

gp91phox-Dependent Expression of Platelet CD40 Ligand

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Background—CD40 ligand (CD40L) expression on platelets is mediated by agonists, but the underlying mechanism is still unclear.

Methods and Results—CD40L expression was measured in platelets from healthy subjects both with and without the addition of antioxidants or a phospholipase A2 (PLA2) inhibitor and in platelets from 2 patients with an inherited deficiency of gp91phox. Immunoprecipitation analysis was also performed to determine whether normal platelets showed gp91phox expression. Unlike catalase and mannitol, superoxide dismutase inhibited agonist-induced platelet CD40L expression in healthy subjects. Immunoprecipitation analysis also showed that platelets from healthy subjects expressed gp91phox. In 2 male patients with inherited gp91phox deficiency, collagen-, thrombin-, and arachidonic acid-stimulated platelets showed an almost complete absence of superoxide anion (O_2^-) and CD40L expression. Incubation of platelets from healthy subjects with a PLA2 inhibitor almost completely prevented agonist-induced O_2^- and CD40L expression.

Conclusions—These data provide the first evidence that platelet CD40L expression occurs via arachidonic acid-mediated gp91phox activation. (*Circulation*. 2004;110:1326-1329.)

Key Words: CD40 ligand ■ NADPH oxidase ■ oxidative stress ■ platelets

CD40 ligand (CD40L), a member of the tumor necrosis factor ligand family, is a transmembrane protein with proinflammatory and prothrombotic properties on interaction with its receptor CD40.¹ CD40L was found primarily on the immune system cells and subsequently on macrophages, smooth muscle cells, endothelial cells, and platelets.¹ On agonist stimulation, platelets express CD40L, which is then cleaved on the membrane surface and finally circulates in the soluble form.² It has been calculated that >95% of circulating soluble CD40L (sCD40L) originates from platelets.² The mechanism accounting for CD40L expression by activated platelets is still unclear. Previous studies demonstrated that platelets produce reactive oxidant species, which in turn act as intracellular signaling molecules that amplify platelet response to agonists.³ In this study, we sought to determine whether reactive oxidant species play a role in platelet CD40L expression.

Methods

Materials

Anti-CD40L Ab (CD154 immunoglobulin (Ig) G1 mouse, clone TRAP-1), anti-CD62P (IgG1 mouse, clone CLBThromb/6), and isotypic control (IgG1 mouse, clone 679.IMC7) were obtained from Beckman Coulter. Anti-91phox Ab (polyclonal IgG goat) and a normal goat IgG (sc 2028 polyclonal) as irrelevant antibody (IRR) were obtained from Santa Cruz. Immunoprecipitation products were

obtained from Amersham Pharmacia. Any other nonspecified products were obtained from Sigma Aldrich.

Platelet Isolation From Whole Blood

Blood samples were mixed with 0.13 mol/L sodium citrate (9:1 ratio). Washed platelets and platelet-rich plasma were prepared as previously described.³

Flow Cytometric Analysis

CD40L and CD62P expression on platelet membrane was analyzed with specific fluorescein isothiocyanate conjugated-labeled monoclonal antibodies (Mab). An irrelevant isotype-matched antibody (anti-IgG1) was used as a negative control.

Mab (20 μ L) was added to platelets (200 μ L, 2×10^8 /mL) previously fixed with 2% paraformaldehyde in PBS (0.1% bovine serum albumin) and incubated for 60 minutes at 4°C. The unbound Mab was removed by addition of 0.1% bovine serum albumin PBS and centrifugation at 500g for 3 minutes (twice). Fluorescence intensity was analyzed on an Epics XL-MCL Cytometer (Coulter Electronics) equipped with an argon laser at 488 nm. For every histogram, 50 000 platelets were counted to evaluate the percentage of positive platelets. Antibody reactivity is reported as mean fluorescence according to this formula: Mean fluorescence of specific antibody minus mean fluorescence of control antibody. Platelets were incubated 10 minutes at 37°C with the antioxidants, AACOCF3 (a PLA2 inhibitor), or control medium before stimulation.

