HMGB1 binds to activated platelets via the receptor for advanced glycation end products and is present in platelet rich human coronary artery thrombi

Ingo Ahrens[1,2]; Yung-Chih Chen[2*]; Danijal Topcic[2*]; Michael Bode[2,3]; David Haenel[1]; Christoph E. Hagemeyer[2]; Hannah Seeba[1]; Daniel Duerschmied[1]; Nicole Bassler[2]; Karin A. Jandeleit-Dahm[4]; Matthew J. Sweet[5]; Alex Agrotis[6]; Alex Bobik[6]; Karlheinz Peter[2]

[1]Department of Cardiology and Angiology I, Heart Centre, University of Freiburg, Germany; [2]Atherothrombosis and Vascular Biology Laboratory, Baker IDI, Melbourne, Australia; [3]Division of Cardiology, University of North Carolina, Chapel Hill, North Carolina, USA; [4]Diabetes Complications Division, Baker IDI, Melbourne, Australia; [5]Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia; [6]Vascular Biology and Atherosclerosis Laboratory, Baker IDI, Melbourne, Australia

Summary

High mobility group box 1 (HMGB1) acts as both a nuclear protein that regulates gene expression, as well as a pro-inflammatory alarmin that is released from necrotic or activated cells. Recently, HMGB1-expression in human atherosclerotic plaques was identified. Therapeutic blockade of HMGB1 reduced the development of diet-induced atherosclerosis in ApoE knockout mice. Thus, we hypothesised an interaction between HMGB1 and activated platelets. Binding of recombinant HMGB1 to platelets was assessed by flow cytometry. HMGB1 bound to thrombin-activated human platelets (MFI 2.49 vs 25.01, p=0.0079). Blood from wild-type, TLR4 and RAGE knockout mice was used to determine potential HMGB1 receptors on platelets. HMGB1 bound to platelets from wild type C57Bl6 (MFI 2.64 vs 20.3, $p<0.05$), and $TLR4^{-/-}$ mice (MFI 2.11 vs 25.65, $p<0.05$) but failed to show binding to platelets from $RAGE^{-/-}$ mice ($p>0.05$). RAGE expression on human platelets was detected by RT-PCR with mRNA extracted from highly purified platelets and confirmed by Western blot and immunofluorescence microscopy. Platelet activation increased RAGE surface expression (MFI 4.85 vs 6.74, $p<0.05$). Expression of HMGB1 in human coronary artery thrombi was demonstrated by immunohistochemistry and revealed high expression levels. Platelets bind HMGB1 upon thrombin-induced activation. Platelet specific expression of RAGE could be detected at the mRNA and protein level and is involved in the binding of HMGB1. Furthermore, platelet activation up-regulates platelet surface expression of RAGE. HMGB1 is highly expressed in platelet-rich human coronary artery thrombi pointing towards a central role for HMGB1 in atherothrombosis, thereby suggesting the possibility of platelet targeted anti-inflammatory therapies for atherothrombosis.

Keywords

HMGB1, platelets, RAGE, ACS

Correspondence to:
PD Dr. Ingo Ahrens
Heart Center, University of Freiburg
Cardiology and Angiology I
Hugstetter Str. 55
79106 Freiburg, Germany
E-mail: ingo.ahrens@universitaets-herzzentrum.de

* Equally contributing authors.

Received: December 29, 2014
Accepted after major revision: June 9, 2015
Epub ahead of print: July 23, 2015

http://dx.doi.org/10.1160/TH14-12-1073
Thromb Haemost 2015; 114: ■■■

Introduction

High mobility group box 1 (HMGB1) is a DNA binding protein comprised of three domains (A box, B box, and an acidic C-terminal tail, ►Figure 1). It facilitates DNA replication, recombination, transcription and repair. HMGB1 can also act as a cytokine eliciting pro-inflammatory effects (1, 2). It is passively released from necrotic, damaged or apoptotic cells (3) and can also be actively secreted from circulating activated immune cells such as macrophages (4).

HMGB1 has been implicated in the pathophysiology of pathogen-associated diseases like sepsis, and antibody-mediated blockade of HMGB1 reduced lethality in a mouse model of sepsis (4). HMGB1 has also been implicated in the pathophysiology of sterile inflammation (2) and autoimmune disorders such as rheumatoid arthritis (5). Atherosclerosis is also an inflammatory disease with no clear links to pathogen-associated inflammation (6). We and others found high expression of HMGB1 in atherosclerotic plaques (7, 8) and demonstrated that antibody-mediated blockade of HMGB1 reduced diet-induced atherosclerosis in a mouse model, thereby establishing a link between HMGB1 and the pathophysiology of atherosclerosis (9).

