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Original Article

Characterization of Soluble CD40 ligand Released from Human Activated Platelets

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We report here that soluble CD40 ligand (sCD40L) is released from human platelets when activated with collagen or thrombin. The sCD40L was detectable in the culture supernatants of platelets within 30 min after stimulation in vitro, and reached maximal levels in 3 h. The release was blocked by the metalloproteinase inhibitor, KB8301, indicating that the soluble CD40L is made by cleaving the membrane bound CD40L expressed on activated platelets. The sCD40L was undetectable in the supernatant of the activated platelets obtained from patients with X-linked hyper IgM syndrome (XHIM), who have defects in CD40L gene. Since sCD40L has been shown to have biologic function on the activation of vascular endothelial cells and B cells, these findings suggest that platelets play some roles in both inflammation and humoral immune response by releasing soluble CD40L.

Key words: soluble CD40 Ligand, activated platelets, metalloproteinase inhibitor, Hyper IgM syndrome

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Introduction

CD40 ligand (CD40L, CD154) is a type II transmembrane protein that belongs to TNF super-family. CD40L was first described as a key molecule in the T cell helper function, which induces B cell proliferation, Ig class switching and memory B cell development[1–4]. The importance of CD40L in the human immune system has been further shown by the finding that X-linked hyper IgM syndrome, a human primary immune deficiency with defective class switching, is caused by the mutation of CD40L gene[5–7].

Subsequently, CD40L was found to be expressed on a variety of cells including mast cells, basophils[8], eosinophils[9], B cells and platelets[10]. It has been demonstrated that CD40L expressed on platelets is involved in the initiation of inflammatory response of the vessel wall by inducing expression of adhesion molecules and secretion of chemokines in vascular endothelial cells[10].

In addition to the membrane bound CD40L molecule, the soluble type of CD40L (sCD40L) was observed in the culture supernatant of activated T cells[11]. By biochemical analysis, the sCD40 L was determined to be the 18 kDa protein that consists of extracellular domain of CD40L. Furthermore, sCD40L was detectable in the sera of normal human individuals, and the amounts of sCD40L were increased in the sera of patients with SLE[12,13]. The sCD40L in the sera of SLE patients was biologically functional, since it induced CD54 (ICAM-1) on the Ramos B cell line, and the induction of this molecule was specifically blocked by the anti-CD40L neutralizing mAb.

In this study, in order to clarify whether sCD40L could be produced by human platelets, we investigated the sCD40L production of platelets when they were stimulated with thrombin, collagen, and thrombopoietin. We found that activated platelets secrete sCD40L, and the secretion was made by the cleavage of membrane bound CD40L by metalloproteinase(s). Roles of sCD40L produced by activated platelets are discussed.

Materials and Methods

Platelets preparation

Human blood samples were drawn from healthy volunteers or three patients with XHIM by venipuncture under the informed consent. The CD40L mutations in the XHIM are as follows; 1) ATAG deletion at position +178 to +181 of exon 2, 2) G to A nonsense mutation at position +441 (TGG to TGA), leading Trp 144 to stop codon, 3) G to A missense mutation at position +697 (GGA to AGA), leading Gly 226 to Arg amino acid change. None of three patients expressed CD40L protein on the activated T cells when they were stained with 5c8, 106, or 7, which are three kinds of anti-CD40L antibodies. Blood samples were collected into 1/10 volume of 3.8% (wt/vol) trisodium citrate and were gently mixed by inversion. Platelet rich plasma (PRP) was prepared by centrifugation of whole blood at 200 g for 15 min. The PRP was spun at 800 g to form a soft platelet pellet. The pellet was resuspended in 1 ml modified Hepes-Tyrode buffer supplemented with 3 U/ml apirase and incubated for 10 min at 37°C. The modified Hepes-Tyrode buffer contains 138 mM, sodium chloride (NaCl), 2.9 mM potassium chloride (KCl), 0.5 mM sodium dihydrogenphosphate (NaH_2PO_4), 1 mM magnesium dichloride ($MgCl_2$), 1 mM dextrose, and 20 mM Hepes (pH 6.7), supplemented with 0.035 g/l albumin. After the incubation, 10 ml of the Hepes-Tyrode buffer was added to the samples and washed twice. Platelets (2×10^8 cells/ml) were then resuspended with the modified Hepes-Tyrode buffer (pH 7.35) supplemented with 0.35 g/l albumin, and were used for experiments.

Cell culture

The Jurkat T cell line (JLN34 clone), which constitutively expresses CD40L, was cultured in the RPMI1640 culture medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% of heat inactivated fetal calf serum (Gibco). They were incubated with various concentrations of metalloproteinase inhibitor KB8301 (kind gift by Dr. H. Yagita, Juntendo University School of Medicine, Tokyo, Japan). The cells were suspended at a concentration of 2×10^6 cells/ml/well. Supernatants were harvested and assayed for sCD40L by ELISA.

