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Haematologica 2020 [Epub ahead of print]

Citation: Anastasia Kyselova, Sven Zukunft, Deborah Puppe, Ilka Wittig, W. Alexander Mann, Imke Dornauf, Ingrid Fleming, and Voahanginirina Randriamboavonjy. Human platelets are a source of collagen I.
Haematologica. 2020; 105:xxx

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Human platelets are a source of collagen I

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\textbf{Text word count}: 1115

\textbf{Number of figures}: 2

\textbf{Number of table}: 1

\textbf{Number of references}: 15

\textbf{Disclosure statement}: The authors have nothing to disclose.

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Platelets are involved in primary hemostasis but also affect or modulate inflammatory and immune responses as well as angiogenesis\(^1\) and cancer metastasis\(^2\). Activated platelets can affect the function of other cells either through direct cell-cell interaction or through the release of soluble factors and microparticles. Moreover, despite being anucleate, platelets contain mRNA and are able to synthesize proteins\(^3\). However, the platelet protein landscape has not been fully explored and there is evidence of dynamic changes in the platelet proteome upon activation. To investigate potential mechanisms underlying platelet hyper-reactivity, platelets from healthy subjects and patients with polycystic ovary syndrome (PCOS) were studied. The later syndrome is a common endocrinopathy affecting women of reproductive age and is associated with abnormal platelet responses\(^4\). Here we report, for the first time, that human platelets express pro-collagen I mRNA and protein, which is secreted in response to platelet activation and processed by thrombin to mature collagen I, which then binds to platelet membrane and decorates the platelet surface. The experimental evidence relating to the expression and processing of collagen I is briefly presented here, but the wider implications these findings can have for PCOS patients and other metabolic conditions are the subject of a separate study.

Amino acid profiling revealed that platelets from PCOS patients contained significantly more hydroxyproline than platelets from non-PCOS subjects (Figure 1A), with no consistent differences in levels of the other amino acids evaluated (heatmap, Supplementary Figure 1 & amino acid concentrations, Supplementary Table 1). Hydroxyproline is a proteinogenic amino acid that is produced by the hydroxylation of proline and a major component of fibrillary collagen\(^5,6\), making it a reliable indicator of collagen levels. As platelets are known to respond to collagen\(^7\), but not to generate it, different techniques were used to verify platelet expression of collagen. Using an assay based on the acid hydrolysis of samples to form hydrolysates and hydroxyproline, that is specific for collagen, it was possible to confirm that platelets express collagen. Moreover, platelets from the PCOS collective expressed significantly more collagen than platelets from healthy individuals (Figure 1B). Mass spectrometry identified collagen I, particularly the \(\alpha_1\) and \(\alpha_2\) subunits, as the most abundant isoform (Table 1). This fits well with the detection of mRNA encoding pro-collagen I \(\alpha_1\) and \(\beta_2\) in human platelets (Figure 1C), implying that collagen I could be synthesized de novo by platelets. Western blotting confirmed the expression of collagen in platelets and revealed that the platelet activation associated with PCOS increased platelet collagen I expression (Figure 1D), even though mRNA levels were comparable in platelets from the healthy and PCOS donors. The anti-collagen I antibody used did not bind to samples that had been pre-treated with collagenase, confirming the specific binding of the antibody to collagen I (Figure 1E).
Finally, immunohistochemistry of platelets fixed on poly-L-lysine confirmed the presence of collagen I on the surface of non-permeabilized platelets (Figure 1F).

Collagens are synthesized from pro-collagens that are secreted into the extracellular space where they undergo proteolysis to remove the N- and C-terminal extension peptides before being cross-linked and assembled into collagen fibrils.\(^8\) We next assessed whether platelets are able to release pro-collagen I and to process it into mature collagen I. Procollagen I was detectable in the supernatant of unstimulated platelets but levels increased significantly after stimulation with thrombin (Figure 2A) whereas platelet stimulation with the thromboxane A\(_2\) analogue; U46619, exerted a much weaker effect. No significant differences were observed between platelets from healthy and PCOS subjects suggesting the storage of similar amounts of pro-collagen in platelets from healthy and PCOS patients. These findings implied that the higher levels of mature collagen I detected in platelets from PCOS patients may be linked to the accelerated conversion of pro-collagen I to mature collagen I. Indeed, in the absence of a platelet agonist, pro-collagen I was only detected in Triton X-100 permeabilized platelets i.e. pro-collagen I was stored and sequestered intracellularly. However, after stimulation with thrombin, pro-collagen was released to decorate the platelet surface and could be detected in the absence of Triton X-100 (Figure 2B). The finding that platelets express pro-collagen I mRNA and are able to release pro-collagen I protein in response to platelet agonists convincingly demonstrates that platelets are a novel source of pro-collagen I. Although the presence of enzymes involved in collagen biosynthesis in human platelets was reported more than 40 years ago,\(^9\) this study presents the first evidence of pro-collagen I release by platelets and the presence of mature collagen on platelet surface.

