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Original Contribution

Vitamin C inhibits platelet expression of CD40 ligand

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Abstract

Upon stimulation with agonists, platelets express CD40 ligand (CD40L), a transmembrane protein implicated in the initiation and progression of atherosclerotic disease. We have recently discovered that oxidative stress plays a major role in platelet CD40L expression. In this study, we sought to determine whether vitamin C, a known antioxidant, is able to influence platelet CD40L expression. In vitro experiments were done by stimulating platelets with collagen in the presence or absence of vitamin C (50–100 μM) or vehicle as control. An in vivo study was done in 10 healthy subjects who were randomized to intravenous infusion of placebo or 1 g vitamin C for 45 min in a crossover design. At the end of infusion platelet CD40L and O_{2^-} were measured. The in vitro study demonstrated that vitamin C dose dependently inhibited platelet CD40L expression without affecting agonist-induced platelet aggregation. In subjects treated with placebo no changes of platelet CD40L and O_{2^-} were observed; conversely, vitamin C infusion caused a significant and parallel decrease of platelet O_{2^-} (−70%, $P < 0.001$) and CD40L (−68%, $P < 0.001$). Platelet aggregation was not modified by either treatment. This study suggests that water-soluble antioxidants, which scavenge superoxide radicals, may reduce platelet CD40L expression.

Keywords: Vitamin C; Platelets; Free radicals/free-radical scavengers; Reactive oxygen species

Introduction

CD40 ligand (CD40L), a member of the tumor necrosis factor ligand family, is a transmembrane protein with proinflammatory and prothrombotic properties upon interaction with its receptor CD40 [1]. CD40L was primarily found on the immune system cells, and subsequently on macrophages, smooth muscle cells, endothelial cells, and platelets [1]. Upon agonist stimulation, platelets express CD40L that is then cleaved on the membrane surface and finally circulates in the soluble form [2]; it has been calculated that over 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].

We have recently demonstrated that platelet production of O_2^- is implicated in the expression of CD40L, as platelet incubation with SOD, which dismutases O_2^- to H_2O_2, significantly decreased agonist-induced CD40L expression [4]. These findings were confirmed by an in vivo study performed in patients with a deficit of gp91phox, the central core of NADPH oxidase. These patients showed a CD40L down-regulation, suggesting that O_2^- generated by NADPH oxidase activation elicits an up-regulation of CD40L [4].

These data induced us to seek whether natural antioxidants play a role in modulating platelet CD40L expression.

Abbreviations: CD40L, CD40 ligand; sCD40L, soluble CD40L; PRP, platelet-rich plasma; FITC, fluorescein isothiocyanate; Mab, monoclonal antibodies; PBS, phosphate-buffered saline; BSA, bovine serum albumin; AU, arbitrary unit.

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We first performed an in vitro study to assess if vitamin C, a water-soluble molecule that scavenges superoxide radicals [5], was able to influence platelet CD40L expression. For the first time, we herein report that vitamin C inhibits CD40L expression in vitro and in vivo.

Methods

Platelet preparation and activation

Blood samples were taken from healthy subjects who had fasted at least 12 h and were mixed with 0.13 M Na citrate. Platelet-rich plasma (PRP) was prepared as previously described [3]. Unless otherwise reported PRP was stimulated at 37°C for 3 min with collagen [4 μg/ml) or thrombin (0.1 U/ml). Agonist-induced platelet aggregation (Born's method) was measured by light transmission (LT%) difference between PRP and platelet-poor plasma.

O_2^- detection

Superoxide anion (O_2^-) production by PRP stimulated with agonists was measured by lucigenin (5 μM) [3] and expressed as stimulation index (SI = mean level of stimulated platelet luminescence/average level of luminescence in unstimulated platelets).

CD40L and CD62P expression

CD40L and CD62P expression on platelet membrane was analyzed using specific FITC-labeled monoclonal antibodies (Mab) as previously reported [4]. An irrelevant isotype-matched antibody (anti-IgG1) was used as a negative control.

Mab (20 μl) was added to platelets (200 μl, 2×10^8/ml) previously fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) (0.1% BSA) and incubated for 60 min at 4°C. The unbound Mab was removed by addition of 0.1% BSA phosphate-buffered saline and centrifugation at 500*g* for 3 min (twice). Fluorescence intensity was analyzed on an Epics XL-MCL Cytometer (Coulter Electronics, FL) 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 has been reported in an arbitrary unit (AU) obtained by multiplying the number of positive events obtained after platelet stimulation by the mean fluorescence observed when the specific Mab was used, and correcting against the values obtained in the unstimulated sample treated with the same antibody.

Analysis of sCD40L

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

In vitro study

In the in vitro study, platelet suspension (3×10^9 plt/ml) was treated with vitamin C (ascorbic acid, 25–100 μM) or solvent as control (30 min at 37°C) before activation for O_2^-, CD40L, and sCD40L analysis (see Methods above).

In vivo study

We investigated whether a short-term administration of vitamin C could affect agonist-induced platelet CD40L and O_2^-. To calculate the sample size of the study, we first measured agonist-induced platelet CD40L expression in 44 healthy subjects (24 males, 20 females; age, 32 ± 4 years); in this population, platelet CD40L expression was 32 AU ± 3.1. Assuming that vitamin C reduced platelet CD40L by 50%, at least 9 patients for each group had to be evaluated ($\alpha = 0.05$, 1-$\beta = 0.80$).We planned a randomized, double-blind, placebo-controlled crossover study in 10 healthy volunteers (5 females and 5 males; mean age, 30.5 ± 1.0 years, range 24–36 years) who provided their informed consent to participate in the study. All subjects were nonsmokers, had no risk factors for atherosclerosis, and had not taken any antiplatelet agents or antioxidant drugs in the previous month; a 10-day washout was scheduled between the two phases of the study. Collagen-induced platelet CD40L, sCD40L, O_2^-, CD62P, and aggregation were determined at baseline and immediately after 45 min of intravenous infusion of vitamin C at 24 mg/min or placebo.

Statistical analysis

Unless otherwise reported data are expressed as means ± SD. The 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^- formation was evaluated using the linear correlation analysis followed by ANOVA test.

Results

Compared to unstimulated platelets, agonists such as collagen and thrombin significantly increased platelet O_2^- formation and CD40L expression (Fig. 1); O_2^- and CD40L were significantly correlated ($r = 0.93$, $P = 0.006$) with collagen (4 μg/ml, $n = 4$) and ($r = 0.85$, $P = 0.005$) with thrombin (0.1 U/ml, $n = 4$). A similar correlation was detected using several concentrations of collagen (1 to 8 μg/ml) or thrombin (0.01 to 1 U/ml) (data not shown). Vitamin C dose dependently inhibited O_2^- and CD40L (Figs. 1A–D).