heterotetrameric complexes via non-classical pathways for the secretion of FGF-1 and IL-1α, both of which lack a signal peptide for classical secretion[25–27]. S100A13 has been implicated in angiogenesis[35–37], tumor development[38], and chronic inflammation[39]. S100A13 is released by stress, including heat shock[29] and serum depletion[30]. The receptor for advanced glycation end products (RAGE) has been shown to be a receptor for S100A13. We did not observe surface expression of S100A13 in washed and suspended CASMCs (Fig 8A) or in adherent CASMCs (S3 Fig). It is noteworthy that the same washed and suspended CASMCs (Fig 3A) or adherent CASMCs (Fig 3B) were obviously associated with hCLEC-2-rFc2 and stimulated granule release from platelets or thrombus formation under flow. These findings suggest that although we identified S100A13 as the CLEC-2 ligand in VSMCs, this protein is not responsible for stimulating granule release from platelets or thrombus formation under flow and that CLEC-2 ligands other than S100A13 are present. However, exogenously added S100A13 did bind to the CASMC surfaces (Fig 8A). In addition, oxidative stress induced surface expression of S100A13 (Fig 8B). Moreover, we observed that S100A13 was expressed in the luminal area and can be exposed to blood flow in atherosclerotic lesions (Fig 9) and that immobilized S100A13 potentiated thrombus formation on collagen-coated surfaces (Fig 10). Based on these findings, we suggest that S100A13 is released from CASMCs and bound to their surface under some pathological conditions, which may result in interactions between platelets and CASMCs *in vivo*. It has been reported that prominent staining of MAC-1, IL-1α, and S100A13 was observed at the site of neointimal thickening after balloon injury and that Cu^{2+} chelation inhibited formation of S100A13 heterotetrameric complexes and IL-1α release, thereby reducing neointimal thickening[40]. Therefore, S100A13 may be released and adhered to the surface of VSMCs, and stimulate platelets as the CLEC-2 ligand on the surface of VSMCs at the site of atherosclerotic lesions and stent insertion.

What is the pathophysiological significance of the CLEC-2-VSMC association? We suggest that this association plays a role in thrombosis at the site of stent implantation and plaque erosion, where VSMCs are exposed to blood flow. Upon stent implantation, VSMCs are exposed to blood flow before endothelialization occurs, particularly in the case of drug-eluting stents (DES), for which late stent thrombosis remains a significant problem. Recent findings utilizing intravascular ultrasound and optical coherence tomography suggested that delayed healing and neoatherosclerosis are important mechanisms for very late stent thrombosis[41]. The binding of CLEC-2 to a ligand in VSMCs may trigger stent thrombosis. Patients who undergo DES implantation have an almost lifelong risk of stent thrombosis and need to take dual antiplatelet therapy over an extended period because DES delays endothelialization. However, we have previously reported that CLEC-2-deficient bone marrow chimeric mice did not show a significantly increased bleeding tendency. Therefore, we speculate that an anti-CLEC-2 agent that inhibits the association between CLEC-2 and VSMCs has beneficial effects in preventing stent thrombosis by reducing the quantity of anti-platelet drugs required without increasing the frequency of bleeding complications. Plaque erosion likely occurs at smooth muscle cell/proteoglycan-rich plaques without a large lipid core, and is found in sudden death from coronary thrombosis, comprising 44% of all cases[42]. The association between CLEC-2 and its ligands in VSMCs may also be involved in this process. Blocking of the binding between CLEC-2 and VSMCs may be effective to prevent plaque erosion, which is more often observed in younger individuals and women.

Supporting Information

S1 Fig. CHO cells transfected with human podoplanin, but not CASMCs, induced platelet aggregation. Platelet aggregation by CHO cells transfected with human podoplanin (upper

panels) or CASMCs (lower panels) was monitored by light transmission. Final concentrations of the cells were 2.5×10^5 cells/ml (left panels) and 1.0×10^6 cells/ml (right panels).
(TIF)

S2 Fig. Internal media lamina was lacerated in $FeCl_3$-injured femoral arteries, but not in photochemically-injured femoral arteries. A) Victoria blue-HE staining of non-injured murine femoral artery. Arrowheads indicates internal media lamina. B) Victoria blue-HE staining of photochemically-injured murine femoral artery. C) Victoria blue-HE staining of $FeCl_3$-injured murine femoral artery. Laceration of internal media lamina is indicated by an arrow. The asterisks indicate thrombi.
(TIF)

S3 Fig. S100A13 was expressed in the cytoplasm of adherent CASMCs, but not on the surface. CASMCs cultivated on a 24-well culture dish were fixed and incubated with control mouse IgG (upper panels) or anti-S100A13 antibody (63Y) (middle and lower panels), followed by anti-mouse IgG labeled with Alexa Fluor 488. Where indicated, cells were treated with 0.1% Triton X-100 before addition of the antibodies (lower panels). Cells were visualized by phase-contrast Microscopy (left panels) or fluorescence microscopy (right panels).
(TIF)

S4 Fig. Co-staining for S100A13 and smooth muscle actin in the atherosclerotic lesion in mice. Frozen-thawed sections of the mouse abdominal aorta from ApoE-deficient mice fed a high fat diet were incubated with goat anti-α smooth muscle actin (left panels) or rabbit anti-S100A13 antibody (middle panels), followed by anti-rabbit IgG-Alexa Fluor 488 and anti-goat IgG-Alexa Fluor 597, respectively. Right panels are merged images of left and middle panels. Lower panels are magnified images of the square area from upper panels.
(TIF)

S5 Fig. Oxidative stress induced surface expression of S100A13, but not that of podoplanin in CASMCs. Surface expression of endogenous S100A13 or podoplanin was analyzed by flow cytometry. CASMCs pretreated with vehicle (upper panels) or 1 mM H_2O_2 (lower panels) were incubated with control mouse IgG (filled, left panels), anti-S100A13 antibody (line, left panels), control rat IgG (filled, right panels), or anti-human podoplanin (NZ-1, line, right panels) followed by Alexa Flour 488-conjugated anti-mouse IgG.
(TIF)

S6 Fig. Immunohistochemistry for S100A13 and smooth muscle actin in the femoral artery injured by $FeCl_3$. Frozen-thawed sections of the mouse femoral artery injured by $FeCl_3$ were incubated with anti-smooth muscle actin (SMA) antibody (A), anti-S100A13 (B) followed by visualization using anti-goat IgG Alexa Flour 546 and anti-rabbit IgG Alexa Flour 488, respectively. A and B were merged (C). The phage contrast image are shown in E. Nuclei were counter-stained by DAPI (F).
(TIF)

S7 Fig. Immobilized S100A13 did not increase thrombus formation under flow when immobilized with collagen in CLEC-2-deficient blood. A) Wild type murine whole blood (WT, i and ii) or CLEC-2-deficient murine whole blood (KO, iii and iv) stained with $DiOC_6$ was perfused into capillaries with collagen (i and iii) or collagen plus S100A13 (ii and iv) for 5 min at a shear rate of 1500 s^{-1}. Adherent platelets were visualized by confocal laser microscopy. B) The z-stack data were quantified. The thrombus volume was expressed as the cIFI per image (404374 μm^2). The graph illustrates the percentage of the control (wild type whole blood)

cIFI ± SE (n = 3–4).
(TIF)

Acknowledgments

We are grateful to Chiaki Komatsu, Hisaichiro Nakazawa, Uina Fukuda, Tsutomu Yuminamochi, and Kumiko Nakazawa for excellent technical assistance.

Author Contributions

Conceived and designed the experiments: KS-I OI. Performed the experiments: OI K. Hokamura TS MO NT K. Hatakeyama KS-I. Analyzed the data: KS-I OI. Contributed reagents/materials/analysis tools: KU YA. Wrote the paper: KS-I YO.

References

1. Suzuki-Inoue K, Fuller GL, Garcia A, Eble JA, Pohlmann S, Inoue O, et al. A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. Blood. 2006; 107(2):542–9. Epub 2005/09/22. 2005-05-1994 [pii] doi: 10.1182/blood-2005-05-1994 PMID: 16174766.
2. Suzuki-Inoue K, Kato Y, Inoue O, Kaneko MK, Mishima K, Yatomi Y, et al. Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells. J Biol Chem. 2007; 282(36):25993–6001. Epub 2007/07/10. M702327200 [pii] doi: 10.1074/jbc.M702327200 PMID: 17616532.
3. Kato Y, Kaneko MK, Kunita A, Ito H, Kameyama A, Ogasawara S, et al. Molecular analysis of the pathophysiological binding of the platelet aggregation-inducing factor podoplanin to the C-type lectin-like receptor CLEC-2. Cancer Sci. 2008; 99(1):54–61. Epub 2007/10/20. CAS634 [pii] doi: 10.1111/j.1349-7006.2007.00634.x PMID: 17944973.
4. Tsuruo T, Fujita N. Platelet aggregation in the formation of tumor metastasis. Proc Jpn Acad Ser B Phys Biol Sci. 2008; 84(6):189–98. Epub 2008/10/23. JST.JSTAGE/pjab/84.189 [pii]. PMID: 18941298.
5. Bertozzi CC, Schmaier AA, Mericko P, Hess PR, Zou Z, Chen M, et al. Platelets regulate lymphatic vascular development through CLEC-2-SLP-76 signaling. Blood. 2010; 116(4):661–70. Epub 2010/04/07. blood-2010-02-270876 [pii] doi: 10.1182/blood-2010-02-270876 PMID: 20363774.
6. Suzuki-Inoue K, Inoue O, Ding G, Nishimura S, Hokamura K, Eto K, et al. Essential in vivo roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. J Biol Chem. 2010; 285(32):24494–507. Epub 2010/06/08. M110.130575 [pii] doi: 10.1074/jbc.M110.130575 PMID: 20525685; PubMed Central PMCID: PMC2915686.
7. Hughes CE, Navarro-Nunez L, Finney BA, Mourao-Sa D, Pollitt AY, Watson SP. CLEC-2 is not required for platelet aggregation at arteriolar shear. Journal of thrombosis and haemostasis: JTH. 2010; 8 (10):2328–32. Epub 2010/08/11. JTH4006 [pii] doi: 10.1111/j.1538-7836.2010.04006.x PMID: 20695981.
8. Finney BA, Schweighoffer E, Navarro-Nunez L, Benezech C, Barone F, Hughes CE, et al. CLEC-2 and Syk in the megakaryocytic/platelet lineage are essential for development. Blood. 2012; 119(7):1747–56. Epub 2011/12/22. blood-2011-09-380709 [pii] doi: 10.1182/blood-2011-09-380709 PMID: 22186994.
9. Osada M, Inoue O, Ding G, Shirai T, Ichise H, Hirayama K, et al. Platelet Activation Receptor CLEC-2 Regulates Blood/Lymphatic Vessel Separation by Inhibiting Proliferation, Migration, and Tube Formation of Lymphatic Endothelial Cells. Journal of Biological Chemistry. 2012; 287(26):22241–52. Epub 2012/05/05. M111.329987 [pii] doi: 10.1074/jbc.M111.329987 PMID: 22556408.
10. May F, Hagedorn I, Pleines I, Bender M, Vogtle T, Eble J, et al. CLEC-2 is an essential platelet-activating receptor in hemostasis and thrombosis. Blood. 2009; 114(16):3464–72. Epub 2009/07/31. blood-2009-05-222273 [pii] doi: 10.1182/blood-2009-05-222273 PMID: 19641185.
11. Bender M, May F, Lorenz V, Thielmann I, Hagedorn I, Finney BA, et al. Combined in vivo depletion of glycoprotein VI and C-type lectin-like receptor 2 severely compromises hemostasis and abrogates arterial thrombosis in mice. Arterioscler Thromb Vasc Biol. 2013; 33(5):926–34. doi: 10.1161/ATVBAHA.112.300672 PMID: 23448972.
12. Morton LF, Hargreaves PG, Farndale RW, Young RD, Barnes MJ. Integrin alpha 2 beta 1-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaternary

(polymeric) structures are sufficient alone for alpha 2 beta 1-independent platelet reactivity. Biochem J. 1995; 306 (Pt 2):337–44. Epub 1995/03/01. PMID: 7534064; PubMed Central PMCID: PMC1136526.

13. Shin Y, Morita T. Rhodocytin, a functional novel platelet agonist belonging to the heterodimeric C-type lectin family, induces platelet aggregation independently of glycoprotein Ib. Biochem Biophys Res Commun. 1998; 245(3):741–5. Epub 1998/05/20. S0006-291X(98)98516-3 [pii] doi: 10.1006/bbrc.1998.8516 PMID: 9588185.

