.001) (Fig. 1B).

A potential role of leukocytes in sCD40L release in shed blood was considered to be unlikely because lymphomonocytes incubated with thrombin, collagen, or their combination

Fig. 2. Platelet-induced endothelial cells activation in vitro. VCAM-1 expression (A) and IL-8 release (B) by human umbilical vein endothelial cells (HUVEC) coincubated for 4 h with resting and thrombin (Thr)-stimulated platelets and effect of an anti-CD40L blocking antibody. Maximal HUVEC activation was obtained by incubating HUVEC with LPS (20 µg/ml) for 4 h. The line indicates the values of platelet-surface CD40L expression corresponding to the HUVEC VCAM-1 expression columns. Similar levels of VCAM-1 expression were obtained when HUVEC were detached with 2 mM EDTA instead of 0.05% trypsin and when platelets were stimulated with human α-thrombin instead of bovine thrombin (data not shown). VCAM-1 expression (C) and IL-8 release (D) by human aortic endothelial cells (HAEC) coincubated for 4 h with resting and thrombin-stimulated platelets and effect of an anti-CD40L blocking antibody. Maximal HAEC VCAM-1 expression was obtained by incubating HAEC with LPS (20 µg/ml), whereas maximal IL-8 release was obtained by incubating HAEC with thrombin (0.5 U/ml). The line indicates the values of platelet-surface CD40L expression corresponding to the respective HAEC VCAM-1 expression columns. E: VCAM-1 expression by HUVEC coincubated for 4 h with venous blood-derived or bleeding-time-blood-derived platelets. The line indicates the values of platelet-surface CD40L expression corresponding to the HUVEC VCAM-1 expression columns. *P < 0.05; #P < 0.01 vs. basal, §P < 0.05 vs. thrombin 0.1 U/ml + anti-CD40L. No VCAM-1 expression increase was observed when HUVEC were coincubated with bleeding-time-blood-derived platelets preincubated with 20 µg/ml of the CD40L-blocking mAb TRAP1 (data not shown). PRP, platelet-rich plasma.
do not express CD40L for up to 12 h (basal: 4.09 ± 0.01%; 12-h collagen stimulation: 4.99 ± 0.9%; 12-h thrombin stimulation: 5.26 ± 0.7%; 12-h collagen + thrombin stimulation: 5.3 ± 0.7%). When the stimulus was LPS (50 μg/ml), CD40L started to be expressed only after 1-h incubation (basal: 4.09 ± 0.01%; 10-min stimulation: 4.3 ± 0.1%; 1-h stimulation: 12.64 ± 3%).

Platelet-induced endothelial cell activation. To determine whether the degree of expression of CD40L detected on the surface of platelets activated in vivo upon exposure to a damaged vessel wall is sufficient to induce endothelial cell activation, platelets were stimulated in vitro with increasing amounts of bovine thrombin and then the samples showing a CD40L expression level comparable to that reached in vivo were coincubated with HUVEC or HAEC; IL-8 release and VCAM-1 surface expression by endothelial cells were then assessed.

Mildly activated platelets induced HUVEC and HAEC activation, as documented by a significant increase of VCAM-1 expression and IL-8 release. Platelet-induced HUVEC and HAEC activation was inhibited by preincubation with the CD40L blocking mAb TRAP1 (Fig. 2, A–D).

VCAM-1 expression on HUVEC was also significantly higher when cells were coincubated with in vivo-activated human blood platelets (i.e., platelets recovered from the bleeding-time blood) than when they were coincubated with nonactivated, peripheral venous blood-derived platelets. This is consistent with increased CD40L expression by platelets in the bleeding-time blood as shown by flow cytometry of CD40L on platelets and by the blockade of VCAM-1 expression on HUVEC upon preincubation of bleeding-time-blood-derived platelets with the anti-CD40L-blocking mAb TRAP1 (Fig. 2E).

CD40L expression on platelets in blood emerging from the skin-bleeding-time wound: effect of aspirin. Aspirin intake prolonged the bleeding time from 5.9 ± 0.8 to 11.4 ± 1.2 min (P < 0.01), abolished serum TxB2 production (from 370 to 0.37 ng/ml P < 0.01), and significantly reduced P-sel expression on the surface of platelets in blood emerging from the skin wound (Fig. 5A). On the contrary, platelet CD40L expression in skin-wounded-blood was not inhibited by aspirin, and it increased significantly during the bleeding-time procedure despite aspirin ingestion (Fig. 5B).

DISCUSSION

Platelets exert an inflammatory activity by interacting with other cells, especially endothelial cells and monocytes. In particular, they were shown in in vitro studies to initiate inflammatory responses on endothelial cells, such as the expression of adhesion receptors and the production of chemo- kines, through CD40-CD40L interactions (21). Platelet-expressed CD40L is shed in plasma in a soluble form, but, from in vitro studies, it is still controversial whether sCD40L can exert procoagulant, prothrombotic, and proinflammatory activities (2, 22, 36).