To determine whether pro-collagen I could be converted to collagen I upon platelet stimulation, platelets from healthy donors were stimulated with thrombin and levels of collagen were assessed by Western blotting. This revealed a clear thrombin-induced decrease in pro-collagen I and increase in collagen I levels suggesting that collagen I was generated in response to platelet activation (Figure 2C). A number of different peptidases have been implicated in the generation of collagen,\(^10\) including bone morphogenic protein (BMP)-1 which is the main procollagen C-proteinase.\(^11\) There is no evidence of BMP-1 expression in platelets (PlateletWeb database\(^12\)) and the BMP-1 inhibitor; UK383367, failed to affect the thrombin-induced increase in collagen I levels. These observations suggested that an alternative protease could target platelet pro-collagen I and as thrombin was suggested to cleave pro-collagen in cultured fibroblasts,\(^13\) collagen cleavage was studied in platelets stimulated with thrombin or the thromboxane A\(_2\) analogue. Thrombin and U46619 both elicit platelet activation, however, only thrombin led to the cleavage of pro-collagen and the appearance of a ~60 kD product recognized by the pro-collagen antibody (Figure 2D). To confirm this finding, a truncated recombinant pro-collagen α1 (~80 kD) that is a substrate
for procollagen N- and C-proteinases was incubated with thrombin in vitro. This procedure resulted in the appearance of 3 additional peptides (~60, ~40 and ~28 kD), and was prevented by the thrombin inhibitor; hirudin (Figure 2E).

Taken together, the results of the present study demonstrate that platelets are a source of circulating collagen. Using different techniques i.e mass spectrometry, a specific assay for total collagen, RT-qPCR, immunoblotting and immunohistochemistry it was possible to show that platelets express pro-collagen I mRNA and protein. The latter is secreted in response to platelet activation and can undergo thrombin-mediated proteolysis to generate mature collagen I that binds to platelet surface. The platelet receptor that binds pro-collagen I was not characterized here, but procollagen I very likely attaches to platelets via α2β1 integrin.14 The functional consequences of the enhanced collagen I levels on the surface of the pre-activated platelets from PCOS patients remains to be demonstrated. However, the collagen-coating on activated platelets may well act as an agonist for other cells, to link platelet activation with the enhanced atherothrombosis risk in these patients.15

**Funding:** This work was supported by the Deutsche Forschungsgemeinschaft (RA 2435/3-2 to VR and FL 364/7-1 to IF).

**Author Contributions.** A.K., D.P, I.W and S.Z performed experiments, acquired the data and analyzed results. W.A.M. and I.D. characterized the patients, V.R. conceived and designed the research and wrote the manuscript and I.F. co-designed the study and wrote the manuscript.
References


10. Prockop DJ, Sieron AL, Li SW. Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. Matrix Biol. 1998;16(7):399-408.


Table 1. Collagen isoforms detected in human platelets

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Figure legends

Figure 1. Hydroxyproline and collagen levels in human platelets. A. Hydroxyproline levels in platelets from healthy donors (H) and PCOS subjects, n=9-13 (Student’s t test, ***P<0.001). B. Collagen levels in platelets from healthy donors (H) and from PCOS subjects, n=10 (Student’s t test, *P<0.05). C. Expression of mRNA encoding collagen I α1 and β2 in platelets from healthy donors and PCOS patients, n=7-8 (ANOVA, Tukey’s multiple comparisons post-test, p>0.1). D. Representative blots (upper panel) and quantification (lower panel) showing collagen I α1 subunit in platelets from healthy donors (H) and PCOS subjects, n=5-7 (Student’s t test, *P<0.05). E. Representative blots (upper panel) and quantification (lower panel) showing collagen I α1 expression in platelet lysates from PCOS subjects treated with solvent or collagenase (ColG), n=4 (Student’s t test, ***P<0.001). F. Representative images (left panel) and quantification (right panel) of collagen I (red) and Gp1b (green) in unstimulated non-permeabilized platelets from healthy donors (H) or PCOS subjects; bar = 10 µm; n=6 (Student’s t test, ***P<0.001).