Platelets were aliquoted into 0.25 ml modified Hepes-Tyrode buffer (pH 7.35) at a concentration of 2×10^8 cells/ml, and were stimulated with the agonist thrombin (4 U/ml) (Sigma Diagnostics, St Louis, MO, USA), collagen (10 μg/ml) (Sigma) or thrombopoietin (TPO) (3.2 ng/ml) (KIRIN, Takasaki, Japan) for various hours at 4°C. After incubation, the culture supernatants were collected by centrifugation.

Metalloproteinase inhibitor, KB8301 (1.0 μM), was added simultaneously with thrombin (4 U/ml) into the platelet culture. The culture supernatants were isolated after incubation for 1 h, 3 h, and 6 h. They were stored at –80°C and subjected to sCD40L assay.

Soluble CD40L detection by ELISA

The amounts of soluble CD40L were quantified using specific ELISA (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions.

Statistical analysis

The mean concentrations for sCD40L released from activated platelets of healthy individuals and those of patients were compared using the Mann-Whitney *U* test.

Results

Activated platelets release soluble CD40L

It has been recently reported that activated platelets express CD40L on their surface[10]. We, therefore, examined whether activated platelets could release soluble CD40L extracellularly. As shown in Fig. 1, thrombin as well as collagen stimulation induced release of sCD40L from human platelets. The amounts of sCD40L released from platelets stimulated by thrombin or collagen was significantly higher than those released from unstimulated platelets (thrombin: 11.833 ± 0.764 ng/ml, collagen: 16.125 ± 1.315 ng/ml, no stimulation: 3.483 ± 1.173 ng/ml, mean ± SD) ($P < 0.05$). The amounts of sCD40L released from platelets cultured with TPO (4.575 ± 0.435 ng/ml) were similar with those observed from unstimulated platelets, indicating that TPO does not induce the release of sCD40L from platelets.

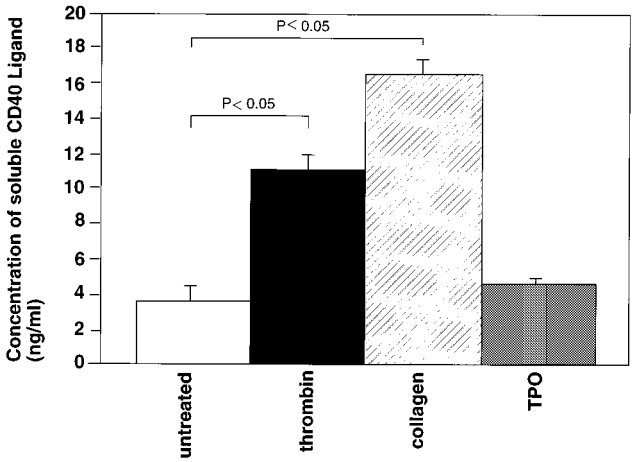

Figure 1. Release of soluble CD40L from activated platelets. Platelets (2×10^8 cells/ml) were isolated from 4 healthy individuals and stimulated with human thrombin (4 U/ml), collagen (10 μg/ml) or TPO (3.2 ng/ml) for 1 h at 4 °C. The amounts of sCD40L in the supernatant were detected by specific ELISA. The result represents the mean ± SD of triplicate detection of one typical result of the four independent experiments.

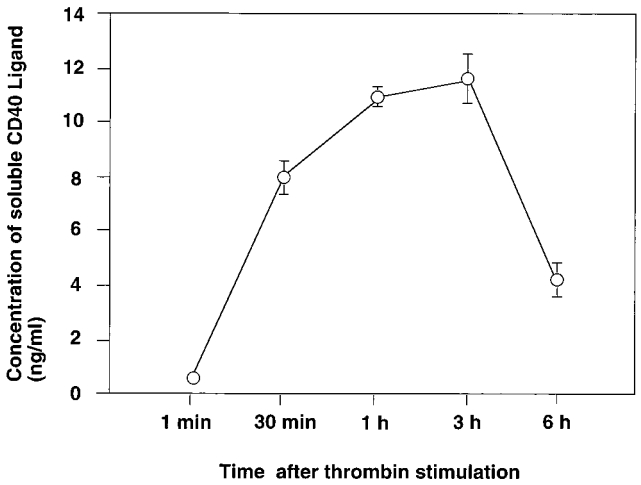

Figure 2. Time course of soluble CD40L release from activated platelets. Platelets (2×10^8 cells/ml) isolated from 4 healthy adults were stimulated with thrombin (4 U/ml) and were incubated for 1 min, 30 min, 1 h, 3 h, and 6 h at 4 °C. The amounts of soluble CD40L in the supernatant were measured by ELISA. The result represents the mean ± SD of triplicate detection of one typical result of the four independent experiments.