14. Hatakeyama K, Asada Y, Marutsuka K, Sato Y, Kamikubo Y, Sumiyoshi A. Localization and activity of tissue factor in human aortic atherosclerotic lesions. Atherosclerosis. 1997; 133(2):213–9. PMID: 9298681.

15. Ishikawa T, Hatakeyama K, Imamura T, Ito K, Hara S, Date H, et al. Increased adrenomedullin immunoreactivity and mRNA expression in coronary plaques obtained from patients with unstable angina. Heart. 2004; 90(10):1206–10. doi: 10.1136/hrt.2003.017921 PMID: 15367525; PubMed Central PMCID: PMC1768472.

16. Sharmin S, Sakata K, Kashiwagi K, Ueda S, Iwasaki S, Shirahata A, et al. Polyamine cytotoxicity in the presence of bovine serum amine oxidase. Biochem Biophys Res Commun. 2001; 282(1):228–35. doi: 10.1006/bbrc.2001.4569 PMID: 11263996.

17. Suzuki-Inoue K, Inoue O, Frampton J, Watson SP. Murine GPVI stimulates weak integrin activation in PLCgamma2-/- platelets: involvement of PLCgamma1 and PI3-kinase. Blood. 2003; 102(4):1367–73. Epub 2003/05/06. doi: 10.1182/blood-2003-01-0029 2003-01-0029 [pii]. PMID: 12730118.

18. Kikuchi S, Umemura K, Kondo K, Saniabadi AR, Nakashima M. Photochemically induced endothelial injury in the mouse as a screening model for inhibitors of vascular intimal thickening. Arterioscler Thromb Vasc Biol. 1998; 18(7):1069–78. PMID: 9672067.

19. Inoue O, Suzuki-Inoue K, McCarty OJ, Moroi M, Ruggeri ZM, Kunicki TJ, et al. Laminin stimulates spreading of platelets through integrin alpha6beta1-dependent activation of GPVI. Blood. 2006; 107 (4):1405–12. Epub 2005/10/13. 2005-06-2406 [pii] doi: 10.1182/blood-2005-06-2406 PMID: 16219796; PubMed Central PMCID: PMC1895394.

20. Suzuki-Inoue K, Yatomi Y, Asazuma N, Kainoh M, Tanaka T, Satoh K, et al. Rac, a small guanosine triphosphate-binding protein, and p21-activated kinase are activated during platelet spreading on collagen-coated surfaces: roles of integrin alpha(2)beta(1). Blood. 2001; 98(13):3708–16. Epub 2001/12/12. PMID: 11739176.

21. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W Jr., et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation. 1995; 92(5):1355–74. PMID: 7648691.

22. Munnix IC, Strehl A, Kuijpers MJ, Auger JM, van der Meijden PE, van Zandvoort MA, et al. The glycoprotein VI-phospholipase Cgamma2 signaling pathway controls thrombus formation induced by collagen and tissue factor in vitro and in vivo. Arteriosclerosis, Thrombosis, and Vascular Biology. 2005; 25 (12):2673–8. Epub 2005/10/29. 01.ATV.0000193568.71980.4a [pii] doi: 10.1161/01.ATV.0000193568.71980.4a PMID: 16254207.

23. Morowski M, Vogtle T, Kraft P, Kleinschnitz C, Stoll G, Nieswandt B. Only severe thrombocytopenia results in bleeding and defective thrombus formation in mice. Blood. 2013; 121(24):4938–47. doi: 10.1182/blood-2012-10-461459 PMID: 23584880.

24. Hirata Y, Umemura K, Kondoh K, Uematsu T, Nakashima M. Experimental intimal thickening studies using the photochemically induced thrombosis model in the guinea-pig femoral artery. Atherosclerosis. 1994; 107(1):117–24. PMID: 7945554.

25. Mohan SK, Yu C. The IL1alpha-S100A13 heterotetrameric complex structure: a component in the non-classical pathway for interleukin 1alpha secretion. J Biol Chem. 2011; 286(16):14608–17. doi: 10.1074/jbc.M110.201954 PMID: 21270123; PubMed Central PMCID: PMC3077658.

26. Mouta Carreira C, LaVallee TM, Tarantini F, Jackson A, Lathrop JT, Hampton B, et al. S100A13 is involved in the regulation of fibroblast growth factor-1 and p40 synaptotagmin-1 release in vitro. J Biol Chem. 1998; 273(35):22224–31. PMID: 9712836.

27. Mandinova A, Soldi R, Graziani I, Bagala C, Bellum S, Landriscina M, et al. S100A13 mediates the copper-dependent stress-induced release of IL-1alpha from both human U937 and murine NIH 3T3 cells. J Cell Sci. 2003; 116(Pt 13):2687–96. doi: 10.1242/jcs.00471 PMID: 12746488.

28. Ridinger K, Schafer BW, Durussel I, Cox JA, Heizmann CW. S100A13. Biochemical characterization and subcellular localization in different cell lines. J Biol Chem. 2000; 275(12):8686–94. PMID: 10722710.

29. Kathir KM, Gao L, Rajalingam D, Daily AE, Brixey S, Liu H, et al. NMR characterization of copper and lipid interactions of the C2B domain of synaptotagmin I-relevance to the non-classical secretion of the

human acidic fibroblast growth factor (hFGF-1). Biochim Biophys Acta. 2010; 1798(2):297–302. doi: 10.1016/j.bbamem.2009.09.024 PMID: 19835837; PubMed Central PMCID: PMC3628535.

30. Matsunaga H, Ueda H. Stress-induced non-vesicular release of prothymosin-alpha initiated by an interaction with S100A13, and its blockade by caspase-3 cleavage. Cell Death Differ. 2010; 17(11):1760–72. doi: 10.1038/cdd.2010.52 PMID: 20467443.

31. Blanc A, Pandey NR, Srivastava AK. Synchronous activation of ERK 1/2, p38mapk and PKB/Akt signaling by H2O2 in vascular smooth muscle cells: potential involvement in vascular disease (review). Int J Mol Med. 2003; 11(2):229–34. PMID: 12525883.

32. Hatakeyama K, Kaneko MK, Kato Y, Ishikawa T, Nishihira K, Tsujimoto Y, et al. Podoplanin expression in advanced atherosclerotic lesions of human aortas. Thrombosis Research. 2012; 129(4):e70–6. Epub 2012/01/31. S0049-3848(12)00007-2 [pii] doi: 10.1016/j.thromres.2012.01.003 PMID: 22283975.

33. Hayashi K, Saga H, Chimori Y, Kimura K, Yamanaka Y, Sobue K. Differentiated phenotype of smooth muscle cells depends on signaling pathways through insulin-like growth factors and phosphatidylinositol 3-kinase. Journal of Biological Chemistry. 1998; 273(44):28860–7. Epub 1998/10/24. PMID: 9786887.

34. Cosemans JM, Angelillo-Scherrer A, Mattheij NJ, Heemskerk JW. The effects of arterial flow on platelet activation, thrombus growth, and stabilization. Cardiovasc Res. 2013; 99(2):342–52. doi: 10.1093/cvr/cvt110 PMID: 23667186.

35. Massi D, Landriscina M, Piscazzi A, Cosci E, Kirov A, Paglierani M, et al. S100A13 is a new angiogenic marker in human melanoma. Mod Pathol. 2010; 23(6):804–13. doi: 10.1038/modpathol.2010.54 PMID: 20208480; PubMed Central PMCID: PMC2882157.

36. Li C, Zhang F, Wang Y. S100A proteins in the pathogenesis of experimental corneal neovascularization. Mol Vis. 2010; 16:2225–35. PMID: 21139687; PubMed Central PMCID: PMC2994359.

37. Landriscina M, Schinzari G, Di Leonardo G, Quirino M, Cassano A, D'Argento E, et al. S100A13, a new marker of angiogenesis in human astrocytic gliomas. J Neurooncol. 2006; 80(3):251–9. doi: 10.1007/s11060-006-9189-y PMID: 16773219.

38. Pierce A, Barron N, Linehan R, Ryan E, O'Driscoll L, Daly C, et al. Identification of a novel, functional role for S100A13 in invasive lung cancer cell lines. Eur J Cancer. 2008; 44(1):151–9. doi: 10.1016/j.ejca.2007.10.017 PMID: 18061437.

39. Lambros MP, Parsa C, Mulamalla H, Orlando R, Lau B, Huang Y, et al. Identifying cell and molecular stress after radiation in a three-dimensional (3-D) model of oral mucositis. Biochem Biophys Res Commun. 2011; 405(1):102–6. doi: 10.1016/j.bbrc.2010.12.135 PMID: 21216227.

40. Mandinov L, Mandinova A, Kyurkchiev S, Kyurkchiev D, Kehayov I, Kolev V, et al. Copper chelation represses the vascular response to injury. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100(11):6700–5. doi: 10.1073/pnas.1231994100 PMID: 12754378; PubMed Central PMCID: PMC164510.

41. Siddiqi OK, Faxon DP. Very late stent thrombosis: current concepts. Curr Opin Cardiol. 2012; 27(6):634–41. Epub 2012/10/19. doi: 10.1097/HCO.0b013e3283587c7e 00001573-201211000-00012 [pii]. PMID: 23075822.

42. Farb A, Burke AP, Tang AL, Liang TY, Mannan P, Smialek J, et al. Coronary plaque erosion without rupture into a lipid core. A frequent cause of coronary thrombosis in sudden coronary death. Circulation. 1996; 93(7):1354–63. PMID: 8641024.

platelets

http://informahealthcare.com/plt
ISSN: 0953-7104 (print), 1369-1635 (electronic)

Platelets, Early Online: 1–9
 DOI: 10.3109/09537104.2015.1021319

informa
healthcare

METHODS PAPER

Measurement of soluble C-type lectin-like receptor 2 in human plasma†

Fuminori Kazama[1]*, Junya Nakamura[2]*, Makoto Osada[1]*, Osamu Inoue[3], Mitsuru Oosawa[2], Shogo Tamura[1,4], Nagaharu Tsukiji[1], Kaoru Aida[5], Akio Kawaguchi[5], Soichi Takizawa[5], Masahiro Kaneshige[5], Shoichiro Tanaka[5], Katsue Suzuki-inoue[1], & Yukio Ozaki[1]

[1]*Department of Clinical and Laboratory Medicine, Faculty of Medicine, University of Yamanashi, Chuo, Yamanashi, Japan,* [2]*Department of Antibody Group, Narita R&D Department, Research and Development Division, LSI Medicine Corporation, Takomachi, Katori-gun, Chiba, Japan,* [3]*Faculty of Medicine, Infection Control Office, University of Yamanashi Hospital, University of Yamanashi, Chuo, Yamanashi, Japan,* [4]*Japan Society for the Promotion of Science, Tokyo, Japan, and* [5]*Department of Internal Medicine III, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Chuo, Yamanashi, Japan*

Abstract

Detection of platelet activation *in vivo* is useful to identify patients at risk of thrombotic diseases. Platelet factor 4 (PF4) and β-thromboglobulin (β-TG) are used for this purpose; however, they are easily released upon the minimal platelet activation that occurs during sampling. Soluble forms of several platelet membrane proteins are released upon platelet activation; however, the soluble form of C-type lectin-like receptor 2 (sCLEC-2) has not yet been fully investigated. Western blotting with an anti-CLEC-2 antibody showed that sCLEC-2 was released from washed human platelets stimulated with collagen mimetics. To detect sCLEC-2 in plasma, we established a sandwich enzyme-linked immunosorbent assay (ELISA) using F(ab')2 anti-CLEC-2 monoclonal antibodies. Although plasma mixed with citrate, adenosine, theophylline and adenosine (CTAD) is needed for the PF4 and β-TG assays, effects of anti-coagulants (EDTA, citrate and CTAD) on the sCLEC-2 ELISA were negligible. Moreover, while special techniques are required for blood sampling and sample preparation for PF4 and β-TG assay, the standard blood collections procedures used in daily clinical laboratory tests have shown to suffice for sCLEC-2 analysis. In this study, we found that two forms of sCLEC-2 are released after platelet activation: a shed fragment and a microparticle-bound full-length protein, both of which are detected by the sCLEC-2 ELISA. The average concentration of sCLEC-2 in the plasma of 10 healthy individuals was 97 ± 55 pg/ml, whereas that in the plasma of 25 patients with diabetes mellitus (DM) was 149 ± 260 pg/ml. A trend towards an increase in sCLEC-2 concentration in the DM patients may reflect *in vivo* platelet activation in the patients, suggesting that sCLEC-2 may have clinical significance as a biomarker of *in vivo* platelet activation.