Figure 2. Release of pro-collagen by activated platelets and processing by thrombin. A. Procollagen released from platelets from healthy donors or PCOS patients treated with solvent (Sol), thrombin (Thr, 1 U/ml, 10 minutes) or the thromboxane A₂ analogue U46619 (U46, 1 µmol/L, 10 minutes); n=4-5 (ANOVA and Tukey’s multiple comparisons post-test, ***P<0.001). B. Pro-collagen I (red), and Gp1b (green) expression in non-permeabilized (-TX-100) and permeabilized (+TX-100) platelets from healthy donors, treated with solvent (Sol) or thrombin (1U/ml, 10 minutes); bar = 5 µm. Comparable results were obtained in 5 additional experiments. C. Representative blots (left panel) and quantification (right panel) showing collagen I in platelets from healthy donors stimulated with either solvent (sol) or thrombin (Thr) in the presence or in the absence of the BMP inhibitor UK383367 (UK), n=6 (ANOVA and Tukey’s multiple comparisons post-test, ***P<0.001). D. Representative blot (upper panel) and quantification (lower panel) showing pro-collagen (PC) and its cleavage product (ΔPC) in platelets treated with solvent, thrombin (Thr, 1U/ml) or U46619 (U46, 1 µmol/L) for up to 60 minutes. The bar graph shows the quantification of cleaved pro-collagen (ΔPC) after 60 minutes, n=8 (ANOVA and Tukey’s multiple comparisons post-test, ***P<0.001). E. Representative silver gel (upper panel) and quantification (lower panel) showing the cleavage of a 80 kD recombinant pro-collagen (rPC) by thrombin (Thr, 1U/ml, 30 minutes) in the absence or in the presence of hirudin (Hir, 1U/ml), n=4 independent experiments (ANOVA and Tukey’s multiple comparisons post-test, ***P<0.001).
Figure 1
Figure 2
Research Design and Methods

Reagents

Thrombin was from Hemochrom Diagnostica (Essen, Germany). The anti-collagen I antibodies were from Cell Signaling (New England Biolabs, Frankfurt, Germany). All other compounds were from Merck (Darmstadt, Germany).

Study subjects

A total of 32 PCOS patients diagnosed according the Rotterdam criteria, attending the Endokrinologikum Frankfurt for fertility problems or for routine control were included in the present study. Thirty-five age-matched female subjects without PCOS, diabetes or insulin resistance served as the control group. None of the participants took any medication known to interfere with platelet aggregation for at least 10 days before blood sampling. The study protocol was approved by the ethics committee of the Goethe University Hospital (No. E 61/09 Geschäfts Nr 86/09) and the Landesärztekammer Hessen. All of the participants gave written informed consent.

Platelet isolation

Human platelets were isolated as described 1 and samples were either directly used for further experiments or snap frozen and stored at -80°C until use.

Quantification of free amino acids

Platelets were lysed in Triton X-100 lysis buffer and lysates containing 100 µg protein were used for amino acid analysis. Sample preparation was performed using the EZ:faast Liquid chromatography (LC) /mass spectrometry (MS) free amino acid analysis kit (Phenomenex, Aschaffenburg, Germany) according to the manufacturer’s instructions, with minor modifications. Internal standards (10 µL) were applied to all samples and to the standard curve. Analysis of metabolites was performed by LC-MS/MS using the EZ:faast AAA-MS HPLC column on an Agilent 1290 Infinity LC system (Agilent, Waldbronn, Germany) coupled to a QTrap 5500 mass spectrometer (Sciex, Darmstadt, Germany). Electro spray ionization in positive mode was employed. The intensity of the measured metabolite was normalized to internal standards. Analyst 1.6.2 and MultiQuant 3.0 (Sciex, Darmstadt, Germany), were used for data acquisition and analysis, respectively.

Mass spectrometry

Platelet lysates were subjected to SDS-PAGE and the gel was stained with coomassie blue. Each lane was cut into 16 slices equal fractions and collected in 96 filter well plates (30-40 µm PP/PE, Pall Corporation). The gel pieces were destained in 60% Methanol, 50 mmol/L ammoniumbicarbonate (ABC). Proteins were reduced in 10 mmol/L DTT, 50 mmol/L ABC for


one hour at 56°C and alkylated for 45 minutes in 30 mM iodoacetamide. Samples were
digested for 16 hours with trypsin (sequencing grade, Promega) at 37°C in 50 mmol/L ABC,
0.01% Protease Max (Promega) and 1 mmol/L CaCl₂. Peptides were eluted in 30% ACN and
3% formic acid, centrifuged into a fresh 96 well plate, dried in speed vacuum and resolved in
1% acetonitrile and 0.5% formic acid.

LC-MS/MS was performed using a Thermo Scientific Q Exactive Plus equipped with an ultra-
high performance LC unit (Thermo Scientific Dionex Ultimate 3000) and a Nanospray Flex Ion-
Source (Thermo Scientific). Peptides were loaded on a C18 reversed-phase precolumn
(Thermo Scientific) followed by separation on a 2.4 µm Reprosil C18 resin (Dr. Maisch GmbH)
in-house packed picotip emitter tip (diameter 100 µm, 15 cm long from New Objectives), using
a gradient from 4% ACN, 0.1% formic acid to 50 % eluent B (99% acetonitrile, 0.1% formic
acid) for 30 minutes with a flow rate 300 nL/min and washout with 99% B for 5 minutes.