Development of atherosclerosis has been closely associated with the deposition of activated platelets at the site of endothelial dysfunction (10). Furthermore platelets have also been implicated in late stages of atherosclerosis during the transition of stable to unstable plaques as well as plaque rupture (11, 12). Beside their primary haemostatic functions, platelets are actively contributing to pro-inflammatory processes thereby facilitating the progression of atherosclerosis (13). It has recently been demonstrated that acti-

vated platelets contribute to inflammation in the vessel wall by the recruitment and activation of leukocytes through platelet molecules released either by shedding (CD40L) or exocytosis (serotonin) (14, 15). Expanding from the observations mentioned above, we hypothesised that the DNA binding cytokine HMGB1, which plays a critical role in the development of atherosclerosis (16), interacts with activated platelets and that this interaction may add to the inflammatory functions of platelets. To the best of our knowledge we are the first to describe binding of HMGB1 to activated platelets via the platelet-expressed receptor for advanced glycation endproducts (RAGE).

Materials and methods

Materials

The polyclonal RAGE-specific antibody N16 (sc-8230) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The PE-labelled anti-P-selectin mAb (clone AK-4) and the IgM PAC-1 were from BD Pharmingen (San Jose, CA, USA). Monoclonal antibodies (anti-HMGB1 and control) were purified from culture media of growing hybridomas producing the anti-HMGB1 and anti-*Keyhole Limpet* hemocyanin (control) antibodies using Mep HyperCel (Pall Life Sciences, Cerg, France) and DEAE cellulose chromatographies (17). Monoclonal Alexa-Fluor-488–labelled anti-His tag antibody was purchased from Qiagen (Hilden, Germany). Secondary FITC labelled rabbit-anti-goat antibody for flow cytometry was purchased from Santa Cruz Biotechnology (sc-2777). The secondary HRP-conjugated antibody for detection of RAGE via Western blot (donkey-anti-goat-IgG-HRP, sc-2020) was also from Santa Cruz Biotechnology.

Expression and purification of recombinant HMGB1 in mammalian cells

The pMT/BIP/-HMGB1-V5-His construct was kindly provided by Dr Marco Bianchi, Milano, Italy. This construct was used as a template to generate a full length HMGB1 cDNA containing an AscI restriction site at the 5'-end, and his(6x)-tag and BamHI restriction site at the 3'-end of the gene. The PCR amplification was performed with the following primers for HMGB1:5'-GGCGCGCCATGGGCAAAGGAGATCCTAAG-3' (sense) and 5'-GGATCCTCAATGGTGATGGTGATGATG-3' (antisense), using PlatinumTaq DNA polymerase (Life Technologies, Paisley, UK), according to the manufacturer's protocol. The PCR product was subcloned into pSecTag2A mammalian expression vector (Life Sciences) and the final product was confirmed by sequencing.

The FreeStyle 293-F cells (Life Technologies, Paisley, UK) were cultured in suspension flasks containing 1L of the FreeStyle 293 Expression Medium (Life Technologies). The flasks were maintained on a platform shaker in a humidified incubator at 37 °C with 5% CO_2 and 95% air, and an agitation rate of 150 rpm. The pSecTag2A construct (1 mg) was transiently transfected into 2.5×10^9 of FreeStyle 293-F cells using polyethylenimine (PEI) (25 kDa linear PEI, Polysciences, Warrington, PA, USA). The transfection complex was formed at the DNA:PEI ratio of 1:5 (w/w) in 150 mM NaCl and 20 mM HEPES (pH 7.5), with 20-minute (min) incubation at room temperature (RT) prior to the addition to the culture. The conditioned medium containing the recombinant protein was collected six days post-transfection and dialysed in no less than 10 litre of 1xPBS (pH 7.4) for 16 hours (h) at 4 °C. The protein purification was undertaken using fast protein liquid chromatography (FPLC), whereby the dialysed medium containing over-expressed recombinant 6xHis-tagged HMGB1 was applied to Ni-NTA column (Qiagen, Hilden, Germany) at a flow rate of 1 ml/min. The column was washed with buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 40 mM imidazole (pH 8.0) until the baseline absorbance was reached. The recombinant HMGB1 was eluted with buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole (pH 8.0), and then dialysed in 1xPBS (pH 7.4) for 2 h at 4 °C. The concentration of recombinant HMGB1 was determined using BCA assay (Thermo Scientific, Waltham, MA, USA) and the integrity of purified protein was assessed via SDS-PAGE under reducing conditions followed by western blotting onto an Immobilon P membrane (Millipore Corporation, Billerica, MA, USA). After blocking the membrane overnight with phosphate buffered saline containing 0.2% Tween20 (PBS-Tween) and 5% skim milk, a HRP-labelled anti-His(6)-antibody (Roche, Basel, Switzerland) was added (dilution 1:2,000) and incubated for 2 h at RT. The membrane was washed several times with PBS-Tween buffer. Visualisation of peroxidase activity was achieved by addition of SuperSignal® Chemiluminescent Substrate (Pierce, Rockford, IL, USA) on a ChemiDoc XRS® (BioRad, Hercules, CA, USA).