Very recent data show that sCD40L enhances platelet activation and aggregation through a CD40-dependent TNF receptor-associated Factor-2/Rac-1/p38 MAP kinase pathway (47) and that, consistent with platelet activation, there is a positive correlation between platelet aggregation and surface CD40L.
expression in patients with unstable cardiovascular disease (27). However, evidence that sCD40L induces endothelial activation is scanty. Although a proinflammatory role of platelet-released CD40L in vitro has been demonstrated, very little evidence is available for a role in vivo in humans. Some studies have investigated variations in plasma sCD40L in disease and their relevance for endothelial activation in vivo. sCD40L was found to be upregulated in diabetic patients as a consequence of persistent hyperglycemia, and serum from diabetic patients was shown to induce endothelial cell activation and monocyte recruitment to the arterial wall via sCD40L, possibly contributing to the accelerated atherosclerosis of diabetes (7). Similarly, upregulation of sCD40L after percutaneous transluminal coronary angioplasty was correlated with restenosis and with the activation of endothelial cells and the recruitment of monocytes to the arterial wall via sCD40L, possibly contributing to the accelerated atherosclerosis of diabetes (8). It has to be noticed that the minimum concentration of sCD40L found to enhance leukocyte to endothelium interaction in these studies (10 ng/ml) is still far above the maximum concentrations reached in vivo at a localized site of vessel-wall damage (0.6 ng/ml). Differently from the large deal of information on sCD40L in disease, platelet-surface CD40L has been studied mainly in vitro or in animal models, with only a few studies in vivo in humans and none of them evaluating directly its relevance for endothelial activation. Most in vitro studies were carried out in rather artificial conditions, i.e., with nonphysiological stimuli and in the absence of important factors regulating platelet activation in vivo, such as the interaction with the vessel wall or blood flow. Using the bleeding-time test, an established model of localized in vivo activation of the cellular and plasmatic components of hemostasis (6, 18), we have shown that human platelets express CD40L on their surface upon interaction with a damaged vessel wall and release sCD40L during this process. Although the maximum level of platelet CD40L expression attained in vivo platelet activation is significantly lower than the level reached in vitro after stimulation with strong platelet agonists, our results show that it is sufficient to induce endothelial activation. Indeed, platelets only mildly activated in vitro and expressing surface CD40L to an extent similar to that reached in vivo, activated HUVECs and HAECs, leading to the expression of adhesion molecules (VCAM-1) and to the release of inflammatory cytokines (IL-8); therefore, CD40L expression by platelets activated in vivo is sufficient to initiate an inflammatory and procoagulant response by the endothelium.

Although we do not have definitive proof that the sCD40L we measured in the bleeding-time blood was released by platelets rather than leukocytes, some clues strongly suggest this, namely the observation that stimulation with collagen and thrombin does not increase CD40L expression on lymphomonocytes and the highly significant correlation between sCD40L and sP-Sel, another marker of platelet activation, in bleeding-time blood. Moreover, studies on the cellular origin of circulating sCD40L indicate that >95% of it derives from platelets (1).

In our in vivo model, MCP-1, a marker of endothelial activation, but not IL-8 and sVCAM-1, increased significantly in shed blood at the sixth minute of bleeding, suggesting that a relatively longer time of interaction of activated platelets with
endothelial cells is required to induce secretion of this marker and that only when platelets reach their maximal activation can they induce endothelial activation. The levels of MCP-1 significantly correlated with the levels of CD40L expressed by platelets, suggesting that endothelial activation is largely platelet CD40L mediated. On the contrary, sCD40L released by activated platelets at a localized site of vascular injury in vivo does not reach the concentrations required to induce HUVEC activation, possibly because sCD40L release is slow, requiring 30 to 60 min for completion (33, 39). Moreover we showed that physiological or supraphysiological concentrations of sCD40L do not activate endothelial cells even when coincubated with bleeding-time-derived plasma, making it unlikely that the in vivo attained levels of sCD40L may act in concert with other mediators present in plasma to activate endothelium. Therefore, sCD40L seems to represent a reliable marker of platelet activation in vivo and possibly of the prothrombotic activity of platelets, rather than an effector. It must be considered, however, that sCD40L has been reported to display different effects on endothelium depending on its source (7, 22); thus we may not completely exclude that sCD40L may contribute in vivo to endothelial activation.

Our data also show that platelet CD40L expression induced in vivo by exposure to a damaged vascular wall is not inhibited by aspirin. In previous studies aspirin was found to partially inhibit sCD40L release in vitro, in response to collagen, a platelet agonist strictly dependent on TXA2 production (33), but not to ADP or TRAP, whereas it had no effect on the platelet expression of CD40L, either in vitro or in vivo (5, 23). Our data, together with the observations that aspirin nonresponsiveness in patients with coronary artery disease is associated with higher sCD40L levels and that exercise-induced aspirin resistance is related to sCD40L increase (25), suggest a possible contribution of platelet-derived CD40L to aspirin-insensitive platelet activation in vivo.

Indeed, although the evidence that aspirin prevents thrombotic events is overwhelming (3), it is now quite evident that it does not affect the progression of atherosclerosis (15). The expression by platelets of CD40L may represent one mechanism of aspirin-insensitive platelet contribution to the progression of atherosclerosis.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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