Analysis of Superoxide Anion

Superoxide anion (O_2^-) production was measured by lucigenin (5 μ mol/L)³ and expressed as stimulation index (mean level of stimu-

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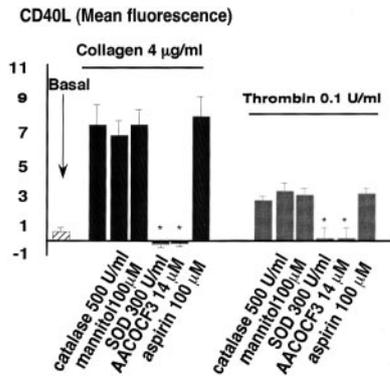


Figure 1. CD40L expression of agonist-stimulated platelets. Collagen- and thrombin- induced platelet CD40L (n=10). * $P < 0.001$ vs agonist- stimulated platelets.

lated platelet luminescence divided by average level of luminescence in unstimulated platelets).

Analysis of sCD40L

After 10 minutes of stimulation with agonists, the reaction was blocked by acidification of the medium with ACD (d-sodium hydrogen citrate, d+glucose, and citric acid), platelet-rich plasma was centrifuged (10 minutes at 360g), and the supernatant was stored at -80°C until use. sCD40L was measured with a commercial immunoassay (Quantikine CD40 Ligand, R&D Systems).

Immunoprecipitation and Western Blotting

The 91phox was immunoprecipitated from platelets (25×10^9 cells, 55 mg protein/0.5 mL) and polymorphonuclear neutrophils (PMN) (5×10^6 cells) as positive controls in denaturated condition using a 91phox Ab polyclonal, size separated on SDS-PAGE (12% gel), blotted, and stained with 91phox polyclonal Ab. Negative controls involved a similar procedure using a goat IRR.⁴

X-Linked Chronic Granulomatous Disease Patient Description

X-linked chronic granulomatous disease (X-CGD), an inherited disorder characterized by the absence or deficiency of phagocyte-NADPH oxidase activity, was diagnosed in 2 male patients (33 and 38 years of age) by demonstrating the absence or manifest deficiency of oxidase activity in stimulated neutrophils.⁵ X-CGD diagnosis was subsequently confirmed by the mutation analysis of the *CYBB* gene encoding the gp91 subunit of phagocyte-NADPH oxidase.⁶ The mutation in patient 1 was identified as a single-base substitution of guanosine to adenosine at residue 252 in exon 3, resulting in a splicing defect. A deletion of thymine 184 in exon 3 was identified for patient 2, resulting in a frame shift.

Platelet Aggregation

Collagen-induced platelet aggregation (Born's method) was measured as previously described.³

Statistical Analysis

Data are reported as mean \pm SD. Comparison between variables in the in vitro study was analyzed by Student's *t* test for unpaired data; the correlation study between CD40L expression and O_2^- and H_2O_2 formation was evaluated by linear correlation analysis, followed by ANOVA.

Results

The flow cytometric analysis showed CD40L expression on the cell surface after agonist stimulation; conversely, CD40L expression was minimally observed in unstimulated platelets (Figure 1). We then tried to determine a possible relationship

between reactive oxidant species and platelet CD40L expression. Compared with unstimulated platelets, collagen and thrombin increased both O_2^- production (stimulation index, 6.1 ± 0.4 and 3.4 ± 0.3 with collagen and thrombin, respectively) and CD40L expression (Figure 1). Using a scalar concentration of collagen (n=3 for each determination) of 0.5 to 16 $\mu\text{g}/\text{mL}$ and thrombin of 0.02 to 0.72 U/mL, we detected a significant correlation between O_2^- and CD40L ($r=0.97$, $P < 0.001$ with collagen; $r=0.95$, $P < 0.007$ with thrombin). Catalase and mannitol did not affect agonist-induced CD40L expression, whereas superoxide dismutase (SOD) significantly decreased it (Figure 1). Conversely, SOD (300 U/mL) did not affect collagen-induced CD62P expression (n=5; mean fluorescence: unstimulated platelets, 0.07 ± 0.03 ; collagen-stimulated platelets, 5.43 ± 0.75 ; collagen plus SOD-treated platelets, 5.31 ± 0.66).

