Molecular Biology Reports (2022) 49:4129–4134
https://doi.org/10.1007/s11033-022-07433-x

SHORT COMMUNICATION

A novel prothrombotic role of proprotein convertase subtilisin kexin 9: the generation of procoagulant extracellular vesicles by human mononuclear cells

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Received: 24 February 2022 / Accepted: 25 March 2022 / Published online: 12 April 2022

Abstract

Background Proprotein convertase subtilisin kexin 9 (PCSK9) is a serin protease synthesized mainly in the liver that binds the receptor of low-density lipoprotein and promotes its degradation in lysosomes. PCSK9 is considered a promising target for the development of new therapies for the treatment of hypercholesterolemia and related cardiovascular diseases. Extracellular vesicles represent a heterogeneous population of vesicles, ranging in size between 0.05 and 1 μm involved in numerous pathophysiological processes, including blood coagulation. We investigated whether PCSK9 stimulation induces the release of procoagulant extracellular vesicles from human mononuclear cells (PBMCs) and THP-1 cells.

Methods and results PBMCs and THP-1 cells were stimulated whit PCSK9, the generation of EV was assessed by the prothrombinase assay and by cytofluorimetric analysis. EV-associated tissue factor activity was assessed by a one-stage clotting assay. PCSK9 induced an increase in extracellular generation by PBMCs and THP-1 cells as well as an increase in extracellular vesicle-associated tissue factor. Pre-treatment with inhibitors of the toll like receptor, TLR4 (C34), and of NF-κB signaling (BAY 11-7082), downregulated PCSK9-induced extracellular vesicle generation and of extracellular- bound tissue factor. Similar effect was obtained by an anti-PCSK9 human-monoclonal antibody.

Conclusions PCSK9-mediated generation of procoagulant EV could contribute to increase the prothrombotic status in patients with cardiovascular diseases.

Keywords PCSK9 · Extracellular vesicles · Tissue factor · Procoagulant activity

Introduction

Proprotein convertase subtilisin kexin 9 (PCSK9) is a serine protease synthesized in the form of a soluble zymogen mainly in the liver which, following an auto-catalytic process in the endoplasmic reticulum, is transformed into proprotein convertase. PCSK9 is secreted into the bloodstream, where it binds the low-density lipoprotein (LDL) receptor in its extra-cellular portion and its degradation products in lysosomes, preventing its recycling on the hepatocyte membrane [1]. PCSK9 gain-of-function mutations are associated with autosomal dominant hypercholesterolemia and premature atherosclerosis [2]. PCSK9 loss-of-function mutations conversely lead to low levels of LDL-cholesterol and cardiovascular protection [3]. Pharmacological inhibition of PCSK9 with monoclonal antibodies leads to a 60% LDL-cholesterol reduction in patients already treated with maximum tolerated statin therapy [4]. High circulating levels of PCSK9 predict cardiovascular events in patients with atrial fibrillation and in those with stable coronary artery disease (CAD), including patients with well-controlled LDL-cholesterol levels [5].

Tissue factor (TF) is an integral membrane protein constitutively expressed in subendothelial tissues and exposed to the blood following injury. TF is a high affinity receptor and essential cofactor for coagulation factor (F) VII(a); upon binding to TF, FVII(a) activates both FX and FIX thus

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initiating a series of reactions that eventually lead to fibrin generation [6]. An inducible form of TF is also synthesized by blood and vascular cells upon stimulation by different agonists. Circulating monocytes are endowed with a potential for synthesizing and expressing substantial amounts of TF [6]. Quite notably, monocytes represent the effector arm of the innate immune system deeply involved in endogenous inflammatory processes [7] and an extensive crosstalk links these two systems, whereby inflammation activates coagulation and coagulation also considerably affects inflammatory activity [8]. Lipopolysaccharide (LPS, endotoxin) is one of the best characterized agonists capable of stimulating TF synthesis by monocytes. LPS is the principal glycolipid component of the outer membrane of Gram-negative bacteria and a well characterized inflammatory stimulus, signaling through the Toll-like (TL) receptor pathway [9].

Extracellular vesicles (EV) are cell membrane-derived vesicles ranging between 0.1 and 1 μm in diameter shed upon activation and/or during apoptosis by virtually all cells including monocytes [10]. The discovery that EV could harbor functional TF [11] has contributed to modify the paradigm of circulating monocytes and endothelial cells as the only sources of intravascular procoagulant material released in response to inflammatory stimuli [12]. Exposure to LPS [13] as well as other agonists, including angiotensin II [14], rosiglitazone [15], environmental particulate matter [16] and leptin [17] causes an increase in procoagulant, monocyte-derived TF-bearing EV.