Keywords

Biomarker, CLEC-2, platelet activation, platelets, thrombosis

History

Received 22 September 2014
Revised 17 January 2015
Accepted 16 February 2015
Published online 10 April 2015

Introduction

Platelet activation by various stimuli plays a key role in pathological thrombosis, as observed in myocardial infarction and stroke; thus, detection of platelet activation *in vivo* could be useful for identifying patients at risk of thrombosis and for monitoring the effects of anti-platelet therapy [1]. For this purpose, plasma levels of the releasates from activated platelets have been measured, including that of β-thromboglobulin (β-TG) and platelet factor 4 (PF4) [2]. These platelet-specific CXC chemokines are stored in platelet α-granules and are released in the extracellular space upon activation. Consequently, elevated levels of these factors in plasma have been used as biomarkers of pre-thrombotic and thrombotic states [2]. Nonetheless, several shortcomings preclude their clinical use, such as the ease at which they are released in response to minimal platelet activation that can be induced by tourniquet application [3]. Moreover, as PF4 is a heparin-binding protein, the plasma concentration of PF4 is altered in the presence of heparin, which is frequently given to patients with atherothrombosis.

In addition, a number of platelet membrane proteins are also shed after platelet activation. The shed forms of these proteins in plasma – such as glycocalicin and glycoprotein VI (GPVI) – are being explored as markers for *in vivo* platelet activation. Although, some authors have reported favourable results, whereas others found limited usefulness of these proteins as biomarkers [4].

CLEC-2 was identified as a platelet receptor for the platelet-activating snake venom, rhodocytin [5]. CLEC-2 belongs to the C-type lectin superfamily and elicits activation signals via Src family kinases and Syk [5]. We also identified podoplanin as an endogenous ligand for CLEC-2 [6,7]. Podoplanin is expressed on the surface of tumour or lymphatic endothelial cells, and facilitates haematogenous tumour metastasis or blood/lymphatic vessel separation during the developmental stages by stimulating

†Some of these results were reported in an abstract for the XXIV Congress of the International Society on Thrombosis and Haemostasis in July 2013.

*These authors contributed equally to this work.

Correspondence: Katsue Suzuki-Inoue, Department of Clinical and Laboratory Medicine, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan. Tel: +81 55 273 9884. Fax: +81 55 273 6924. E-mail: katsuei@yamanashi.ac.jp

platelet activation through CLEC-2 binding [8–10]. CLEC-2 is most highly expressed in platelets and megakaryocytes but is also expressed in neutrophils [11] and macrophages [12] at lower levels in mice. In contrast, expression of CLEC-2 is limited to platelets expressing CLEC-2 in human peripheral blood cells. This highly-specific expression of CLEC-2 in human platelets prompted us to examine whether CLEC-2 is shed after platelet activation and its potential use as a biomarker for platelet activation *in vivo*.

In this study, we found that soluble CLEC-2 (sCLEC-2) is released after platelet activation in both a fragmented shed form and a microparticle (MP)-bound full-length form. Moreover, we developed and validated an enzyme-linked immunosorbent assay (ELISA) system to quantify these two forms of sCLEC-2 in plasma. Significantly, we also assess the possibility for sCLEC-2 to be used as a marker for *in vivo* platelet activation.

Methods

This study was approved by the Ethics Committee of the University of Yamanashi and LSI Medicine Corporation. Written informed consent was provided by participants, according to the Declaration of Helsinki. This study was also approved by the Institutional Animal Care and Use Committee of LSI Medicine Corporation.

Materials

PP2 and its negative control compound, PP3, are from Merck Millipore (Darmstadt, Germany). Rhodocytin was purified as described previously [13]. The GPVI agonist poly(PHG) was a generous gift from the JNC Corp. (Yokohama, Japan) [14]. Collagen-related peptide (CRP) was synthesized by Peptide Institute Inc. (Osaka, Japan) and cross-linked with glutaraldehyde as described previously [15]. The recombinant extracellular domain of human CLEC-2 expressed as a dimeric human IgG Fc fusion protein (hCLEC-2-hFc2) was generated as described previously [6]. Monomeric recombinant human CLEC-2 extracellular domain (hCLEC-2ex) was generated by papain digestion of hCLEC-2-hFc2 using Fab Preparation Kit (Thermo Fisher Scientific Inc.; Waltham, MA) according to the manufacturers' instructions.

Platelet preparation

Venous blood from healthy un-medicated volunteers was collected into 10% sodium citrate. Washed human platelets were then obtained by centrifugation as described previously [16].

Proteolysis of CLEC-2

Washed platelets (100 µl) were pre-incubated with or without inhibitors for 10 minutes at room temperature. Then 200 µl of modified Tyrode's buffer or buffer containing platelet agonists was added to the washed platelets, and the mixture was incubated for 120 minutes at 30 °C. The final concentration of washed platelets was 1×10^9/ml. Final concentrations of inhibitors or platelet agonists were 50 µM PP3, 50 µM PP2, 1 µg/ml poly(PHG), 300 nM rhodocytin and 1 µg/ml CRP. The reaction was terminated by adding 200 µl of 10 mM EDTA. All samples were centrifuged at 20 000*g* for 1 minute and supernatants (90 µl) were collected into new tubes (1st C/F supernatant fraction). Pellets were then disrupted in 1 ml of 1 × lysis buffer by sonication and 90 µl of lysate was transferred to new tubes (1st C/F pellet fraction). The remaining 420 µl of supernatant was ultracentrifuged at 100 000*g* for 3 hours at 4 °C. After ultracentrifugation, supernatants (90 µl) were transferred into new tubes (2nd C/F supernatant fraction). The remaining pellets were sonicated in 120 µl of 1 × lysis buffer and 90 µl of lysate was transferred into new tubes (2nd C/F pellet fraction). All four fractions were dissolved, separated and analysed by western blotting with the anti-human CLEC-2 antibody, 11E6, as described previously [17].

Flow cytometry analysis

The platelet-rich plasma, 1st C/F sup/pellet fraction and 2nd C/F pellet fraction samples were stained with PE anti-human CD42b (clone: HIP1, BD Biosciences, San Jose, CA) or mouse IgG1 PE Platelet Control (BD Biosciences). Samples were diluted to 500 µl with filtered PBS and immediately subjected to analysis using a FACS Calibur (BD Biosciences) flow cytometer. To set the gates for platelet-derived microparticle (PDMP) and platelet populations, CD42b-positive events were gated on scatter diagram with SSC-H and FL2-H. This subset of events was then examined by size on scatter diagram with FSC-H and SSC-H. The CD42b-positive FSC^{low}/SSC^{low} and FSC^{high}/SSC^{high} populations were defined as PDMP and platelets, respectively, as previously reported [18]. Data were processed using BD CellQuest Pro software (BD Biosciences).

For analysis of surface expression of CLEC-2 on stimulated platelets, washed platelets were stimulated with or without 1 µg/ml CRP for 120 minutes, and the reaction was terminated by the addition of EDTA. After centrifugation, pelleted platelets were stained with control mouse IgG-Alexa Flour 488 or anti-CLEC-2 (clone 4A10, see below)-Alexa Flour 488 and analysed on a BD Accuri flow cytometer (BD Biosciences).

For P-selectin analysis, human blood was taken into CTAD-, EDTA- or citrate-containing tubes and centrifuged as described below. Whole blood prior to centrifugation and the lower fraction containing red blood cells and platelets following centrifugation were diluted 10 times with PBS. Diluted samples (10 µl) were then stained with 2.5 µl anti-CD62P (P-selectin) antibody (BD Biosciences) for 10 minutes. After addition of 500 µl PBS, the samples were analysed on a BD Accuri flow cytometer and data were processed using FCS express (De Novo Software, Los Angeles, CA).

Mouse anti-human CLEC-2 antibody

Murine mAbs 11E6, 5D11 and 4A10 were produced against the human CLEC-2 extracellular domain using conventional hybridoma method. Briefly, BALB/cAJcl mice were immunized with a recombinant dimeric human IgG Fc fusion protein containing the extracellular domain of human CLEC-2. Splenocytes were harvested 4 days after the final peritoneal antigen boost, fused with P3X-63 mouse myeloma cells, and cultured.

Measurement of sCLEC-2 using ELISA

A 96-well F8 Maxisorp plate (Nunc, Roskilde, Denmark) was coated with F(ab')2 of 11D5 (10 µg/ml) in coating buffer (0.05 M bicarbonate, pH 9.5), overnight at 4 °C. Wells were washed six times with 100 µl aliquots of 0.1 M borate-buffered saline pH 8.0 (BBS) containing 0.1% (v/v) Tween 20 (BBS-T), blocked with 1% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) in PBS for 1 hour at room temperature, and then washed with BBS-T six additional times prior to addition of samples. Experiments were performed in triplicate and each plate routinely included standards consisting of recombinant human CLEC-2 extracellular domain (hCLEC-2ex, 0–5 ng/ml, final concentration) in 0.3% BSA, 0.1% sodium octanoate and 0.14 M NaCl in 25 mM sodium phosphate buffer (pH 7.2; sample diluent buffer). Test samples of plasma were diluted 4–8 times with the sample diluent buffer. After a 1.5-hour incubation at room temperature, plates were washed six times with BBS-T and 100 µl (1 µg/ml of biotin-labelled F(ab')2 11E6 was added to each well. After a 1 hour of

incubation and three BBS-T washes, AMDEX High-Performance Conjugate (RPN4401V, GE Healthcare, Little Chalfont, UK) was added (100 µl/well; 1/6000 dilution of stock) to the plates for 1 hour, followed by another five washes. Next 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma-Aldrich) was added to each well and the mixture was incubated for 20–30 minutes at room temperature in the dark. To stop the reaction, 100 µl 2N H_2SO_4 was to each well and absorbance was measured within 30 minutes at 450 nm with excitation at 630 nm.

Precision, recovery test and limit of detection for the ELISA system

To determine the precision of the ELISA system, a series of concentrations of hCLEC-2ex were assayed using the standard protocol described earlier. The mean absorbance was calculated with a coefficient of variability (CV%). The CV% of inter-assay variability (triplicate) was measured by dividing the standard deviation of the mean by CV%. A series of concentrations of hCLEC-2ex were measured seven times using the standard protocol described earlier. The limit of detection was determined as the lowest concentration of hCLEC-2ex likely to be reliably distinguished from the blank sample (control). To perform recovery test for the ELISA assay, human plasma samples were analysed with or without the addition of a known amount of hCLEC-2ex (5 ng/ml, final concentration).

Patients

Anti-coagulated blood samples from healthy individuals and diabetes mellitus (DM) patients were obtained with informed consent approved by the University of Yamanashi ethics committee (No. 955). We recruited DM patients whose HbA1c [the Japanese Diabetes Society (JDS) assigned value] levels were more than 8.0% and were followed up by the Department of Internal Medicine III, University of Yamanashi.

Sampling and preparation of human plasma

To obtain CATD (citrate, theophylline, adenosine and dipyridamole) plasma, 4.5 ml of blood from healthy donors were collected, using a syringe with a 20-gauge needle without a tourniquet, into CTAD-containing tubes. Blood in CTAD tubes was then incubated on ice for 15–60 minutes within 2 minutes of collection prior to centrifugation at 2500*g* for 20 minutes. A 0.5-ml aliquot of plasma was removed and transferred to plastic tubes. Concentrations of PF4, β-TG and soluble P-selectin were measured using PF4 TMB ELISA kit (Roche Diagnostics, Basal, Switzerland), β-TG TMB ELISA kit (Roche Diagnostics), CD62P human ELISA kit (Gen-Probe, San Diego, CA), and Human GPVI ELISA Kit (USCN Life Science Inc., Wuhan, China) according to the manufacturers' instructions.

To obtain serum or citrate-/EDTA-treated plasma, blood (2 ml) from healthy donors was collected into vacutainers with or without anticoagulants (citrate or EDTA) using a 21-gauge needle and a tourniquet, followed by immediate centrifugation at 1600*g* for 10 minutes. Where indicated, 2 ml of whole blood in EDTA-coated vacutainers was stimulated with 1 µg/ml of poly(PHG) and incubated at room temperature for the indicated period of time. After centrifugation, 250 µl of plasma was transferred to plastic tubes.

Statistics

Statistical significance was determined with the Games–Howell test using SPSS software (IBM Software, Armonk, NY) or Mann–Whitney test using Graph Pad Prism (GraphPad Software, La Jolla, CA). In each case, p values of <0.05 were interpreted as a minimal indication of statistical significance.

