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Soluble sortilin is released by activated platelets and its circulating levels are associated with cardiovascular risk factors

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ABSTRACT

Objective: Sortilin is involved multilaterally in the development of atherosclerosis. Here, we examine the release of soluble sortilin (sSortilin) from platelets and assess the association between circulating levels of sSoritlin and atherothrombosis such as coronary artery disease (CAD).
Methods and results: sSortilin levels measured in healthy subjects were higher in serum than in plasma (38.4 ± 8.7 vs. 15.8 ± 2.9 ng/mL; $p < 0.0001$). Platelets were shown to contain both membrane-bound sortilin and its soluble form lacking the cytoplasmic tail. Stimulation of platelet-rich plasma with collagen induced sSortilin release concomitantly with platelet aggregation, and the release was suppressed by aspirin. In clinical evaluation, plasma sSortilin was detected at significantly higher levels in cardiovascular risk patients with hypertension, dyslipidemia, and/or diabetes without CAD (non-CAD, 18.7 ± 3.3 ng/mL) than in patients with CAD under aspirin therapy (17.1 ± 3.6 ng/mL; $p < 0.01$) or in healthy controls (16.8 ± 2.9 ng/mL; $p < 0.01$). In these patients, plasma sSortilin levels were significantly correlated with platelet counts ($r_s = 0.33$; $p = 0.0085$) and showed significant positive associations with cardiovascular risk factors: low-density lipoprotein cholesterol ($r_s = 0.37$; $p = 0.0023$), triglycerides ($r_s = 0.28$; $p = 0.023$), and serum uric acid ($r_s = 0.30$; $p = 0.017$) in non-CAD, and γ-glutamyltransferase ($r_s = 0.43$; $p = 0.020$) and high-sensitivity C-reactive protein ($r_s = 0.33$, $p = 0.0022$) in CAD.
Conclusion: Elevated plasma sSortilin levels may be associated with *in vivo* platelet activation and could be a risk factor for atherothrombosis.

1. Introduction

Coronary artery disease (CAD), including angina pectoris and myocardial infarction, is a critical atherosclerotic disease leading to death. Atherosclerosis is a vascular inflammatory condition characterized by plaque formation on injured blood vessel walls through intimal thickening with an accumulation of cholesterol and induction of macrophage foam cells. Disruption of the atherosclerotic plaque induces adhesion and aggregation of activated platelets, which leads to the development of atherothrombosis. Activated platelets also promote the atherogenesis process via the release of inflammatory mediators [1]. Thus platelets play a critical role in the pathogenesis of atherothrombotic disease.

Sortilin is a type-I transmembrane glycoprotein belonging to the vacuolar protein sorting 10 protein (VPS10p) domain receptor family [2] and is expressed in a wide range of tissues including brain, spinal cord, heart, and skeletal muscle in humans [3]. This protein is localized predominantly in the intracellular compartment including the endoplasmic reticulum-Golgi apparatus [3], and transports a wide variety of intracellular proteins as a multiligand receptor between the trans-Golgi network and endosomal/lysosomal compartments or secretory granules [4]. This sorting receptor is also expressed restrictively at the cell surface and acts as an endocytotic receptor for various extracellular ligands in lipid metabolism [5,6] and the neuronal system [7,8] to facilitate

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recycling or lysosomal degradation of the internalized ligands. In addition, sortilin is known to be cleaved around the base of its luminal side by sheddase, and the ectodomain is released as a soluble form into the extracellular space [9,10]. This soluble type of sortilin contains ligand-binding domains and has been demonstrated to have an antagonistic effect against the original membrane type [10–12].

Sortilin, which is encoded by the cardiovascular-risk gene *SORT1* [13–15], has been shown to be involved in several atherogenic events. In lipid metabolism, sortilin in the liver controls plasma cholesterol levels by regulating the secretion of very low-density lipoprotein (VLDL) through the intracellular trafficking of apolipoprotein B100 departed from the trans-Golgi network [14,15]. Hepatic sortilin also functions as a endocytotic receptor for circulating LDL particles to deliver them to the lysosomal degradation pathway [13,16]. In addition, hepatic sortilin is reported to facilitate the secretion of proprotein convertase subtilisin/kexin type 9 (PCSK9), which is involved in the degradation of the LDL receptor [17]. Recent studies have shown that sortilin in macrophages promotes the uptake of native LDL and foam cell formation independently of the LDL receptor [18]. Moreover, sortilin in immune cells including macrophages directly affects atherogenesis independently of lipoprotein metabolism by regulating the secretion of proinflammatory cytokines such as interleukin-6 and interferon-γ [19]. Thus, sortilin has multifaceted roles in the development and progression of atherosclerosis. However, little is known about the participation of the soluble form of sortilin (sSortilin) in atherogenesis and the source of circulating sSortilin, whereas the secretion of endogenous sSortilin is reported in neuronal and tumor cells [9,10].

In the present study, we examined the release of sSortilin from platelets and evaluated plasma sSortilin levels in cardiovascular risk patients.