We recently provided evidence that PCSK9 stimulation elicits TF expression in cells of monocytic lineage through the activation of the TLR4/NF-κB signaling pathway [18]. Based on the above data and on the observation that both PCSK9 and EV are increased in thrombotic diseases [1, 19] we hypothesized that PCSK9 induces prothrombotic, TF-bearing EV generation by monocytes, possibly via TLR4/NF-κB activation.

Materials and methods

Reagent and materials

RPMI-1640, penicillin, streptomycin, trypan blue, Histopaque®-1077, sodium citrate, LPS from *Escherichia coli* O55:B5, hrPCSK9, BAY 11–7082 (BAY), β-mercaptoethanol and Fetal bovine serum (FBS) were obtained from Sigma-Aldrich (Milan, Italy). Human relipidated full length recombinant human TF was from BioMedica Diagnostics (Windsor, Canada). Ultrapure LPS-RS, CLI-095 (CLI) were purchased from InvivoGen (San Diego, California). The human monoclonal antibody anti-PCSK9 was a generous gift from Amgen Inc. C34 was purchased from Tocris Bioscience (Bristol, UK). LAL chromogenic endpoint assay was obtained from Hycult Biotech (Uden, The Netherlands). Annexin V was purchased by Biosciences (Dublin, Ireland). Carboxyfluorescein succinimidyl ester (CFSE) was purchased by Abcam (Cambridge, UK). THP-1 cells were from the European Collection of Authenticated Cell Cultures (ECACC).

Cell cultures

Human peripheral blood mononuclear cells (PBMCs) were obtained as described [18]. Briefly, suspensions derived from single-donor buffy coats were obtained from the local blood bank and represented leftovers otherwise destined to disposal. The procedure was approved by the local ethics committee. The buffy coats were kept at room temperature and utilized within 4 h from withdrawal. PBMCs were isolated by centrifugation of fresh buffy coats on Histopaque®-1077 at 400×*g* for 30 min at 20 °C. Cells collected at the interphase were washed twice in sodium citrate 0.38% and resuspended in RPMI-1640 supplemented with 1% penicillin–streptomycin. The final PBMCs preparations typically contain 25–35% monocytes, negligible proportions of neutrophils (< 5%), 65–75% lymphocytes and some contaminating platelets. Cell viability was assessed by dimethyl thiazolyl diphenyl tetrazolium (MTT) (Sigma-Aldrich, Milan, Italy): high viability (> 85%) was confirmed for all preparation. To account for interindividual variability among donors as well as interferences from copurified lymphocyte- and contaminant platelet-derived TF, cells of the monocytic lineage, THP-1, were also used in selected experiments. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 5% β-mercaptoethanol, 2 mM L-glutamine, 50 U/mL penicillin, and 50 g/mL streptomycin and incubated in a 5% CO_2 humidified atmosphere at 37 °C. Throughout the study procedures, THP-1 cells were maintained in a logarithmic growth phase at a concentration between 3 and 5×10^5 cells/mL.

Cell cultures suspended in polypropylene tubes, PBMCs (3×10^6 cells/mL) and THP-1 (1×10^6 cells/mL), were incubated with PCSK9 (1 and 5 μg/mL; 18 and 4 h for the two cell types, respectively) prior to EV-associated TF-PCA and total EV analysis. LPS at concentrations of 0.1 and 10 μg/mL for PBMCs and THP-1 respectively was used as a positive control in all set of experiments. Antagonists were added to cell culture 30 min prior to stimulation.

To prevent LPS contamination, glassware was exposed to high temperature (200 °C for 4 h). In addition, plasticware, reagents and solutions used for in vitro cell cultures were preliminarily tested on a routine basis with a

sensitive chromogenic LAL assay. Reagents with endotoxin concentrations > 60 pg/mL were discarded.