To determine the role of arachidonic acid in the interaction between O_2^- and CD40L, agonist-stimulated platelets from healthy subjects were investigated with or without the addition of AACOCF3. Compared with controls, platelets treated with AACOCF3 showed a significantly lower expression of O_2^- (-79% with collagen and -66% with thrombin; $P < 0.001$; n=10) and CD40L (Figure 1). Conversely, incubation of platelets with aspirin reduced O_2^- by 17% ($P < 0.05$) with collagen and by 13% ($P = \text{NS}$) with thrombin but did not change CD40L expression (Figure 1); the specific thromboxane receptor inhibitor SQ 29548 did not change either platelet O_2^- or CD40L (not shown).

To determine whether gp91phox is detectable in human platelets, we performed an in vitro study in platelets from healthy volunteers. Neutrophils were tested as positive controls, and an IRR was tested as a negative control. In this experiment, denaturated conditions were used to prevent interference of gp22phox with gp91phox detection on account of their natural complex form (flavocytochrome b558). Leukocyte contamination in platelet suspension was assessed to be $< 0.1\%$ (50 PMN per 50 000 platelets per 1 mL). A leukocyte suspension (1×10^3 PMN/mL diluted to obtain 50 PMN/mL) was immunoprecipitated, showing no gp91phox expression. Immunoprecipitation of the platelet sample allowed us to demonstrate that gp91phox is expressed by human platelets (Figure 2A).

The role of gp91phox in CD40L expression was then investigated in 2 patients affected by X-CGD and in male control subjects matched for age. Compared with control subjects, X-CGD patients showed almost complete suppression of CD40L and O_2^- by collagen- and thrombin- stimulated platelets (Figure 2C and 2D). Similar findings were observed with arachidonic acid, thus suggesting that, in platelets, NADPH oxidase activation and subsequent CD40L expression occur via arachidonic acid pathway (Figure 2C and 2D). In the 2 patients with X-CGD, plasma levels of sCD40L were much lower than those found in healthy subjects and did not increase after platelet stimulation with the agonist (Figure 2B). In patients with X-CGD, collagen (4 $\mu\text{g}/\text{mL}$)-induced platelet aggregation (patient 1: lag phase, 30 seconds; light transmission%, 88; and patient 2: lag phase, 25 seconds; light transmission%, 91%) was comparable to that