MS data were recorded by data-dependent acquisition. The full MS scan range was 300 to
2000 m/z with a resolution of 70000, and an automatic gain control (AGC) value of 3E6 total
ion counts with a maximal ion injection time of 160 ms. Only higher charged ions (2+) were
selected for MS/MS scans with a resolution of 17500, an isolation window of 2 m/z and an
automatic gain control value set to E5 ions with a maximal ion injection time of 150 ms. MS1
Data were acquired in profile mode.

Mass spectrometry data were analysed by MaxQuant v1.6.1.0 2. Proteins were identified using
Acetylation (+42.01) at N-terminus, oxidation of methionine (+15.99) and hydroxyproline
(+15.99) were selected as variable modifications and carbamidomethylation (+57.02) as a
fixed modification on cysteines. The enzyme specificity was set to Trypsin. False discovery
rate (FDR) for the identification of protein and peptides was 1%.

Measurement of collagen content

Total collagen was determined using a commercially available kit (K218-100-BV, Biovision,
Biocat, Heidelberg, Germany), according to manufacturer’s instructions.

Western blotting

Platelets were lysed in triton-X 100 lysis buffer and lysate was subjected to SDS-PAGE
followed by immunoblotting as described 3.

In vitro treatment of pro-collagen with thrombin

Recombinant human pro-collagen 1 (1 µg; R&D system, Bio-Techne, Wiesbaden, Germany)
was incubated with thrombin (1 U/ml) in the presence or not of hirudin (1U/ml) for 30 minutes
at 37°C in phosphate buffered saline. Reactions were stopped by adding reducing SDS-PAGE
buffer and heating for 5 minutes at 95°C. Samples were subjected to SDS-PAGE followed by protein silver-staining.

**RT-qPCR**

Total RNA was isolated using peqGOLD TriFast reagent (VWR, Darmstadt, Germany) and mRNA was reversely transcribed using SuperScript III reverse transcriptase (Life Technologies, Darmstadt, Germany). Real-time qPCR from equal amounts of cDNA was performed using a Magnetic Induction Cycler (Biozym, Hessisch Oldendorf, Germany) and a SYBR Green master mix (Thermo Fisher Scientific, Dreieich, Germany). The relative expression levels of collagen Iα1 and collagen I β2 were calculated using the ΔΔCt method and normalized to 18S ribosomal RNA in each sample. The following primers were used: collagen I α1: forward 5’-CCAAATCTGTCTCCCCAGAA-3’, reverse 5’-TCAAAAACGAAGGGAGATG-3’; collagen I β2 forward 5’-CTGCAAGAACAGCATTGCAT-3’, reverse 5’-GGCGTGATGGCTTATTTGTT-3’; 18S forward 5’-CTTTGGTCGCTCGCTCCTC-3’, reverse 5’-CTGACCGGGTTGTTTTGAT-3’ – all from Biospring (Frankfurt, Germany).

**Platelet immunostaining**

Resting platelets or platelets stimulated with thrombin were fixed with paraformaldehyde and added on poly-L-lysine-coated 8-well chamber microslides (Ibidi, Gräfelfing, Germany). After blocking with BSA (3%) in PBS for 30 minutes at room temperature (RT), platelets were stained overnight at 4°C with anti-pro-collagen I or anti-collagen I antibodies. After washing and staining with fluorescent secondary anti-rabbit or anti-mouse antibodies (Thermo Fischer Scientific, Darmstadt, Germany), and anti-CD42b antibody (BD transduction laboratories, Heidelberg, Germany) platelets were washed and mounted with Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Images were obtained using a Zeiss LSM-510 Meta laser confocal microscope and analyzed using the imaging software AxioVision 4.8 (Zeiss, Jena, Germany). Data was expressed as the mean ratio of collagen-positive platelets to total platelets counted in 3-4 images that were randomly taken from each sample.

**Statistical analysis**

Data are expressed as mean ± SEM and statistical evaluation was performed using either Student’s t test, or two-way ANOVA followed by Tukey’s post-test where appropriate using Prism software (GraphPad 7). Values of P<0.05 were considered statistically significant.

**Supplementary Table 1.** Excel data reporting the concentrations of amino acids (nmol/µg protein) in platelets from healthy donors and from PCOS patients.
References


Supplementary Figure 1: Heatmap showing the 25 most significantly altered amino acids in platelet lysates from healthy donors and from PCOS patients. The color code describes high levels (red) and low levels (blue) of each amino acid; n=9-13.