Assessment of EV generation

EV generation was investigated by two independent methods. The Zymuphen MP-activity kit (Hyphen BioMed, Neuville-sur-Oise, France) measures the concentration of phophatidylserine (PS) based on the rate of prothrombin conversion to thrombin in a solid-phase chromogenic assay in which the availability of PS is the rate limiting step [20]. Thrombin generation is then converted to PS concentration by mean of a standard curve generated with known concentrations of PS, according to the manufacturer's instructions. PS concentration was assessed in the cell-free conditioned medium of PBMC and THP1 cells, after culture in different conditions. Under these experimental conditions, EV are considered the only source of PS. A cytofluorimetric analysis was conducted using a FACScanto™II flow cytometer (BD Biosciences, San Jose, CA, USA). EV were first discriminated by size, using commercially available calibration beads (Megamix Plus—SSC; Stago, Milan, Italy), as events conforming to a light scatter distribution within the 0.16−0.5 µm range in a SSc vs. FSc window and further identified as annexin V^+, to identify medium-large EV expressing phosphatidylserine [10], and $CFSE^+$ events after incubation with allophycocyanin (APC)-annexin V and CFSE, in a APC vs. FITC window. Annexin-V binds to PS and is therefore used as a marker for PS-exposing EV. CFSE was used as a marker of EV integrity [21]. Events acquisition was obtained at low flow rate and stopped after 180 s.

Assessment of EV-associated TF activity

TF activity was measured in EV derived from PBMCs (3×10^6 cells/mL) and THP-1 cells (1×10^6 cells/mL) by a one-stage clotting time test using a STart Max semi-automated Coagulation analyzer (Diagnostica Stago S.A.S., Milan, Italy). The conditioned media were cleared by centrifugation at 16,000×*g* for 5 min at 4° to remove dead cells and big cell fragments that might have detached, and then submitted to further centrifugation at 16,000×*g* for 45 min at 4 °C. The pellets were resuspended in 125 µL normal saline, lysed by three freeze–thaw cycles and added to an equal volume of pooled, EV-free normal human plasma. Time to clot formation upon recalcification with 25 mM $CaCl_2$ was recorded. All experiments were carried out at 37 °C. For each experimental session, calibration curves were generated using recombinant human relipidated TF (pg/mL) (BioMedica Diagnostics-Windsor-NS Canada). In our experimental conditions, clotting times of 627 ± 84 s and 19 ± 2 s (mean ± SD) were obtained with 0.001 pg/mL and 100 pg/mL TF, respectively. Experiments were run in triplicate and averaged.

Statistical analysis

Data are shown as mean ± SEM. Comparisons between two groups were performed using unpaired t-test; comparisons among more than two groups were performed using ANOVA for independent measures followed by Sidak's post-hoc analysis. All tests are two-tailed. Prism software (GraphPad, San Diego, CA, USA) was used for analysis and graph generation.

Results and discussion

First, we investigated whether PCSK9 stimulation induces EV generation by monocytic cells. As sown in Fig. 1A, incubation of PBMCs with PCSK9 (1 µg/mL) causes a significant increase in procoagulant EV generation, as assessed by an assay that measures phosphatidylserine (PS) concentration. Similar results were obtained with cells of the monocytic line, THP-1 (Fig. 1B). To confirm the specificity of the phenomenon, we used evolocumab, an inhibitory human

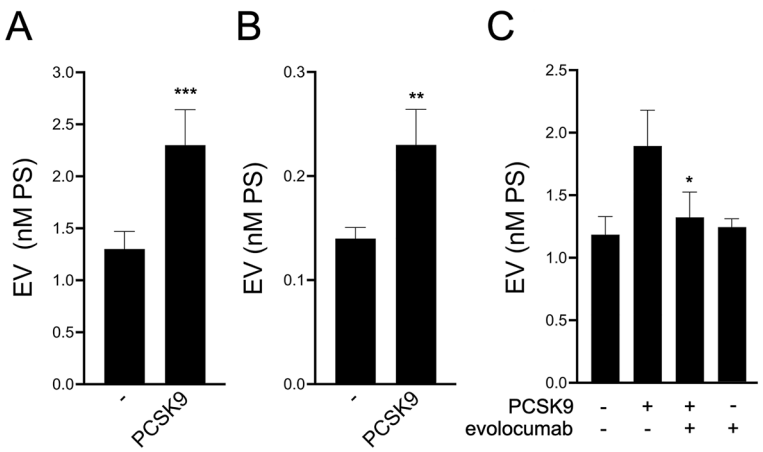

Fig. 1 EV generation, expressed as PS concentration, by PBMCs (**A**) and THP-1 (**B**) incubated in the absence and in the presence of PCSK9. ** and ***p < 0.01 and 0.001, respectively for PCSK9 stimulated cells versus unstimulated cells; n = 13 (**A**) and 11 (**B**); Student's t-test. **C**: Inhibition of PCSK9-induced EV generation by evolocumab; *p < 0.05 for EV generation in the presence PCSK9 and evolocumab vs. PCSK9 alone; n = 5; ANOVA with Sidak's post-hoc analysis.

monoclonal antibody directed against PCSK9. The antibody abolished the upregulation of EV generation by PBMC after incubation with PCSK9 (Fig. 1C).