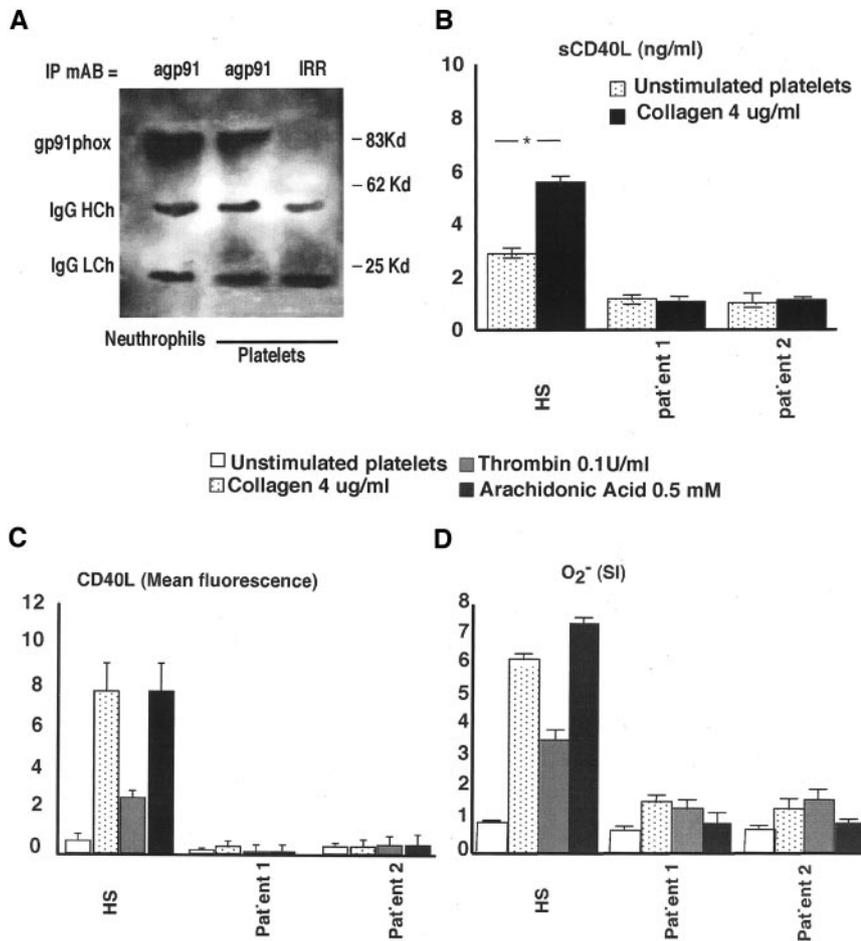


Figure 2. gp91phox and CD40L expression in X-CGD patients and control subjects. A, 91phox immunoprecipitation (IP) in healthy subjects (HS). Platelets and neutrophils were precipitated with anti-gp91phox and IRR. HCh and LCh indicate heavy and light chains of immunoglobulin. Result is representative of 5 separate experiments. B, sCD40L in HS (n=4) and X-CGD patients (4 different determinations). **P*<0.001. C and D, CD40L expression and O₂⁻ formation in X-CGD patients (4 different determination) and HS (n=10).

of healthy subjects (lag phase, 25+5 seconds; light transmission%, 85+9, n=10).

Discussion

This study provides the first evidence that platelet production of O₂⁻ plays a key role in CD40L expression. We observed that platelets from healthy volunteers incubated with SOD, a scavenger of O₂⁻, significantly inhibited CD40L. Moreover, in 2 patients with an inherited deficiency of gp91phox, the catalytic core of NADPH oxidase, O₂⁻ expression and CD40L expression by activated platelets were almost completely suppressed. It should be noted that X-CGD patients, in addition to showing a reduced platelet CD40L expression, also exhibited very low levels of sCD40L in unstimulated platelets and no changes after platelet stimulation.

These findings imply that platelets express gp91phox, an NADPH oxidase subunit that had not been previously detected in platelets. In fact, only the gp22phox, gp47phox, and gp67phox subunits have been found so far.⁷ In this study, we provide the first evidence that platelets express gp91phox and demonstrate that NADPH oxidase has a central role in generating O₂⁻ in platelets, like in phagocytic and nonphagocytic cells.^{8,9}

The intracellular signaling eliciting NADPH oxidase activation by agonist-activated platelets is still unclear. Arachidonic acid has been shown to be an important respiratory burst activator.¹⁰

Previous studies have demonstrated that arachidonic acid synergizes with p47phox phosphorylation in phagocytes to facilitate interaction with p22phox and to induce activation of phagocyte-NADPH oxidase.¹¹ Consistent with these findings, platelet O₂⁻ production by arachidonic acid was almost completely suppressed in 2 patients with an inherited gp91phox deficiency.

Consistent with previous findings,¹² aspirin did not affect platelet CD40L expression; in addition, aspirin marginally influenced platelet O₂⁻ expression, suggesting that NADPH oxidase activation mainly occurs through a COX1-independent mechanism. Several lines of evidence suggest that the CD40L-CD40 dyad is implicated in atherothrombosis. Engagement of CD40L with its receptor stimulates the synthesis of adhesion molecules, chemokines, and tissue factor and activates metalloproteinases.^{1,2} The role of CD40L in atherogenesis is confirmed by the fact that, in hyperlipidemic mice, anti-CD40L antibodies reduced the atherosclerotic lesion.¹³ Oxidized LDL seems to play a key role in pathogenesis of atherosclerosis; in a recent study,¹⁴ oxidized LDL dose dependently increased CD40L in human vascular endothelial cells and smooth muscle cells, suggesting that oxidative stress is implicated in CD40L expression. The fact that oxidative stress also plays a role in platelet CD40L expression suggests that an increase in CD40L expression might be another mechanism through which oxidative stress elicits the atherosclerotic damage.

In conclusion, this study demonstrates that platelet CD40L expression occurs via arachidonic acid-mediated NADPH oxidase activation and suggests that scavenging O_2^- or reducing platelet release of arachidonic acid could represent a novel approach to inhibit CD40L expression.

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