Results

Soluble CLEC-2 is released after platelet activation

We initially tested whether soluble human CLEC-2 was released after platelet activation induced by the CLEC-2 agonist, rhodocytin. sCLEC-2 was defined as CLEC-2 molecules in the supernatants of plasma or buffer after the removal of platelets by centrifugation. Washed platelets stimulated with rhodocytin were centrifuged, and the supernatants (Figure 1Aa, 1st C/F supernatant) and pellets (Figure 1Ab, 1st C/F pellet) were analysed using western blotting with an anti-CLEC-2 monoclonal antibody, 11E6. In unstimulated whole-cell lysates (Figure 1Ab, WCL), 11E6 recognizes double bands that correspond to full-length CLEC-2 protein at 32/40 kDa, depending on the state of glycosylation [5]. In contrast, supernatants from rhodocytin-treated platelets, three bands at 32, 40, and 25 kDa were observed (Figure 1Aa, 1st C/F supernatant, lane 3). However, after ultracentrifugation of the 1st C/F supernatant, only the 25 kDa band was detectable in the supernatants (Figure 1Ad, 2nd C/F supernatant, lane 3), whereas the 32/40 kDa bands were detected in the pellet fraction (Figure 1Ac, 2nd C/F pellet, lane 3).

It is expected that 1st C/F pellet contains intact platelets and 1st C/F supernatant contains molecules released from activated platelets, such as MPs, granule contents and shed membrane proteins. Therefore, the 2nd C/F pellet should contain MPs, while the 2nd C/F supernatant contains shed membrane proteins and released granule contents. To confirm this hypothesis, we analysed these fractions by flow cytometry. We determined the region of intact platelets (R5) using platelet-rich plasma and the region of platelet MPs (R4) (Figure 1B–D) as described previously [18]. We confirmed that intact platelets (platelet-rich plasma) fractioned into R5 and expressed GPIb (Figure 1B). The number of particles that fractioned into the R4 MP region, but not R5, was greatly increased in the 1st C/F supernatant (Figure 1C) and the 2nd C/F pellet after stimulation (Figure 1D). Moreover, the particles localizing in R4 expressed GPIb (Figure 1D, right panel). These findings clearly show that 1st C/F supernatant and 2nd C/F pellet contain platelet MPs, but not remaining intact platelets.

Based on these findings, it is suggested that the 32/40 kDa bands fractioned into 1st C/F supernatant and 2nd C/F pellet are intact CLEC-2 molecules on the surface of platelet MPs and that the 25 kDa soluble band is a CLEC-2 fragment, most likely cleaved by a proteinase. Soluble CLEC-2 occurs in two forms; intact CLEC-2 molecules (32/40 kDa) on platelet MPs and shed CLEC-2 (25 kDa). Hereafter, we named the 25 kDa sCLEC-2 fragment as shed CLEC-2 and 32/40 kDa sCLEC-2 as MP CLEC-2. The release of these forms of sCLEC-2 was also observed after platelet activation by other classical agonists, including collagen, CRP (collagen mimetic; data not shown), poly(PHG) (another collagen mimetic; see Figure 5), and thrombin (data not shown).

The CLEC-2 band in 1st C/F pellet marginally decreased after stimulation (Figure 1Ab), but this was not always apparent. Flow cytometric analysis was able to detect low, but distinct, decrease in the surface expression of CLEC-2 after stimulation (Figure 1E), which is consistent with the release of sCLEC-2 from the surface of activated platelets.

Establishment of ELISA for evaluation of sCLEC-2 in human plasma

To investigate whether sCLEC-2 is detected in human plasma, we developed a sandwich ELISA system. We first generated

anti-human CLEC-2 mouse monoclonal antibodies that recognize the extracellular domain of CLEC-2. Among the 20 hybridomas obtained, we selected two clones with highest activity, immobilized F(ab′)2 of 11D5, and used biotin-labelled F(ab′)2 of 11E6 for detection. To obtain a standard curve, serial dilutions of soluble hCLEC-2ex (0.1–10 ng/ml) in dilution buffer were used (Figure 2A). The dose–response curve (Figure 2A) illustrates the specificity of the assay as reflected by a dose-dependent increase

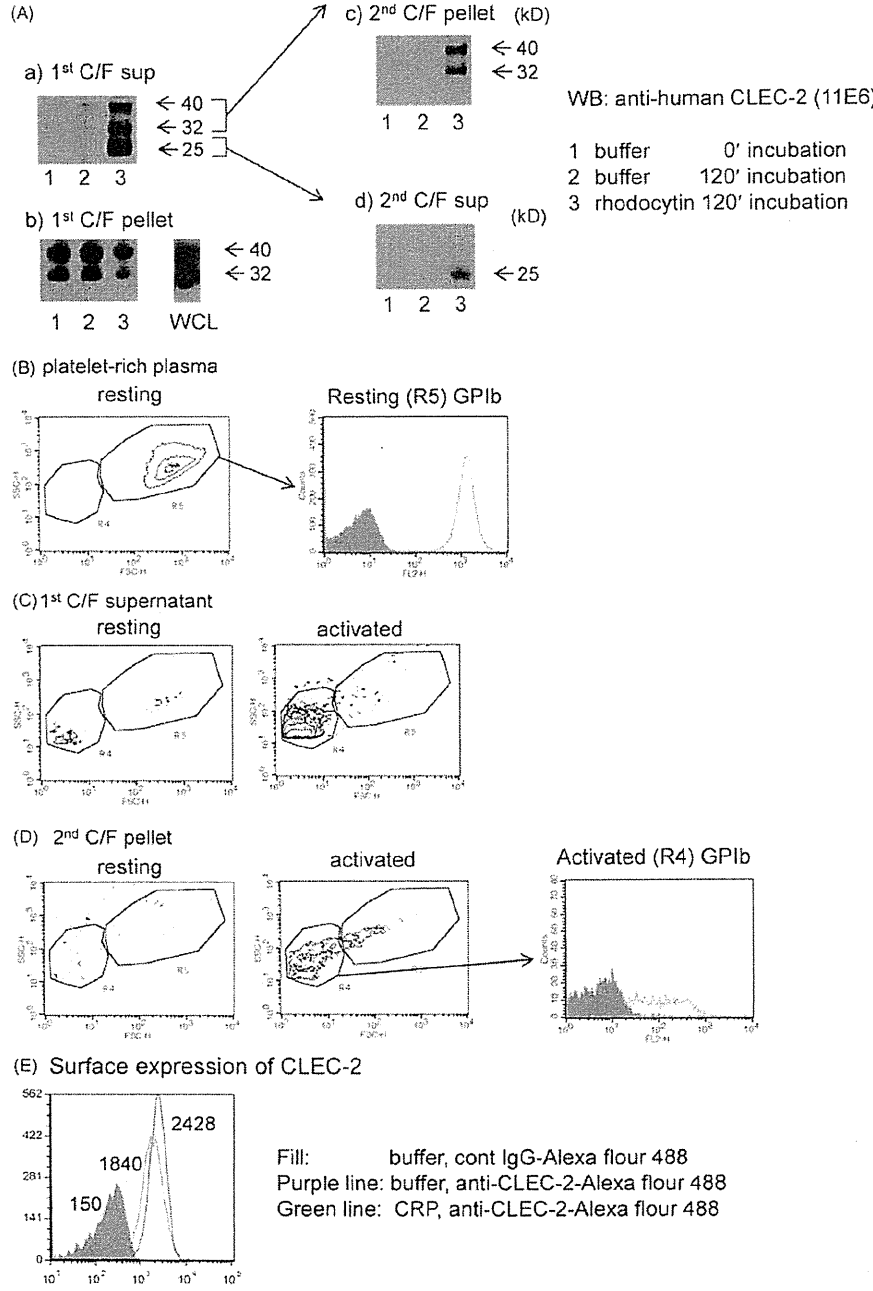
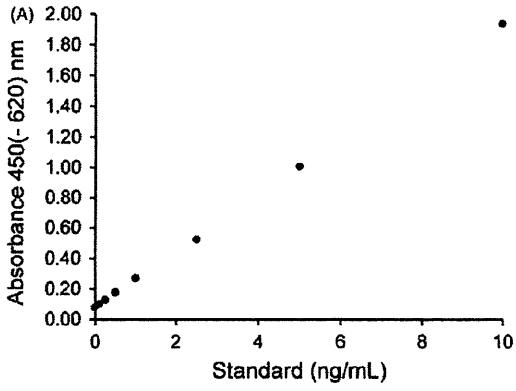


Figure 1. Release of soluble CLEC-2 after platelet activation. (A) Washed platelets were stimulated with or without 300 nM rhodocytin for an indicated period of time, and the reaction was terminated by the addition of EDTA. After centrifugation (C/F), sCLEC-2 in the supernatants (a, 1st C/F sup) and CLEC-2 in the pellet (b, 1st C/F pellet) were visualized by western blotting with an anti-CLEC-2 antibody (11E6). The supernatants were further ultracentrifuged (2nd C/F). Shed CLEC-2 in the resultant supernatants (d, 2nd C/F sup) and full-length CLEC-2 on MPs in the resulting pellet (c, 2nd C/F pellet) were visualized by western blotting with 11E6. WCL denotes whole-cell lysate of human platelets. (B–D) Flow cytometry analysis of platelet-rich plasma (B). Washed platelets were stimulated with or without 1 μg/ml CRP for 120 min, and the reaction was terminated by the addition of EDTA. 1st C/F supernatant (C), 2nd C/F pellet (D) and 1st C/F pellet (E). R4 and R5 denote gates for platelet MPs and intact platelets, respectively. Images of GPIb staining were inseted in (B) and (D). Fill (control mouse IgG-PE), line (anti-GPIb-PE). (E) Median fluorescent intensities were inserted in the image. The data are representative of at least two independent experiments.



(B)

Standard (ng/ml)	Mean (n = 3) (450 nm)	CV (%)
0	0.081	5.24
0.1	0.100	2.13
0.25	0.128	2.77
0.5	0.177	0.00
1	0.271	1.83
2.5	0.523	0.54
5	1.008	2.39
10	1.938	3.69

Figure 2. The dose–response curve and precision for the ELISA assay. (A) Measurement of hCLEC-2ex in a sample diluent using ELISA. The average absorbance ± STD was determined in duplicate. The standard curve was generated using linear regression (Prism). (B) ELISA precision as reflected by inter-assay variability. Eight concentrations of hCLEC-2ex diluted in a sample diluent were assayed in triplicate using the standard ELISA. The mean absorbance and CV% were calculated.

in absorbance and a linear response of approximately 10 ng/ml. The CV% of the inter-assay variability (triplicate) were <6% of the mean (Figure 2B), which is <10%, indicating acceptable precision of the assay.

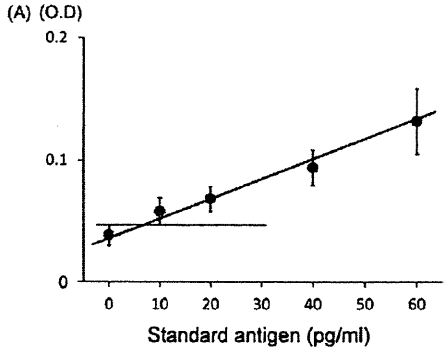
The sCLEC-2 ELISA system was evaluated for sensitivity and specificity when plasma samples were spiked with 5.0 ng/ml of hCLEC-2ex. The limit of detection was 10 pg/ml (Figure 3A). The recovery was between 98% and 121% (Figure 3B).

Effects of blood collection and processing on plasma sCLEC-2 levels

The PF4 and β-TG released during platelet activation are well-characterized markers of *in vivo* platelet activation [19,20]. Nevertheless, PF4 and β-TG are easily released in response to blood sampling and processing procedures [3], and the special conditions required to measure PF4 and β-TG make these assays hard to perform in clinical settings. We next evaluated effects of blood collection and processing procedures on the level of plasma sCLEC-2. Although no significant difference in plasma sCLEC-2 levels was found between CATD-, citrate- and EDTA-containing tubes (Figure 4A), the sCLEC-2 serum concentration was significantly increased compared to that found in plasma, suggesting that platelet activation by thrombin during clotting results in release of sCLEC-2 (Figure 4A). In comparison, concentrations of PF4 and β-TG were significantly increased in EDTA- or citrate-treated plasma, as well as in serum (Figure 4B and C).