We next investigated some of the mechanisms of PCSK9 induced upregulation of EV generation. BAY-117082 (BAY), a compound which inhibits NF-κB by preventing the phosphorylation and subsequent degradation of IκB abolished EV generation upon PCSK9 stimulation both in PBMCs and THP-1 (Fig. 2A, B, respectively). A similar inhibitory effect was induced by the TLR4 inhibitor, C34 (Fig. 2C).

The results described above were obtained analyzing the concentration of PS in the conditioned medium. As this medium is cell-free, EV are likely the only source of PS. However, to confirm the results we also analyzed EV by flow cytometry. EV were defined as events conforming to light scatter distribution within the 0.16–0.5 µm bead range in a SSc vs FSc window and further identified as annexin V positive events, and therefore expressing PS, and carboxyfluorescein diacetate succinimidyl ester (CFSE) positive events. CFSE is a membrane permeant molecule that, upon cleavage by intracellular esterases, produces a fluorescent dye. Accordingly, CFSE positivity confirms that the events detected are closed vesicles with an intact membrane, enclosing esterases rather than cell debris. With this approach, we also confirmed that PCSK9 induces the release of EV by THP-1 (235 ± 18 events $SSC^+/annexinV^+/CFSE^+$ in basal condition vs 565 ± 122 events $SSC^+/annexinV^+/CFSE^+$ after stimulation with PCSK9; mean ± SD, $p < 0.05$).

The EV generated upon PCSK9 stimulation are procoagulant, due to the exposure of PS, a negatively charged phospholipid required for the assembly of the multimolecular complexes that participate in the coagulation cascade. However, EV also might contribute to the coagulation processes through the exposure of TF [12]. Indeed, EV generated by both PBMC and THP1 cells upon stimulation with PCSK9 showed a TF-dependent procoagulant activity as assessed by a one-stage clotting assay (Fig. 3A, B, respectively). Again, direct PCSK9 inhibition with evolocumab and blocking of the of NF-κB-dependent signaling pathway by BAY abrogated PCSK9-induced generation of EV-bound TF-dependent procoagulant activity both in PBMCs (Fig. 3C) and THP-1 cells (not shown).

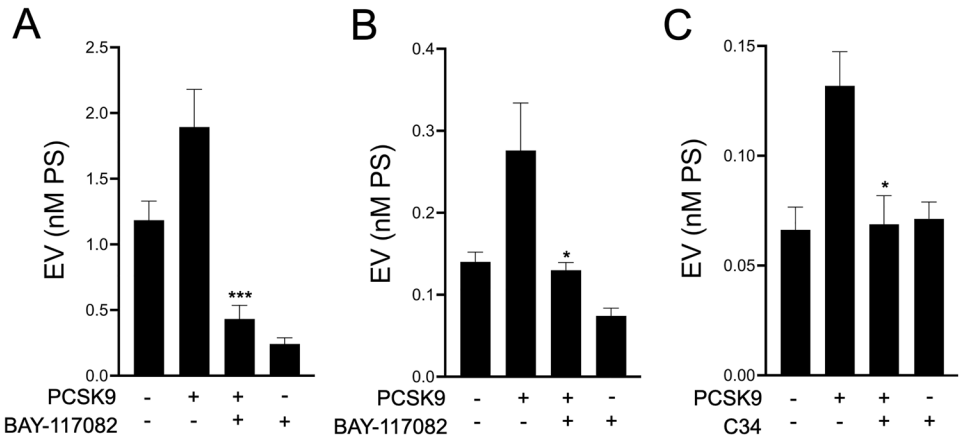

Fig. 2 Inhibition of EV generation, expressed as PS concentration, by PBMCs (**A**) and THP-1 (**B**, **C**) by BAY-117082 (**A**, **B**) and C34 (**C**). * and ***$p < 0.05$ and 0.001, respectively, for inhibited versus unhibited; n = 5 (A, B) and 4 (**D**). ANOVA with Sidak's post-hoc analysis

