Increased soluble and platelet-associated CD40 ligand in essential thrombocytosis and reactive thrombocytosis

Jean-François Viallard, Anne Solanilla, Bruno Gauthier, Cécile Contin, Julie Déchanet, Christophe Grosset, Jean-François Moreau, Vincent Praloran, Paquita Nurden, Jean-Luc Pellegrin, Alan T. Nurden, and Jean Ripoche

CD40 ligand (CD40L) is expressed on activated CD4+ T lymphocytes and at the activated platelet surface. A circulating soluble form of CD40L (sCD40L) is generated by the way of a proteolytic cleavage. We measured sCD40L in the plasma of either healthy subjects; patients with inflammatory disorders and low, normal, or high platelet count (reactive thrombocytosis); or patients with essential thrombocytosis (ET). A tight correlation was found between the platelet count and plasma sCD40L. ET patients had the highest levels of sCD40L. Platelet-associated CD40L was increased in ET and reactive thrombocytosis, conditions associated with increased platelet regeneration. Platelet-associated CD40L was released upon platelet activation. In conclusion, platelets appear as a reservoir of CD40L that may be a major contributor to circulating sCD40L. Platelet-associated CD40L may be a potential marker of platelet regeneration. (Blood. 2002;99:2612-2614)

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Study design

Patients and controls

In this study, 200 Caucasian patients were enrolled. Twenty-seven patients (17-89 years of age, median 63 years; 37 women and 31 men) were diagnosed as having ET. The diagnostic criteria for ET were those recommended by the Polycythemia Vera Study Group. Sixty-eight patients (17-89 years of age, median 63 years; 37 women and 31 men) were diagnosed as having RT (patients with a platelet count above 450 x 10^9/L, without a myeloproliferative disorder, and with an underlying disease likely associated with RT). In each case the platelet count normalized if the acute disease state was no longer present after treatment. Causes of RT were malignancy (25 patients), infection (17), anemia attributed to iron deficiency (7), and inflammatory diseases (19). Thirty-eight patients (16-86 years of age, median 61 years; 22 women and 16 men) without increased platelet count (normal platelet count, 150 x 10^9/L-450 x 10^9/L) but with an underlying inflammatory disorder included 11 patients with malignancy, 10 with infection, and 38 with inflammatory diseases (n = 17). Twenty patients with low platelet levels (< 100 x 10^9/L) (28-80 years of age, median 59 years; 11 women and 9 men) included 8 patients with myelodyplasia, 8 patients with idiopathic thrombocytopenic purpura (ITP), and 4 patients with secondary thrombocytopenias resulting from an autoimmune or lymphoproliferative disorder. Controls were 47 Caucasian healthy blood donors and health care workers (18-78 years of age, median 38 years; 28 women and 19 men) who were not sex- or age-matched with patients.

Blood sampling and plasma preparation

Whole blood was routinely collected into tubes containing ethylenediaminetetraacetic acid (Becton Dickinson, Mountain View, CA) and was immediately centrifuged at 1000g at 4°C. Plasma was stored at −80°C. In experiments performed to control the extent of platelet activation, whole blood was also collected in the presence of inhibitors of platelet activation (theophylline, adenosine, and dipyridamole [CTAD] tubes, Becton Dickinson). To assess platelet activation, flow cytometry was performed on whole blood with the following monoclonal antibodies: VH10, recognizing P selectin; PAC-1 (Immunotech, Marseille, France); and AP6 (gift from Dr

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Submitted September 4, 2001; accepted November 14, 2001.

Supported by the Association pour la Recherche Médicale en Aquitaine and the Association pour la Recherche contre le Cancer.

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T.S. Kunicki, recognizing the active conformation of glycoprotein IIb-IIIa. Similar concentrations of sCD40L were found in ethylenediaminetetraacetic acid or CTAD anticoagulated tubes, suggesting that sCD40L was not occurring during sample preparation.

**Platelets**

**Platelet preparation.** Blood was collected into CTAD tubes. Platelet-rich plasma and washed platelets were prepared as described. Platelets were resuspended in HEPES Tyrode buffer. 10

**Thrombin-induced platelet activation.** Isolated platelets at a concentration of $250 \times 10^9$/L (250 000/μL) were incubated in the presence or absence of 0.5 U/mL thrombin (Ortho Diagnostic, Raritan, NJ) for 10 minutes at 37°C. Supernatants were obtained after centrifugation at 1200g and were frozen at −80°C before use.