2. Materials and methods

2.1. Subjects

Blood samples for controls were collected at the BML Clinical Reference Laboratory (Saitama, Japan) from 189 healthy subjects (aged 22–68 years; 100 males and 89 females) who had fasted overnight. EDTA-plasma was isolated by centrifugation at 2000*g* for 20 min and stored at −80 °C until use. Serum was prepared similarly after clotting for 30–90 min at 20–25 °C. For clinical investigation, Ninety-one patients (aged 46–88 years; 76 males and 15 females) with CAD including angina pectoris and myocardial infarction were recruited from outpatients of the Cardiology Department at the Nihon University Itabashi Hospital (Tokyo, Japan). CAD was diagnosed by detection of >70% stenosis in at least one vessel by coronary angiography and all CAD patients were treated with low-dose aspirin (<100 mg/day). Sixty-four patients (aged 47–87 years; 29 males and 35 females) who had cardiovascular risk factors such as hypertension, dyslipidemia, and/or type 2 diabetes mellitus with neither symptoms nor previous diagnosis of cardiovascular disease were also recruited as non-CAD patients from outpatients of the same hospital. The non-CAD group was confined to the patients not taking aspirin. EDTA-plasma samples were collected from these patients after overnight fasting at the same hospital. The study was approved by the local ethics committee and all subjects gave informed consent.

2.2. Preparation of recombinant sortilin

cDNA encoding the human sortilin ectodomain (NCBI reference sequence: NP_002950.3, amino acids 1–755) that was fused to a 6 × His tag at the C-terminus was inserted into the pcDNA plasmid (Life Technologies, Carlsbad, CA) to yield the pcDNA/sSORT1 construct. CHO-K1 cells stably transfected with pcDNA/sSORT1 were cultured in serum-free medium CHO-S-SFM II (Life Technologies), and recombinant sortilin (rSortilin) was purified from the culture medium by metal affinity column chromatography using Talon metal affinity resin (Clontech, Mountain View, CA).

2.3. Generation of monoclonal antibodies against sSortilin

Anti-sSortilin monoclonal antibodies (mabs) were generated by gene-immunization [20]. Briefly, BALB/c mice were immunized with 30 μg of pcDNA/sSORT1 in PBS subcutaneously at the tail every 2 weeks. At last, an immunized mouse was administered with 20 μg of rSortilin intraperitoneally and three days later, the spleen was fused with SP2/O-Ag8 myeloma cells. Hybridoma cells producing anti-sSortilin mabs were screened by sandwich enzyme-linked immunosorbent assay (ELISA) with an anti-sortilin polyclonal antibody (R&D systems, Cat.AF3154, Minneapolis, MN), and three clones, A9E, C3G, and E12A, were established. The IgG was purified from mouse ascitic fluids using protein A-Sepharose FF (GE Healthcare, Little Chalfont, UK). These mabs were all of the IgG1 subclass (kappa light chain) and specifically recognized sortilin, but did not react with the any other members of the VPS10p family in western blot analysis (Supplemental Fig. 1) using recombinant proteins, SorCS-1, -2, and -3 (R&D systems, Cat. 3457, 4238, and 3326, respectively) and SorLA (VPS10p domain prepared in-house).

2.4. sSortilin ELISA

We developed an ELISA for the quantification of sSortilin. Mab A9E (0.3 μg/mL in PBS) was coated onto a microplate (Nunc Maxisorp) by incubation at 4 °C overnight. The wells were then blocked with PBS containing 1% BSA for 2 h at 25 °C. After the plate had been washed with PBS containing 0.1% Tween20 (PBST), the calibrator (rSortilin) and plasma/serum samples (1:100) diluted with PBST containing 0.3% BSA were added and incubated for 2 h at 25 °C. After plate washing, horseradish peroxidase-labeled mab E12A (0.03 μg/mL in PBST containing 0.3% BSA) was reacted for 1 h at 25 °C. After washing, 3,3′,5,5′-tetramethylbenzidine substrate solution (Dako, Glostrup, Denmark) was added and incubated for 0.5 h. The reaction was then stopped by the addition of 0.5 M sulfuric acid. The absorbance (ABS) was measured at 450 nm with a microplate reader.

2.5. Immunoprecipitation and immunoblot analysis

Antigens in samples were precipitated using anti-mouse IgG-paramagnetic beads (Life Technologies) coupled with anti-sSortilin mabs or a control mouse IgG, subjected to SDS-PAGE, and then transferred onto a PVDF membrane (Millipore, Billerica, MA). The membrane was probed with horseradish peroxidase-labeled mab A9E, or anti-sortilin C-terminal fragment (Abcam, Cat. ab16640, Cambridge, UK) followed with peroxidase-conjugated goat anti-rabbit IgG (Life Technologies, Cat. 65–6120). Signal bands on the membrane were detected using a chemiluminescence reagent.

2.6. Platelet aggregation assay

The platelet aggregation rate was determined by the change in optical density [21]. Blood samples from healthy volunteers were collected with 3.8% sodium citrate at a proportion of 1:10 (vol:vol) as an anticoagulant, centrifuged immediately at 190*g* for 10 min, and then platelet-rich plasma (PRP) was collected from the upper phase. Remaining samples were further centrifuged at 2000*g* for 10 min to collect the platelet-poor plasma. For assaying, the PRP