We next examined the kinetics of the shedding of soluble CD40L after thrombin stimulation. We found that the platelets released significant amounts of soluble CD40L into the supernatant on thrombin stimulation within 30 min (9.985 ± 0.898 ng/ml) (Fig. 2). Soluble CD40L was not induced in 1 min. Maximal level of sCD40L was achieved in 3 h (13.600 ± 1.556 ng/ml), thereafter, sCD40L significantly declined in 6 h (6.170 ± 0.948 ng/ml) ($P < 0.05$).

Effect of metalloproteinase inhibitor KB8301 on the sCD40L release from Jurkat T cell line (JLN34 clone)

Previous work has shown that soluble CD40L was released from the activated T cells[11]. We next examined if sCD40L is released from the Jurkat T cell line (JLN34 clone) that constitutively expresses CD40L, and if the release of sCD40L could be inhibited by metalloproteinase inhibitor KB8301 treatment. KB8301 was added under the various concentrations; 0.1 μM, 0.5 μM, 1.0 μM, 5.0 μM, and 10.0 μM. As shown in Fig. 3, sCD40L was detectable in the culture supernatant of JLN34 cell line (1.890 ng/ml). Addition of KB8301 significantly down-regulated the CD40L release in a dose-dependent manner, and almost complete inhibition was obtained in the dose of 1.0 μM (< 0.08 ng/ml). The data indicate that metallproteinase inhibitor KB8301 effectively suppress the release of sCD40L from Jurkat T cell line (JLN34 clone).

Reduction of soluble CD40L level released from platelets by metalloproteinase inhibitor KB8301 treatment

To examine if KB8301 treatment down-regulates sCD40L secretion from thrombin stimulated platelets, we analyzed the sCD40Ls level released from platelets treated with KB8301 (1.0 μM). As shown in Fig. 4, sCD40L release was significantly reduced by KB8301 treatment at 1 h and at 3 h ($P < 0.05$).

Soluble CD40L level released from the platelets for X-linked Hyper IgM syndrome patients

Platelets were isolated from 3 patients with X-linked Hyper IgM syndrome and 3 healthy control individuals. The platelets were stimulated with thrombin (4 U/ml) for 1 h. The soluble CD40L was undetectable in samples from all three XHIM patients (Table 1). These data confirm that this ELISA system specifically measures sCD40L.

Discussion

In this study, we demonstrated that human platelets release sCD40L if stimulated with collagen or thrombin. The sCD40L was detectable in the culture supernatants within 30 min after the stimulation. Consistent with this finding, Henn et al reported that platelets express membrane bound CD40L within only 1 min

Table 1. Soluble CD40L released from platelets of XHIM patients and healthy individuals. The platelets isolated from 3 patients with X-linked HIGM and 3 healthy control adults were treated with thrombin (4 U/ml) for 1 h at 4 °C. The levels of CD40L in the supernatants from stimulated platelets were measured with ELISA. Data are shown as mean ± SD of triplicate detection of each experiment.

XHIM Mean ± SD (n=3)*		Healthy Controls Mean ± SD (n=3)*	
Patient 1	0±0	Control 1	6.913±0.634
Patient 2	0±0	Control 2	10.323±0.864
Patient 3	0±0	Control 3	11.850±1.481

*The results indicate the mean concentration for triplicate detection of every one experiment ± SD about the mean

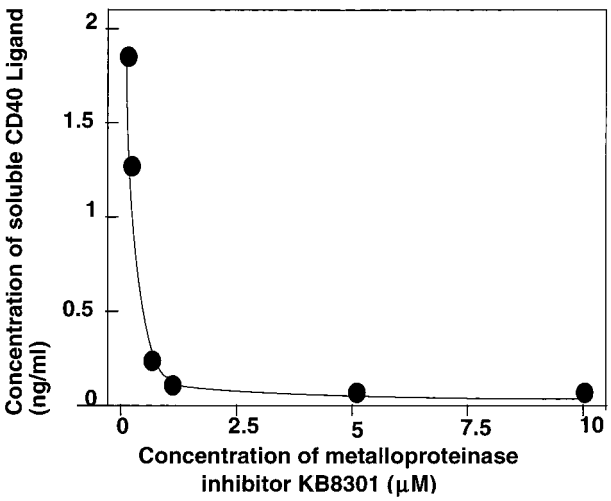

Figure 3. Blocking effect of metalloproteinase inhibitor KB8301 on shedding of soluble CD40L from JLN34 T cell line. JLN34 Jurkat T cell line was treated with various concentrations of the proteinase inhibitor KB8301. Supernatants were harvested after 24 h of culture, and sCD40L levels were assayed by ELISA.