**Platelet lysates.** Platelets at a concentration of $250 \times 10^9$/L (250 000/μL) were lysed (30 minutes, room temperature) in 1% (wt/vol) Triton X-100 (Sigma, Saint-Quentin Fallavier, France) containing leupeptin (Sigma) (20 μg/mL). Lysates were then centrifuged 10 minutes at 1200g.

**Soluble CD40L ELISA**

Quantification of sCD40L in plasma and in platelet lysates was performed with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Bender MedSystems, Vienna, Austria). For some samples, measurement was also performed with the R&D (Abingdon, United Kingdom) ELISA kit.

**Detection of CD40L on T-lymphocyte membrane by flow cytometry**

Peripheral blood mononuclear cells were isolated on Ficoll gradient, and $10^6$ cells per milliliter were stimulated with phorbol myristate acetate and ionomycin as described. 11 Cells were stained with cyanine 5–conjugated anti-CD3, fluorescein isothiocyanate–conjugated anti-CD8, and phycoerythrin-conjugated anti-CD40L monoclonal antibodies (all from Beckman-Coulter, Marseille, France) for 30 minutes at 4°C. After washing, cells were analyzed on a FACScan flow cytometer (Becton Dickinson). Lymphocytes were gated according to their forward and side scatter characteristics. CD40L expression then was analyzed on the CD3+, CD8– subpopulation. Results were expressed as mean fluorescence intensity (MFI) ratio: (MFI stimulated)/MFI for isotypic control.

**Statistical analysis**

Comparisons were done using the nonparametric Mann-Whitney U test, the level of significance being set at .05. Correlations were analyzed with the Spearman test.

**Results and discussion**

All patients with high platelet count had significantly raised levels of sCD40L when compared with controls (Figure 1A). Highest levels of sCD40L were seen within the ET population.

CD40L expression was studied by flow cytometry on activated CD4+ T lymphocytes in 11 patients with ET, 6 patients with RT, and 11 controls. CD4+ T lymphocytes from ET patients showed an increase in the expression of CD40L following activation compared with control lymphocytes (median values 148, 54; extreme values 30-310, 26-215, respectively), but the difference was not statistically significant ($P = 0.09$). There was no difference between patients with RT (median value 60, extreme values 8-159) and controls ($P = 0.61$) or ET patients ($P = 0.11$).

Platelet counts and sCD40L concentrations were highly correlated (Figure 1B), reinforcing the overall picture of a direct link between platelet count and plasma sCD40L. Further evidence for the link between platelets and sCD40L was provided by the...
follow-up of sCD40L in patients with ET before and after treatment. In each case, sCD40L concentrations lowered with treatment following the correction of the platelet count (Table 1).

In order to understand the relation between platelet count and plasma sCD40L, we looked for the presence of CD40L associated with platelets. As expected,6,12 CD40L was found to be present in platelets. A recent report demonstrates that platelets express CD40 and that the binding of CD40L to platelet CD40 results in the release of sCD40L in the fluid phase,13 also supporting the contribution of platelets to plasma sCD40L. Platelets from ET and RT patients had a higher content in CD40L, and they release more CD40L after stimulation with thrombin (Figure 1C). Correlation (Spearman test \( r = 0.89, \ P = .001 \)) was found between the content in platelet-associated CD40L and the amount of sCD40L released after activation.

In conclusion, this study shows a direct correlation between the platelet count and the concentrations of plasma sCD40L. Elevation of platelet-associated CD40L in conditions associated with an increased megakaryocytopenia makes it a potential marker of platelet regeneration. A possibility that deserves further investigation is that younger platelets are richer in CD-40L than those that have been in the circulation for several days, with a shedding of sCD40L occurring throughout the platelet lifespan. In accordance with this hypothesis, we recently found a very high CD40L content in platelets from patients with immune thrombocytopenic purpura, a condition that is associated with intense platelet regeneration (unpublished results, October 2001). Furthermore, results from knock-out mice strongly suggest that CD40L plays a role in thrombus formation at high shear rate.14 As such, CD40L may be an active factor in promoting the known prothrombotic and proinflammatory states known to be exhibited by some patients with high platelet count and as studied here.

References

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