We next examined the platelet activation state in these samples by the surface expression of P-selectin, a membrane protein contained within α-granules and expressed on the platelet surface upon activation. Small aliquots of whole blood anti-coagulated with CTAD, EDTA or citrate were removed before and after centrifugation and immunostained with anti-P-selectin antibody to the whole blood. P-selectin expression was then assessed by flow cytometry in the gated platelet population. As shown in Figure 4D, P-selectin expression by platelets from EDTA- or citrate-treated blood, but not that in CTAD-treated blood, was

Figure 3. Limit of detection and recovery test for the ELISA system. (A) Limit of detection for the ELISA assay was investigated. Concentrations of hCLEC-2ex (0, 0.025, 0.05 and 0.1 ng/ml) were measured seven times using ELISA. The limit of detection was determined as the lowest concentration of hCLEC-2ex likely to be reliably distinguished from a blank sample, and at which concentrations detection is feasible. (B) Recovery test for the ELISA system was assessed. EDTA-treated plasma was analysed with the addition of a known amount of hCLEC-2ex (5 ng/ml. final concentration).



(B)

	Spiked (ng/ml)	Measured (ng/ml)	CV (%)	Expected (ng/ml)	Recovery (%)
Plasma 1	0	0.48	16.7	-	-
	5.0	6.09	1.2	5.48	111.2
Plasma 2	0	0.40	3.8	-	-
	5.0	6.54	0.8	5.4	121.1
Plasma 3	0	0.59	1.3	-	-
	5.0	5.50	13.3	5.59	98.4

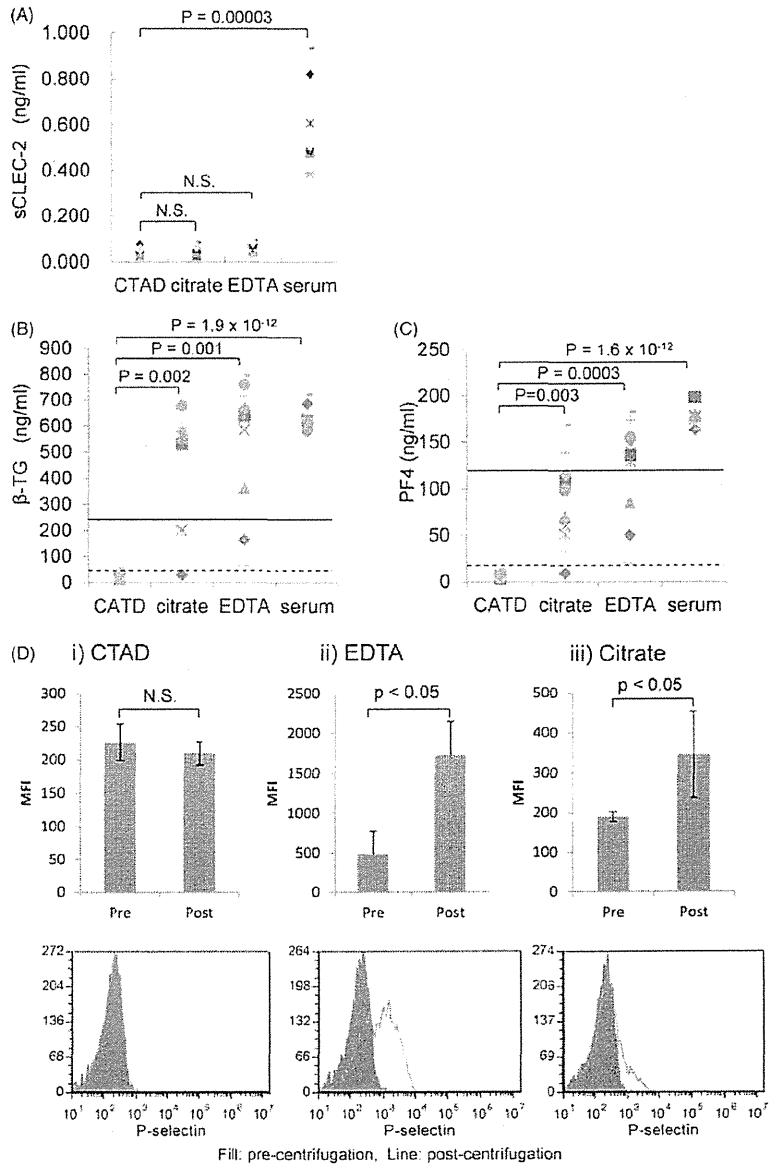


Figure 4. Concentrations of sCLEC-2, β-TG and PF4 in CATD-, citrate-, or EDTA-treated plasma or in serum. Concentrations of sCLEC-2 (A), β-TG (B) and PF4 (C) in CATD-, citrate-, or EDTA-treated plasma or in serum from 10 healthy donors were measured using ELISA. Statistical significance was evaluated by means of Games–Howell test. N.S. denotes "not significant". Dotted lines in the graphs B and C indicated upper limits of reference range and lines in the graphs B and C indicated upper limits of measurement range. (D) P-selectin expression on the surface of platelets in anti-coagulated blood with CTAD (i, upper panel), EDTA (ii, upper panel) and citrate (iii, upper panel) before and after centrifugation was expressed as mean fluorescent intensity ± SE ($n = 3–5$). Representative flow cytometric data were shown in the lower panels of (i)–(iii).

significantly increased after centrifugation. These findings support that a substantial activation of platelets in centrifuged blood treated EDTA or citrate anti-coagulants, which caused release of contents of α granules. These findings suggest that the sampling procedure complying with the manufacturers' instructions is essential for an accurate analysis of PF4 and β-TG concentration and that the sampling procedure for sCLEC-2 measurement is facile. We decided to use blood collected into EDTA-containing vacutainers using a 21-gauge needle and a tourniquet, because these are the most frequently used procedures in daily clinical laboratory tests.

Detection of two sCLEC-2 forms by ELISA

To determine whether our ELISA system detects increases in sCLEC-2 upon platelet activation, we stimulated anti-coagulated whole blood with or without the platelet agonist poly(PHG) and measured the resulting sCLEC-2 concentration in plasma. In this experiment, we used poly(PHG) to simulate platelets because rhodocytin is in finite supply. As shown in Figure 5A, the sCLEC-2 concentration increased in response to poly(PHG) stimulation in a time-dependent manner. In comparison, the sCLEC-2 concentrations in unstimulated whole blood were unchanged at 2 and 4 hours (Figure 5B). These findings suggest that the sCLEC-2 ELISA system can detect increases in sCLEC-2 after platelet activation *in vitro*.

Soluble CLEC-2 consists of shed CLEC-2 and MP CLEC-2 (Figure 1); therefore, we examined whether the ELISA system could detect both forms. For this purpose, washed platelets were stimulated with or without poly(PHG) and pelleted by centrifugation. Supernatants were then analysed using western blotting with 11E6 (1st C/F supernatant, Figure 5Ci). The 1st C/F

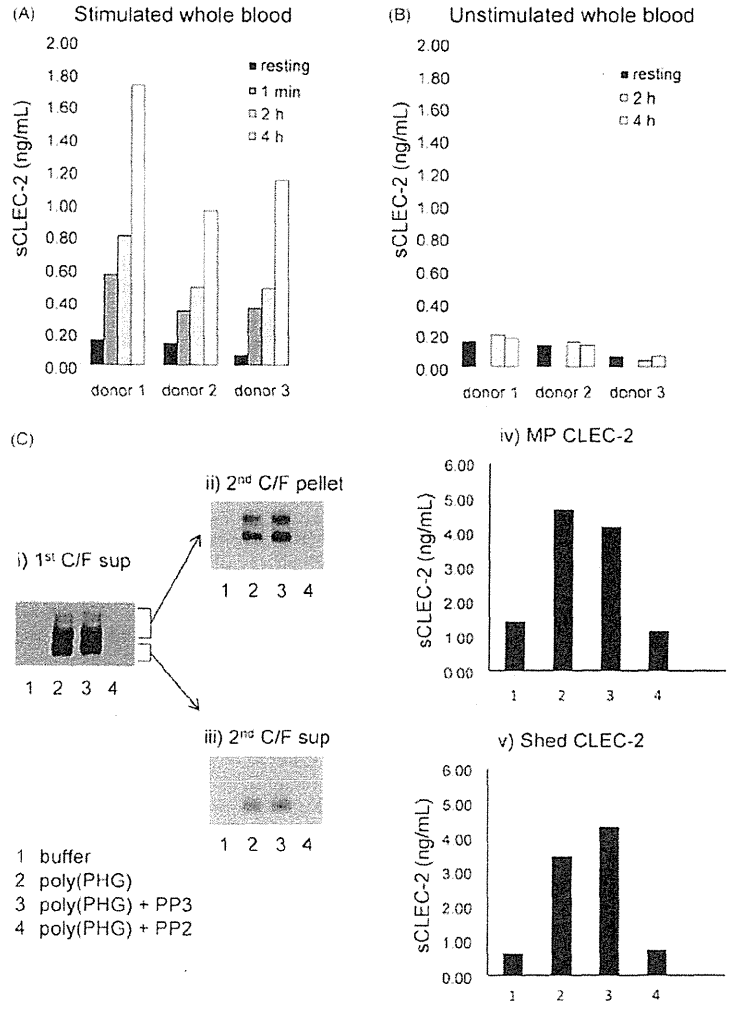


Figure 5. sCLEC-2 concentrations were increased depending on platelet activation. (A and B) EDTA-treated blood from three healthy donors was stimulated with (A) or without (B) 1 μg/ml of poly(PHG) for the indicated periods of time. After centrifugation at 1600*g* for 10 min, 250 μl of plasma was removed and sCLEC-2 was quantified by means of ELISA. (C) Washed platelets (final concentration 10^9/ml) were preincubated with or without 50 μM PP2 or 50 μM PP3 for 10 min and were then stimulated with or without 1 μg/ml of poly(PHG) for indicated periods of time; the reaction was terminated by the addition of EDTA. After centrifugation (C/F), sCLEC-2 in the supernatants (1st C/F sup, i) were visualized by western blotting with an anti-CLEC-2 antibody (11E6). The supernatants were then ultracentrifuged. Shed CLEC-2 in the resultant supernatants (2nd C/F, iii) and the full-length CLEC-2 protein on MPs in the resultant pellet (2nd C/F, iv) were visualized by western blotting with 11E6. Concentrations of sCLEC-2 in aliquots of the sample (ii) and (iii) were measured using the sCLEC-2 ELISA and shown as (iv) and (v), respectively.

supernatant was further ultracentrifuged, and the resulting supernatants (2nd C/F supernatant) and pellets (2nd C/F pellet) were analysed by the same method, and confirmed that the supernatants and pellets contain shed CLEC-2 (Figure 5Ciii) and MP CLEC-2 (Figure 5Cii), respectively. Notably, our ELISA system showed fourfold to fivefold increase in both the shed CLEC-2 (Figure 5Cv, 1–2) and MP CLEC-2 (Figure 5Civ, lanes 1, 2) fractions after stimulation. These results demonstrate that both forms of sCLEC-2 are detected by our ELISA system. It is well known that GPVI-mediated platelet activation is completely inhibited by a specific Src kinase inhibitor, PP2 [21]. PP2, but not its negative control PP3, completely inhibited the generation of both forms of sCLEC-2 (Figure 5Ci–iii). Concomitantly with the western blot analysis, sCLEC-2 concentrations of shed CLEC-2 and MP CLEC-2 decreased to basal level in the presence of PP2 (Figure 5Civ, v, lanes 3, 4), further confirming that sCLEC-2 release is dependent upon platelet activation.

Trend of increased sCLEC-2 concentrations in DM patients

Concentrations of sCLEC-2 and sGPVI in the plasma of healthy volunteers and DM patients were measured. The average concentration of sCLEC-2 in plasma from healthy individuals was 97 ± 55 pg/ml, whereas an average of 149 ± 260 pg/ml was found in patients with DM. Although this difference was not statistically significant, the observed trend towards increased sCLEC-2 concentration in DM patients may reflect *in vivo* platelet activation (Figure 6A). This data further support the potential use for sCLEC-2 as a biomarker of *in vivo* platelet activation. It has been reported that soluble GPVI (sGPVI) is released upon platelet activation *in vivo* in recent years [22]. Plasma sGPVI in the DM patients (13.9 ± 13.5 ng/ml) was significantly higher than plasma sGPVI in healthy individuals (4.98 ± 2.72 ng/ml) (Figure 6B), suggesting that there may be difference in occasion when levels of sCLEC-2 and sGPVI are increased *in vivo*.

Discussion

In this study, we demonstrated that two soluble forms of CLEC-2 are released upon platelet activation – a 25-kDa shed CLEC-2 and MP CLEC-2. We also provided the details of an ELISA-based system that was developed to detect and measure both forms of sCLEC-2 in human plasma. Furthermore, we found that patients with DM trend towards an increase in plasma sCLEC-2 levels compared to healthy subjects.