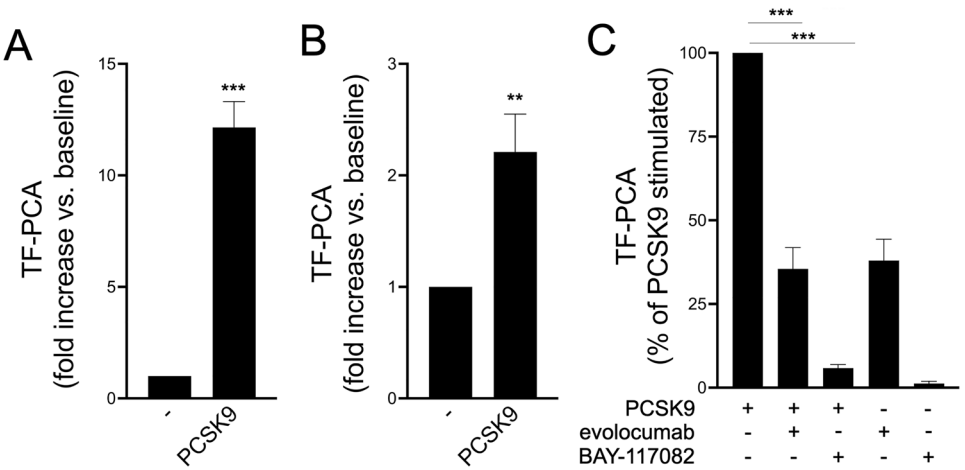

Fig. 3 Effect of PCSK9 on the induction of TF-dependent procoagulant activity by PBMC (**A**) and THP-1 (**B**). ** and ***$p < 0.01$ and 0.001, respectively, n = ; Student's t-test. **C** Inhibition of PCSK9 induced TF-dependent procoagulant activity by evolocumab and BAY117082. ***$p < 0.001$; n = 5 (**A**, **C**), and 8 (**B**); ANOVA followed by Sidaks post-hoc analysis

Our data indicate that PCSK9 induces the generation of prothrombotic, TF-bearing EV by cells of monocytic lineage. A specific monoclonal antibody to PCSK9, evolocumab, blocked the phenomenon, confirming its specificity and effectively ruling out, for example, a potential role of LPS contamination of the reagents. Inhibition with BAY-117982 and C34 suggest that PCSK9-induced stimulation of EV shedding is mediated through NF-κB and TLR4.

The role of EV in numerous pathophysiologic phenomena has gained much attention over the last several years. Specifically in the field of coronary heart diseases, increased numbers of EV have been demonstrated in the peripheral blood of patients with acute coronary syndromes; this observation has been linked, at least in part, to the procoagulant role of PS and TF on their surface [12].

PCSK9 has been extensively studied for its tole as a key regulator of cholesterol metabolism [1]. However, it has become clear over the years that the effects of this molecule in the pathogenesis of atherothrombosis are more complex. Indeed, PCSK9 promotes vascular inflammation through TLR4/NF-κB-mediated modulation of proinflammatory cytokine release [22], an activity that this molecule shares with LPS [23].

We have recently shown that PCSK9 also induces TF expression by monocytes, again via a TLR4/NF-κB pathway [18]; this observation adds to the general understanding of PCSK9 as an agonist with pleiotropic proinflammatory and prothrombotic effects. Our current data further expand on the same line.

The analysis of EV is notoriously complex and none of the available methods (that include, among others, flow cytometry, nanotrack analysis, electron microscopy, PS analysis) is considered sufficient to clearly identify and enumerate specific vesicles [10]. In the current work we have used two independent approaches. One approach measures PS concentration based on the kinetics of thrombin generation in a chromogenic assay in which PS is the rate limiting component. The approach has the advantage of being insensitive to the dimensions of the EV but does not distinguish between EV and cell fragments. In contrast, flow cytometry does recognize closed vesicles enclosing intact cytoplasm via the use of CFSE, a molecule that fluoresces only upon cleavage by intracellular esterases; flow cytometry, however, does not recognize events smaller than approximately 200 nm in size [24, 25]. The use of both approaches increases the reliability of the results.

We had originally planned to perform most experiments with PBMC in parallel with those with THP-1; however, the SARS-CoV2 pandemic has prevented us from accessing blood-derived products for research purposes.

In conclusion, we demonstrate that PCK9 might contribute to the atherothrombotic process through the induction of EV expressing both PS and TF and therefore capable of activating the coagulation cascade. Further studies will investigate more rigorously the mechanisms of PCSK9-induced EV generation.

Author contributions The study conception was performed by AC, CS, DN, VS and TN. material preparation, data collection and analysis were performed by SL, VS and TN; The first draft of the manuscript was written by AC, RP, VS and TN and. All authors read and approved the final manuscript.

Funding This research was funded by the Dipartimento di Patologia Chirurgica, Medica, Molecolare e dell'Area Critica, Università di Pisa.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Pisa University Hospital (Protocol code 558).

Consent to participate Buffy coats obtained from healthy donors used for this study were, "waste of the sample examined" not used for the dosage of biochemical analytes. Data security was maintained by the impossibility of tracing the patient's identity and, therefore, any further information.

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