after thrombin stimulation[10]. Interestingly, it is known that T cells are induced to express membrane bound CD40L several hours after stimulation. This difference between T cells and platelets concerning the kinetics of membrane bound CD40L expression has been explained by the evidence that CD40L was preformed within the platelet cytoplasm and was translocated to the cell surface by the thrombin stimulation, while CD40L is newly synthesized in T cells after stimulation. In addition, release of sCD40L from activated T cells is reported to reach maximal levels in 10 h after stimulation then declined[11]. We observed that in platelets, the maximal levels of sCD40L reached in 3 h after stimulation and declined in 6 h. This observation is also consistent with the rapid expression of membrane bound CD40L on platelets after stimulation.

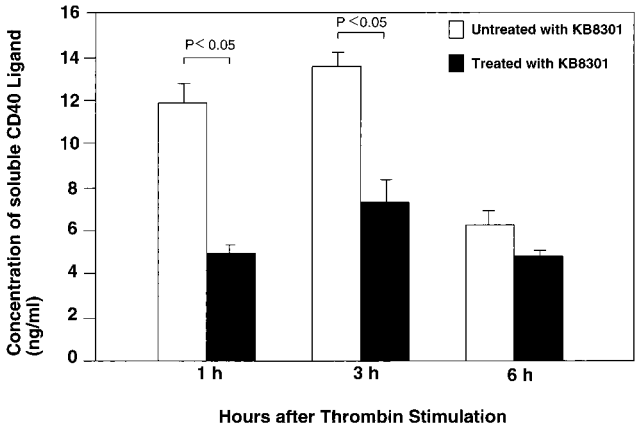

Figure 4. Blocking effect of metalloproteinase inhibitor KB8301 on shedding of soluble CD40L from activated platelets. Platelets isolated from 4 healthy individuals were stimulated with thrombin (4 U/ml) and either treated or untreated with KB8301 at the concentration of 0.2 μM The culture supernatants were harvested for indicated hours. The amounts of sCD40L were assayed by ELISA. Black bars indicate platelets treated with KB8301, white bars indicate platelets untreated with KB8301. The result represents the mean ± SD of triplicate detection of one typical result of four independent experiments.

We observed that metalloproteinase inhibitor blocks the release of sCD40L from Jurkat T cell line and from activated platelets, indicating that membrane bound CD40L is cleaved by metalloprotease, as has been demonstrated in the generation of soluble Fas ligand and TNF-α[14–17]. In contrast, Graf et al reported that they observed no effect of proteinase inhibitors on sCD40L release[11]. The reason of these different findings is currently unknown. One possible explanation is that the metalloproteinase inhibitor used in our study is different from those used in the study by Graf et al, and may have more potent inhibitory effect. In addition, our finding that sCD40L was not released in 1 min after thrombin stimulation supports our hypothesis that CD40L is first expressed as a membrane bound form, and then cleaved by metalloproteinase. MMP-2 (matrix metalloproteinase-2) and Jararhagin, a 52-kDa snake venom metalloproteinase have been reported to possess functions on platelets[18,19].

The physiological role of sCD40L has not yet been clarified. However, the increase of sCD40L in sera has been reported in the patients with SLE[12]. In the report, it has been indicated that the vasculitis is co-related with the levels of sCD40L in sera, suggesting that sCD40L released from platelets is involved in vascular inflammation. Since sCD40L also activates B cells[11], the increase of sCD40L in these disorders may be involved both in their polyclonal B cell activation and

vasculitis.

It has been shown that membrane bound CD40L produced by activated T cells and platelets has the same molecular structure and has the same functional activity[10]. Since soluble CD40L is made by metalloproteinase cleavage both in T cells and platelets, it is likely that sCD40L released from platelets is structurally same as that released from T cells. Membrane bound CD40L induce ICAM-1, VCAM-1, IL-8, and MCP-1 expression of vascular endothelial cells. Furthermore, the sCD40L in sera of SLE patients was verified to possess functional ability to induce B cell activation by induction of CD54 (ICAM-1) expression. Thus sCD40L produced by platelets has possibility to induce B cell activation. Similarly, sCD40L released by T cells may influence the growth or activation of vascular endothelial cells. Our study indicates that the amounts of sCD40L released by 2×10^8 platelets are more than 10 ng. Since the 100 times numbers of platelets to lymphocytes (platelets: 2.5×10^8 cells/ml, lymphocytes: 2.5×10^6 cells/ml) are present in the periphery, it is intriguing to speculate that platelets are another source of sCD40L detectable in normal human serum other than T cells. In this context, platelets may play some roles in the B cell immune system as well as vascular inflammation by secreting sCD40L.

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