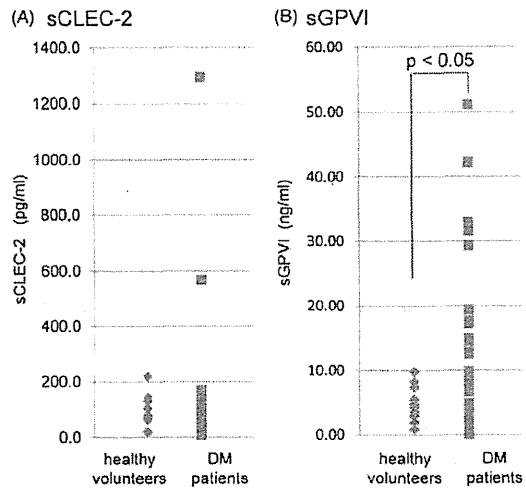


Figure 6. Concentrations of sCLEC-2 in EDTA-treated plasma in healthy donors and DM patients. The sCLEC-2 (A) and sGPVI (B) concentrations in EDTA-treated plasma obtained from 10 healthy donors and 25 DM patients were measured using sCLEC-2 ELISA or sGPVI ELISA, respectively. Statistical significance was evaluated by means of Mann–Whitney test.

The 25-kDa and 32/40-kDa bands were detected by western blotting in the supernatants of stimulated washed platelets after removing intact platelets by centrifugation (1st C/F supernatant) (Figure 1A). A 25-kDa band was observed in the supernatant after ultracentrifugation of the 1st C/F supernatant (2nd C/F supernatant) (Figure 1A). There was no 25-kDa band observed in the whole-cell lysate of resting platelets (Figure 1Ab, WCL), suggesting that the 25-kDa band is cleaved by a proteinase and not a splice variant released from granules. The 32/40-kDa band, corresponding to the molecular weight of intact CLEC-2, was observed in the 2nd C/F pellet of stimulated, but not resting platelets. In addition, flow cytometry analysis showed that the particles in the 2nd C/F are fractioned into a platelet MP area, R4 (Figure 1B). These findings suggest that majority of the 32/40-kDa band are intact CLEC-2 molecules on the surface of platelet MPs, rather than those on the surface of intact platelets.

During the submission process of this article, Gitz et al. reported that CLEC-2 does not undergo proteolytic cleavage, but are released in an intact form on the surface of MPs upon platelet activation [23], which contradicts our findings. The discrepancy may be due to relatively low sensitivity of their method. Our methods directly detect shed CLEC-2 in the supernatants of activated platelets by western blotting, whereas Gitz et al. estimated shed CLEC-2 by the observable decrease of remaining CLEC-2 on the surface of activated platelets by western blotting and flow cytometry. We believe that this qualitative method is less sensitive when compared to the direct detection of shed CLEC-2.

To measure concentration of sCLEC-2 in human plasma, we developed ELISA system using mouse anti-human CLEC-2 monoclonal antibodies that was sufficient to detect increases in sCLEC-2 concentrations in whole blood treated with anti-coagulants in a stimulation- and time-dependent manner (Figure 5A). We are unable to directly examine whether this sCLEC-2 ELISA system detects platelet activation *in vivo* in human; however, two lines of indirect evidence support this possibility: (1) sCLEC-2 concentrations tended to increase in DM patients whose platelets are reportedly activated *in vivo* [24], and (2) sCLEC-2 concentrations somewhat correlated with soluble P-selectin concentration, which is an established biomarker of *in vivo* platelet activation ($r = 0.459$, data not shown). Furthermore, sCLEC-2 concentrations measured by ELISA appear to be in agreement with the data obtained by western blot (compare Figure 5Cii vs. iv; iii vs. v), suggesting that this assay is able to detect both shed CLEC-2 and MP CLEC-2, although, it is not known if either form of CLEC-2 are increased in human plasma upon platelet activation *in vivo*.

It has been reported that the elimination half-life of erythropoietin (30.4 kDa) in men after intravenous injection is about 8.5 hours [25]. Therefore, we assume that half-life of shed CLEC-2 (25 kDa) is similar. Increased terminal sialylation is known to extend the serum half-life of many glycoproteins [26]. As CLEC-2 is abundantly glycosylated [5], its half-life may be longer than expected from its molecular weight. Moreover, the molecular weight of MP CLEC-2 is much larger than shed CLEC-2 because MPs are covered with plasma membrane where a number of platelet membrane proteins exist, thereby its half-life *in vivo* is presumed to be much longer. Because our ELISA system for sCLEC-2 is able to measure both shed CLEC-2 and MP CLEC-2, we assume that sCLEC-2 (shed CLEC-2 and MP CLEC-2) is still useful as markers.

PF4/β-TG assay may tend to be avoided in clinical settings because of these special procedures required for the assay. This study demonstrates that plasma sampling for sCLEC-2 analysis can be performed using standard blood collection protocols. In this regard, the facile procedure to obtain plasma for the sCLEC-2 ELISA is favourable as a daily laboratory test.

We found that sCLEC-2 concentrations were increased in DM patients compared to that of normal subjects, but the data were not statistically significant. Possibly because we recruited DM patients based on HbA1c (more than 8.0%, JDS) rather than diabetic complications that consist mainly of vascular disorders, which can lead to platelet activation. Indeed, two patients who showed very high sCLEC-2 concentrations (1293.2, 566.9 pg/ml) have diabetic complications (retinopathy, neuropathy, and nephropathy). The trend observed in the DM patients is apparently due to two outliers, whereas all other patients were similar to healthy controls.

We found that plasma sGPVI, another marker of platelet activation, was significantly increased in the DM patients compared with healthy donors (Figure 6B). Moreover, we observed that plasma sCLEC-2 concentrations significantly increased in acute coronary syndrome patients than in the stable angina pectoris patients, whereas plasma sGPVI concentration was not significantly different between the two groups (manuscript submitted). Based on these findings, we suggest that GPVI undergoes shedding upon milder platelet activation, whereas sCLEC-2 is released upon relatively strong platelet activation, thereby leading to no significant differences in sCLEC-2 levels between healthy donors and relatively mild DM patients. We propose that the analysis of both sGPVI and sCLEC-2 may have some clinical benefits in the detection of both relatively weak *in vivo* platelet activation and strong *in vivo* platelet activation (close to cardiovascular events). We are currently investigating which diseases show increased levels of plasma sCLEC-2. A facile procedure to obtain plasma for the sCLEC-2 ELISA would help accumulation of the cases.

Acknowledgements

We thank Hideyuki Tanaka, Chiaki Komatsu, Hisaichiro Nakazawa, and Toshiaki Shirai for technical assistance.

Declaration of interest

We received support in part by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology (20569628, M.O.) and the Japan Society for the Promotion of Science

(JSPS) through the Funding Program for Next Generation World-leading Researchers (NEXT Program) (LS052, K.S.-I.).

Junya Nakamura and Mitsuru Oosawa are employees of Mitsubishi Chemical Medicine Corporation. Yukio Ozaki, Katsue Suzuki-Inoue, Junya Nakamura, and Mitsuru Oosawa have pending patent applications from the last two years related to this report (Japanese patent application No. 2012-215900).

References

1. Wagner DD, Burger PC. Platelets in inflammation and thrombosis. Arterioscler Thromb Vasc Biol 2003;23:2131–2137.
2. Kaplan KL, Owen J. Plasma levels of beta-thromboglobulin and platelet factor 4 as indices of platelet activation *in vivo*. Blood 1981; 57:199–202.
3. Ohkawa R, Hirowatari Y, Nakamura K, Ohkubo S, Ikeda H, Okada M, Tozuka M, Nakahara K, Yatomi Y. Platelet release of beta-thromboglobulin and platelet factor 4 and serotonin in plasma samples. Clin Biochem 2005;38:1023–1026.
4. Gurney D, Lip GY, Blann AD. A reliable plasma marker of platelet activation: Does it exist? Am J Hematol 2002;70:139–144.
5. Suzuki-Inoue K, Fuller GL, Garcia A, Eble JA, Pohlmann S, Inoue O, Gartner TK, Hughan SC, Pearce AC, Laing GD, et al. A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. Blood 2006;107:542–549.
6. Suzuki-Inoue K, Kato Y, Inoue O, Kaneko MK, Mishima K, Yatomi Y, Yamazaki Y, Narimatsu H, Ozaki Y. Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells. J Biol Chem 2007;282:25993–26001.
7. Christou CM, Pearce AC, Watson AA, Mistry AR, Pollitt AY, Fenton-May AE, Johnson LA, Jackson DG, Watson SP, O'Callaghan CA. Renal cells activate the platelet receptor CLEC-2 through podoplanin. Biochem J 2008;411:133–140.
8. Kato Y, Kaneko MK, Kunita A, Ito H, Kameyama A, Ogasawara S, Matsuura N, Hasegawa Y, Suzuki-Inoue K, Inoue O, et al. Molecular analysis of the pathophysiological binding of the platelet aggregation-inducing factor podoplanin to the C-type lectin-like receptor CLEC-2. Cancer Sci 2008;99:54–61.
9. Bertozzi CC, Schmaier AA, Mericko P, Hess PR, Zou Z, Chen M, Chen CY, Xu B, Lu MM, Zhou D, et al. Platelets regulate lymphatic vascular development through CLEC-2-SLP-76 signaling. Blood 2010;116:661–670.
10. Suzuki-Inoue K, Inoue O, Ding G, Nishimura S, Hokamura K, Eto K, Kashiwagi H, Tomiyama Y, Yatomi Y, Umemura K, et al. Essential *in vivo* roles of the C-type lectin receptor CLEC-2: Embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. J Biol Chem 2010;285:24494–24507.
11. Kerrigan AM, Dennehy KM, Mourao-Sa D, Faro-Trindade I, Willment JA, Taylor PR, Eble JA, Reis e Sousa C, Brown GD. CLEC-2 is a phagocytic activation receptor expressed on murine peripheral blood neutrophils. J Immunol 2009;182:4150–4157.
12. Chang CH, Chung CH, Hsu CC, Huang TY, Huang TF. A novel mechanism of cytokine release in phagocytes induced by aggretin, a snake venom C-type lectin protein, through CLEC-2 ligation. J Thromb Haemost 2010;8:2563–2570.
13. Shin Y, Morita T. Rhodocytin, a functional novel platelet agonist belonging to the heterodimeric C-type lectin family, induces platelet aggregation independently of glycoprotein Ib. Biochem Biophys Res Commun 1998;245:741–745.
14. Inoue O, Suzuki-Inoue K, Shinoda D, Umeda Y, Uchino M, Takasaki S, Ozaki Y. Novel synthetic collagen fibers, poly(PHG), stimulate platelet aggregation through glycoprotein VI. FEBS Lett 2009;583:81–87.
15. Morton LF, Hargreaves PG, Farndale RW, Young RD, Barnes MJ. Integrin alpha 2 beta 1-independent activation of platelets by simple collagen-like peptides: Collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for alpha 2 beta 1-independent platelet reactivity. Biochem J 1995;306(Pt 2):337–344.
16. Suzuki-Inoue K, Inoue O, Frampton J, Watson SP. Murine GPVI stimulates weak integrin activation in PLCgamma2−/− platelets: Involvement of PLCgamma1 and PI3-kinase. Blood 2003;102: 1367–1373.
17. Osada M, Inoue O, Ding G, Shirai T, Ichise H, Hirayama K, Takano K, Yatomi Y, Hirashima M, Fujii H, et al. Platelet activation receptor CLEC-2 regulates blood/lymphatic vessel separation by inhibiting proliferation, migration, and tube formation of lymphatic endothelial cells. J Biol Chem 2012;287:22241–22252.
18. Osumi K, Ozeki Y, Saito S, Nagamura Y, Ito H, Kimura Y, Ogura H, Nomura S. Development and assessment of enzyme immunoassay for platelet-derived microparticles. Thromb Haemost 2001;85: 326–330.
19. Cella G, Scattolo N, Girolami A, Sasahara AA. Are platelet factor 4 and beta-thromboglobulin markers of cardiovascular disorders? Ric Clin Lab 1984;14:9–18.
20. Walz DA. Platelet-released proteins as molecular markers for the activation process. Semin Thromb Hemost 1984;10:270–279.
21. Nieswandt B, Watson SP. Platelet-collagen interaction: Is GPVI the central receptor? Blood 2003;102:449–461.
22. Al-Tamimi M, Mu FT, Moroi M, Gardiner EE, Berndt MC, Andrews RK. Measuring soluble platelet glycoprotein VI in human plasma by ELISA. Platelets 2009;20:143–149.
23. Gitz E, Pollitt AY, Gitz-Francois JJ, Alshehri O, Mori J, Montague S, Nash GB, Douglas MR, Gardiner EE, Andrews RK, et al. CLEC-2 expression is maintained on activated platelets and on platelet microparticles. Blood 2014;124:2262–2270.
24. Fukuda K, Ozaki Y, Satoh K, Kume S, Tawata M, Onaya T, Sakurada K, Seto M, Sasaki Y. Phosphorylation of myosin light chain in resting platelets from NIDDM patients is enhanced: Correlation with spontaneous aggregation. Diabetes 1997;46: 488–493.
25. Macdougall IC, Eckardt KU. Novel strategies for stimulating erythropoiesis and potential new treatments for anaemia. Lancet 2006;368:947–953.
26. Raju TS, Lang SE. Diversity in structure and functions of antibody sialylation in the Fc. Curr Opin Biotechnol 2014;30C: 147–152.

doi:10.1111/ncn3.156

CASE REPORT

Fulminant bilateral cerebral infarction caused by paradoxical embolism in a patient with protein S Ala525Val substitution

Yoji Yoshikawa,[1,2] Jiro Kitayama,[1,2] Hiromi Ishikawa,[1,2] Asako Nakamura,[1,2] Fumina Taniguchi,[3] Eriko Morishita,[3,4] Tetsuro Ago,[2] Hiroshi Nakane[1,2] and Takanari Kitazono[2]

[1]Cerebrovascular and Neurology Center, National Hospital Organization Fukuoka Higashi Medical Center, Kyushu University, [2]Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, [3]Department of Clinical Laboratory Science, Kanazawa University Graduate School of Medical Science, Kanazawa University Hospital, and [4]Department of Hematology and Oncology, Kanazawa University Hospital, Kanazawa, Japan

Key words
cerebral infarction, gene mutation, paradoxical embolism, protein S deficiency, thrombophilia.

Accepted for publication 17 November 2014.

Correspondence
Yoji Yoshikawa
Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
Email: yoshi8@intmed2.med.kyushu-u.ac.jp

Abstract

We report a 42-year-old woman who developed sudden fulminant cerebral infarction in the bilateral middle cerebral artery territories, causing status epilepticus and a decreased level of consciousness. Investigation showed thrombus in the right soleus vein and a patent foramen ovale, but no obvious embolic source, such as atrial fibrillation or a carotid or cerebral artery atherosclerotic lesion. Blood coagulation tests showed decreased levels of free protein S (25%) and total protein S (52%), and decreased protein S activity (15%). The patient was diagnosed with cerebral infarction as a result of paradoxical embolism, and type I protein S deficiency. DNA sequencing identified a novel point mutation in the *PROS1* gene, leading to the amino acid substitution, Ala525Val. It should be noted that this protein S mutation can cause thrombophilia and cerebral infarction.

Introduction

Protein C (PC) and protein S (PS) are vitamin K-dependent anticoagulant proteins. Thrombin is the final protease in the blood coagulation cascade, and interacts with antithrombin to form the thrombin antithrombin complex, which converts PC to activated PC (APC). APC inactivates the activated coagulation factors, Va and VIIIa.[1] This important negative feedback mechanism prevents excessive blood coagulation. PS is an indispensable cofactor for APC activity. Deficiency of PC or PS causes a hypercoagulable state leading to thromboembolism including deep venous thrombosis, pulmonary embolism and cerebral venous sinus thrombosis, and is usually an inherited autosomal dominant disorder.[2] Patients with a patent foramen ovale (PFO) have interatrial communication with the potential for right-to-left shunting, and are at risk of paradoxical embolism from deep venous thrombosis causing brain infarction. As PFO occurs in approximately 25% of otherwise healthy individuals, paradoxical embolism should be considered as a cause of brain infarction in patients with thrombophilia.[3] Inherited PC and PS deficiencies are more common in Japan than in Western countries. Many point mutations resulting in PC or PS deficiency have been reported to date.[1] We report a patient with fulminant cerebral infarction in the bilateral middle cerebral artery (MCA) territories, caused by paradoxical embolism. The patient had PS deficiency with a previously unreported gene mutation.

Case Report

A 42-year-old woman was transferred to the National Hospital Organization Fukuoka Higashi Medical Center, Fukuoka, Japan, in an ambulance with status epilepticus and a decreased level of consciousness. She had neither a past history of epilepsy and pregnancy loss nor a family history of thrombotic diseases, and was not taking any medications. She had slept in the passenger seat while her family traveled by car overnight, and when she got out of the car and started to walk, she fell down and immediately lost consciousness. On admission, her blood pressure was 126/64 mmHg, heart rate was 101 b.p.m. regular, temperature was 36.4°C and respiratory rate was 24 breaths/min. She had a decreased level of consciousness, conjugate deviation of her eyes to the right and tonic clonic convulsions of all extremities. Laboratory data were almost normal except for a high D-dimer level of 14.7 μg/mL. Atrial blood gas analysis under oxygen administration on administration was normal, except for hyperoxygenation (pH 7.364, pO_2 520.5 mmHg, pCO_2 46 mmHg and HCO_3^- 25.6 mEq/L). Chest X-ray and contrast computed tomography showed no

signs of pulmonary embolism. Cerebrospinal fluid examination was within the normal limits, with a cell count of 3/μL, protein concentration of 29.4 mg/dL and glucose concentration of 61 mg/dL. Diffusion-weighted magnetic resonance imaging showed increased signal intensity in the bilateral MCA territories, indicating bilateral acute brain infarction (Fig. 1a). Magnetic resonance angiography showed stenosis of the left MCA (Fig. 1b), and magnetic resonance venography showed no abnormality (Fig. 1c). Transesophageal echocardiography showed passage of microbubbles from a peripheral vein through the right atrium to the left atrium, indicating a PFO (Fig. 1d). Contrast-enhanced computed tomography showed thrombus in the right soleus vein (Fig. 1e). Blood coagulation and fibrinolysis tests showed decreased levels of free PS (25%) and total PS (52%), and decreased PS activity (15%). DNA sequencing of the *PROS1* gene identified a previously unreported mutation of GCC to GTC, resulting in the amino acid substitution of Ala525 by Val (Ala525Val; Fig. 1f). The patient was diagnosed with cerebral infarction as a result of paradoxical embolism, and type I PS deficiency. Follow-up magnetic resonance angiography showed recanalization of the left MCA (Fig. 1g), supporting an embolic mechanism. Despite intensive medical therapy, the patient still had total aphasia and quadriplegia when she was transferred to another hospital after 59 days.

Discussion

We report a young woman with sudden fulminant bilateral cerebral infarction resulting in status epilepticus, a decreased level of consciousness, total aphasia and quadriplegia. Extensive investigation did not find an obvious embolic source, but found venous thrombosis in the right lower extremity and PFO. She also had PS deficiency with a previously unreported point mutation. Considering all these findings, she was diagnosed with cerebral infarction caused by paradoxical embolism.

As aforementioned, both PS deficiency and PC deficiency are relatively common causes of thrombophilia in Japan. PS

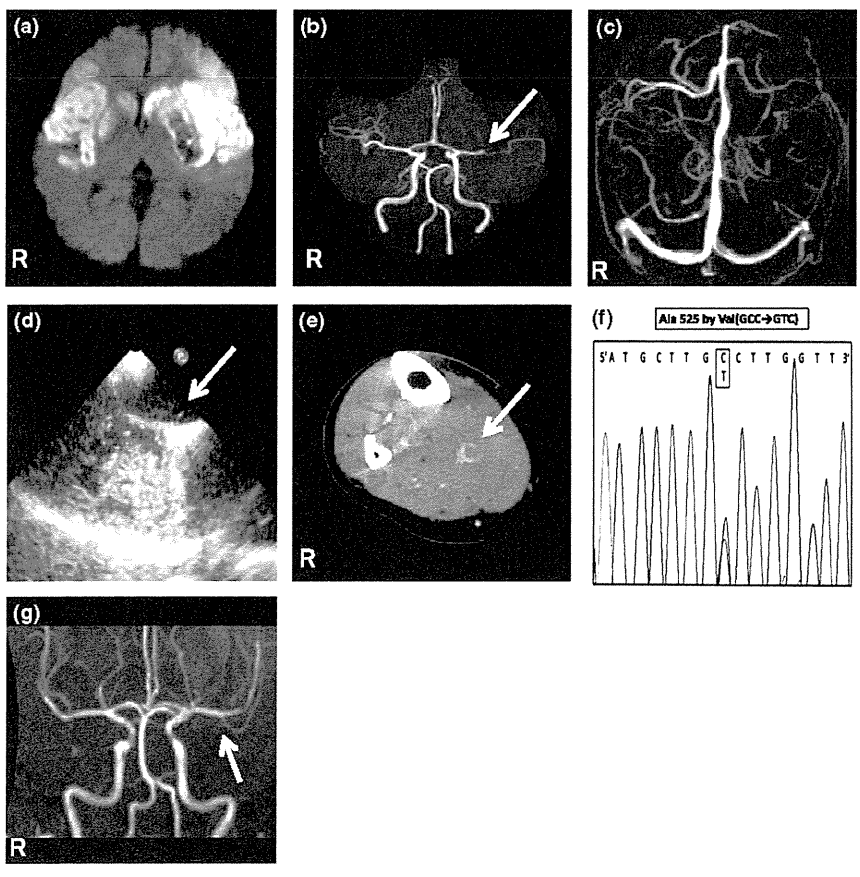


Figure 1 (a) Diffusion-weighted magnetic resonance imaging showed high-intensity lesions in the bilateral middle cerebral artery territories. (b) Magnetic resonance angiography showed stenosis of the left middle cerebral artery (arrow). (c) Magnetic resonance venography showed no abnormality. (d) Transesophageal echocardiography showed a patent foramen ovale (arrow). (e) Contrast-enhanced computed tomography showed thrombus in the right soleus vein (arrow). (f) Direct DNA sequencing identified the mutation GCC→GTC in the *PROS1* gene, resulting in the amino acid substitution of Ala-525 by Val. (g) Follow-up magnetic resonance angiography showed recanalization of the left middle cerebral artery.

circulates free in the plasma, or in a complex with complement protein C4b-binding protein. Only the free form acts as a cofactor to activate APC.[4] PS deficiency is classified into three types. Type I has decreased plasma levels of both free PS and total PS, and decreased PS activity; type II has normal free PS and total PS levels, and decreased PS activity; and type III has a normal total PS level, decreased free PS level and decreased PS activity.[5] Our patient was classified as type I deficiency, because she had decreased levels of free PS (25%) and total PS (52%), and decreased PS activity (15%).

The structure of PS includes an N-terminal Gla domain, thrombin-sensitive region, growth factor region and C-terminal sex-hormone binding globulin (SHBG)-like domain. Ala-525 is a well-conserved amino acid among different species that is located in the SHBG-like domain.[1] Substitution of Ala-525 by Pro, resulting from the point mutation of GCC to CCC, was previously reported to cause type I PS deficiency, similar to the present case.[6] As Ala-525 constitutes a β-strand, substitution of Ala with a more bulky amino acid, such as Val or Pro, might disrupt the structural stability of the protein, thereby decreasing the levels of both free PS and total PS.[1]

In conclusion, we experienced a case of paradoxical cerebral embolism in a patient with a previously unreported mutation of *PROS1*, resulting in the amino acid substitution, Ala525Val, in the SHBG-like domain. It should be noted that this mutation of PS can cause thrombophilia, including severe cerebral infarction in patients with PFO.

References

1 Villoutreix BO, Dahlback B, Borgel D, Gandrille S, Muller YA. Three-dimensional model of the SHBG-like region of anticoagulant protein S: new structure-function insights. *Proteins* 2001; **43**: 203–16.

2 Hayashi T, Nishioka J, Shigekiyo T, Saito S, Suzuki K. Protein S Tokushima: abnormal molecule with a substitution of Glu for Lys-155 in the second epidermal growth factor-like domain of protein S. *Blood* 1994; **83**: 683–90.

3 Roth C, Alli O. Patent foramen ovale and cryptogenic stroke: many unanswered questions. *Cleve. Clin. J. Med.* 2014; **81**: 417–24.

4 Dahlback B. Inhibition of protein Ca cofactor function of human and bovine protein S by C4b-binding protein. *J. Biol. Chem.* 1986; **261**: 12022–7.

5 Nomura T, Suehisa E, Kawasaki T, Okada A. Frequency of protein S deficiency in general Japanese population. *Thromb. Res.* 2000; **100**: 367–71.

6 Borgel D, Jude B, Aiach M, Gandrille S. First case of sporadic protein S deficiency due to a novel candidate mutation, Ala 484–>Pro, in the protein S active gene (PROS1). *Thromb. Haemost.* 1996; **75**: 883–6.

Original Article

Fluvastatin Upregulates the Expression of Tissue Factor Pathway Inhibitor in Human Umbilical Vein Endothelial Cells

Akiko Sekiya[1], Eriko Morishita[1], Keiko Maruyama[2], Hiroki Torishima[1] and Shigeki Ohtake[1]

[1]Department of Clinical Laboratory Science, Kanazawa University Graduate School of Medical Science, Ishikawa, Japan
[2]National Cerebral and Cardiovascular Center, Department of Molecular Pathogenesis, Osaka, Japan

Aim: 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are cholesterol-lowering drugs with a variety of pleiotropic effects including antithrombotic properties. Tissue factor pathway inhibitor (TFPI), which is produced predominantly in endothelial cells and platelets, inhibits the initiating phase of clot formation. We investigated the effect of fluvastatin on TFPI expression in cultured endothelial cells.
Methods: Human umbilical vein endothelial cells (HUVECs) were treated with fluvastatin (0–10 μM). The expression of TFPI mRNA and antigen were detected by RT-PCR and western blotting, respectively. The effects of mevalonate intermediates, small GTP-binding inhibitors, and signal transduction inhibitors were also evaluated to identify which pathway was involved. A luciferase reporter assay was performed to evaluate the effect of fluvastatin on TFPI transcription. The stability of TFPI mRNA was estimated by quantitating its levels after actinomycin D treatment.
Results: Fluvastatin increased TFPI mRNA expression and antigen in HUVECs. Fluvastatin-induced TFPI expression was reversed by co-treatment with mevalonate or geranylgeranylpyrophosphate (GGPP). NSC23766 and Y-27632 had no effect on TFPI expression. SB203580, GF109203, and LY294002 reduced fluvastatin-induced TFPI upregulation. Moreover, fluvastatin did not significantly affect TFPI promoter activity. TFPI mRNA degradation in the presence of actinomycin D was delayed by fluvastatin treatment.
Conclusions: Fluvastatin increases endothelial TFPI expression through inhibition of mevalonate-, GGPP-, and Cdc42-dependent signaling pathways, and activation of the p38 MAPK, PI3K, and PKC pathways. This study revealed unknown mechanisms of the anticoagulant effect of statins and gave a new insight to its therapeutic potential for the prevention of thrombotic diseases.

See editorial vol. 22: 654-655

J Atheroscler Thromb, 2015; 22: 660-668.

Key words: 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, Fluvastatin, Tissue factor pathway inhibitor, Human umbilical vein endothelial cells

Introduction

Activation of the extrinsic coagulation pathway triggers arterial thrombotic events, such as acute coronary syndrome, ischemic stroke, and critical limb ischemia. Thrombin formation is further accelerated at the site of atherosclerotic plaques, where a chronic inflammatory response occurs and collagen and tissue factor (TF) are exposed to circulating blood after the rupture of an atherosclerotic plaque. Tissue factor pathway inhibitor (TFPI), as the major inhibitor of the extrinsic coagulation pathway, regulates arterial thrombosis by binding TF-factor VIIa (TF-FVIIa) and factor Xa (FXa). Recent studies have shown that TFPI attenuates the development of atherosclerosis by

Address for correspondence: Eriko Morishita, Department of Clinical Laboratory Science, Kanazawa University Graduate School, 5-11-80 Kodatsuno, Kanazawa, Ishikawa 920-0942, Japan
E-Mail: eriko86@staff.kanazawa-u.ac.jp
Received: September 30, 2014
Accepted for publication: November 13, 2014

inhibiting endothelial activation and proliferation, and by diminishing monocyte recruitment[1]. Alternative splicing at the 3′ end of the TFPI gene results in the production of two major isoforms of TFPI, TFPIα and TFPIβ, which have different domain structures and tissue distributions. TFPIα circulates in the plasma or bound to the endothelium via its interaction with endothelial glycosaminoglycan. Another pool of TFPIα is released from endothelial cells and platelets after stimulation (i.e., thrombin). Thus, TFPIα acts as a very early phase inhibitor at the locus of clot formation. TFPIβ binds directly to the endothelium surface and contributes to constant anticoagulation on the vascular endothelium[2, 3].

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have been suggested to reduce the risk of cardiovascular events and death. Besides their predominant effects on cholesterol reduction, statins have shown a number of beneficial effects including the improvement of endothelial function, suppression of inflammation, and plaque stabilization[4]. These "pleiotropic" effects are independent of its effect on cholesterol reduction and mainly through inhibition of mevalonic acid synthesis, leading to the decreased synthesis of isoprenoids. Isoprenoids, farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), mediate the posttranslational modification of small GTP-binding proteins of the Ras/Rho family[4]. Recent studies have shown the anticoagulant properties of statins[5]. The downregulation of TF and plasminogen activator inhibitor type 1 and the upregulation of thrombomodulin through inhibition of small GTP-binding proteins by statins were demonstrated *in vitro*[6-8]. Meanwhile, it remains unclear if statins affect TFPI expression. Although some reports, including ours, described the association of statin administration and plasma TFPI concentration[9-11], plasma TFPI does not reflect the amount of TFPI pooled in platelets and on the endothelium. Thus, we considered it beneficial to clarify if statins affect endogenous TFPI production *in vitro*.

In this report, we examined the effects of fluvastatin, a lipid-soluble statin, on TFPI expression in human endothelial cells and investigated its underlying mechanisms.

Methods

Materials

Human umbilical vein endothelial cells (HUVECs) and conditioned medium (EGM-2 Bullet Kit) were purchased from Lonza (Walkersville, MD, USA). EGM-2 was added with all of the attached supplements and 2% fetal bovine serum (FBS). Fluvastatin, mevalonate, FPP, and GGPP were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). FPP and GGPP were dissolved in methanol/10 mmol/L NH_4OH (vol/vol, 7/3). Y-27632 and NSC23766 were purchased from Calbiochem (San Diego, CA, USA). SB203580, U0126, SP600125, LY294002, and GF109203 were purchased from Alexis Biochemicals, Inc. (San Diego, CA, USA) and dissolved in dimethyl sulfoxide (DMSO). Actinomycin D was purchased from Sigma Aldrich Co. and dissolved in DMSO. An anti-TFPI polyclonal antibody was purchased from Haematologic Technologies, Inc. (Essex Junction, VT, USA). Polyclonal antibodies against p38 mitogen-activated protein kinases (MAPK), phospho-p38 MAPK, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Cell Culture

HUVECs (3–8 passages) were grown to confluence in EGM-2 at 37℃ in a humidified atmosphere of 5% CO_2. A human umbilical cell line (EA.hy926) was grown in Dulbecco's modified Eagle's medium containing 10% FBS at 37℃ in a humidified atmosphere of 5% CO_2.

Quantification of mRNA

Total RNA was extracted from cultured cells by using a NucleoSpin kit (NIPPON Genetics, Inc., Tokyo, Japan). The cDNA was synthesized by reverse transcription with a PrimeScript RT-PCR Kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. The cDNA was subjected to the following PCR conditions to amplify TFPI mRNA: 30 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 30 s. The following primers were used: TFPI forward 5′-TGGATGCCTGGGCAATATGA-3′ and reverse 5′-TATTCCAGCATTGAGCTGGGTTC-3′; and GAPDH forward 5′-GCACCGTCAAGGCTGAGAAC-3′ and reverse 5′-ATGGTGGTGAAGACGCCAGT-3′. PCR products were subjected to electrophoresis in a 3% agarose gel and the intensity of the bands was measured using a Typhoon9200 imager (GE Healthcare, Buckinghamshire, UK). The band intensity of the TFPI PCR products was normalized to that of GAPDH in the same samples.

Western Blot

HUVECs were lysed in a buffer comprising 50 mM Tris-HCl, pH 7.5, 1% bovine serum albumin, 2 mM EDTA, 100 U/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 200 mmol/L phenylmethanesulfonyl fluoride. HUVEC lysates were electrophoresed in a 10% SDS/polyacrylamide gel and transferred to a nitrocellulose membrane (Hypond™-P; GE Healthcare). Immunoblotting was performed using primary antibodies against TFPI, followed by secondary antibodies conjugated with horseradish peroxidase. The protein bands were visualized with Immobilon Western HRP Detection Substrate (Merck Millipore, Darmstadt, Germany).

Plasmid Construction for the Luciferase Reporter Assay

According to the TFPI promoter sequence reported by Petit *et al.*, a 1,524 bp (-1,246 and +278) DNA fragment of the 5′-flanking region of the TFPI gene was PCR-amplified using human genomic DNA as a template and the following specific primers containing restriction sites[12]: 5′-GGCTGCTAGCTTTGATTGTG-3′ (containing an *Nhe*I restriction site) and 5′-GCCAGGTACTCACAAGTAAGATCT-3′ (containing a *Bgl*II restriction site). This fragment was digested at the restriction sites and cloned between the unique corresponding sites of the pGL3 basic vector (Promega, Madison, WI, USA).

DNA Transfection and Luciferase Assays

EA.hy926 cells (2.0×10^5 cells/well) were allowed to grow on 12-well plates until they were approximately 80% confluent. The cells were then transiently co-transfected with 0.5 μg TFPI promoter constructs and 0.1 μg internal control vector pRL-TK (Promega) by using the Lipofectin reagent (Invitrogen, Carlsbad, CA, USA). After incubation for 6 h, the medium was replaced by fresh medium and the cells were allowed to grow for another 20 h prior to fluvastatin treatment. Luciferase activity in the cell lysates was determined using the dual-luciferase reporter assay system (Promega) and a luminometer (Atto, Tokyo, Japan) after treatment of the cells with fluvastatin (0–10 μM) for 24 or 48 h.

MTT Assay

The cells were seeded into the wells of 96-well microplates at a density of 5,000 cells/well. After incubation in the designated condition, the medium was replaced with 100 μL fresh culture medium. Then, 10 μL MTT stock solution (5 mg/mL in sterile phosphate-buffered saline) were added to each well. After 3 h, the unreacted dye was removed, and the formazan crystals were dissolved in 150 μL DMSO. After gentle agitation for 5 min, absorbance was read at 570 nm using a Microplate Reader (Tecan, Kanagawa, Japan). Cells incubated in medium without supplementation were considered as controls, and the viability of the control cells was set to 100%.

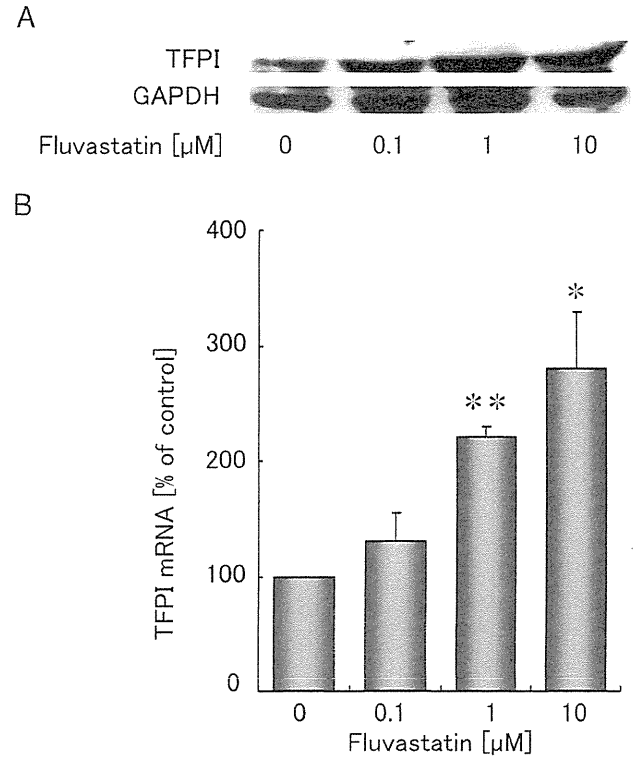


Fig. 1. Fluvastatin upregulates the TFPI antigen and mRNA expression in HUVECs.

HUVECs were incubated with various concentrations (0–10 μM) of fluvastatin for 24 h. mRNA and protein levels were determined by RT-PCR (B) and western blotting (A), respectively. The data represent the mean ± SD of 3 separate experiments. $^{*}p<0.05$ vs. control; $^{**}p<0.01$ vs. control.

Statistical Analysis

Each experiment was performed in triplicate and the results are expressed as the mean ± standard deviation (SD). Student's *t* test and one-way analysis of variance were used for statistical analyses. Microsoft Excel 2007 was applied for all analyses. A value of $p<0.05$ was considered statistically significant.