Blood sampling

Citrated human whole blood was taken with informed consent from healthy donors. With the approval of the AMREP Animal Ethics Committee, mouse whole blood was taken by direct heart puncture of anesthetised mice and blood was anticoagulated with heparin at a final concentration of 40 U/ml. Citrated human blood and serum was taken with informed consent (Ethics approval number 287/12) 6 ± 2 h after coronary angiography from patients with ST-elevation myocardial infarction (STEMI) and from patients undergoing coronary angiography for other reasons than (STEMI) in the Heart Center of the University of Freiburg.

Animals and housing conditions

Wild-type C57BL/6, RAGE knockout and TLR4 knockout mice (both on a C57BL/6 background) were obtained from the Precinct Animal Facility, of the Alfred Medical and Research Educational Precinct (AMREP), Melbourne, Australia. All mice were bred and housed in accordance with good animal practice and following the local institutional regulations of the AMREP Precinct Animal Facility. Male mice were used for all experiments, which were performed according to institutional guidelines of the AMREP animal ethics committee.

Assessment of platelet activation by flow cytometry

Whole blood was diluted 1/20 in modified Tyrode's buffer (150 mmol/l NaCl, 2.5 mmol/l KCl, 12 mmol/l $NaHCO_3$, 2 mmol/l MgCl2, 2 mmol/l $CaCl_2$, 1 mg/ml bovine serum albumin, 1 mg/ml dextrose, pH 7.4) and aliquots of 40 µl were incubated with the platelet agonist thrombin (0.1 U/ml for human and 1.0 ml/U for mouse blood, respectively) as a positive control for platelet activation or with recombinant HMGB1 in a final volume of 50 µl. Samples were then incubated with the PE-labelled anti-P-selectin mAb (clone AK-4, BD Biosciences, San Jose CA, USA) to detect the expression of the platelet activation surface marker P-selectin or with the FITC-labelled GPIIb/IIIa activation specific antibody PAC-1 (BD Biosciences) for 15 min at RT. For isotype controls, IgG1-FITC (Clone 679,1Mc7, Beckman Coulter, Brea, CA, USA) and IgG-PE (Clone 679,1Mc7, Beckman Coulter) were used. Samples were fixed with 450 µl CellFix (BD Biosciences) and analysed in the platelet gate on a FACSCalibur system (BD Biosciences).

Measurement of HMGB1 binding to platelets

Whole blood was diluted 1/20 in modified Tyrode's buffer (150 mmol/l NaCl, 2.5 mmol/l KCl, 12 mmol/l $NaHCO_3$, 2 mmol/l MgCl2, 2 mmol/l $CaCl_2$, 1 mg/ml bovine serum albumin, 1 mg/ml dextrose, pH 7.4) and aliquots of 40 µl were incubated with recombinant his-tagged HMGB1 (rHMGB1) with or without the platelet agonist thrombin (0.1 U/ml for human and 1.0 ml/U for mouse blood, respectively) for 20 min at 37 °C in a final volume of 50 µl. Thereafter samples were incubated with a monoclonal Alexa-Fluor-488–labelled anti-His tag antibody for 15 min at RT in the dark. Samples were fixed with 450 µl CellFix (BD Biosciences) and analysed in the platelet gate on a FACSCalibur system (BD Biosciences).

Detection of platelet RAGE expression by RT-PCR

Human platelets were purified from PRP via gel filtration over a sepharose CL-2B column (Sigma, St. Louis, MO, USA) on a polypropylene column (Thermo Scientific, Rockford, IL, USA) followed by three washing steps in platelet wash buffer. Thereafter the platelet suspension was applied to a discontinuous gradient centrifugation step for further purification to yield highly purified platelets (18). Platelet mRNA was extracted via TRIzol (Merck Millipore) following the manufacturer's instructions and then resuspended in RNAse free water. RT-PCR was carried out using AMV RT transcriptase (Promega, Madison, WI, USA). The sequences of the human RAGE specific primers were: forward (hRAGE-F) 5'ATGGAAACTGAACACAGGCC3' and reverse (hRAGE-R) 5'CACACATGTCCCCACCTTAT3'. Purity control primers to detect leukocyte mRNA were: forward (hCD45-F) 5'GCTCAGAATGGACAAGTA3' and reverse (hCD45-R) 5'CTCTGTCTCTCACACAAACA3'.

Detection of platelet RAGE protein by Western blot

Human platelets were purified from PRP as described above. Platelet pellets were resuspended in platelet lysis buffer (50 mM Tris-HCL, 100 mM NaCl, 1 % Triton X-100, 2 mM EDTA, 50 mM NaF,

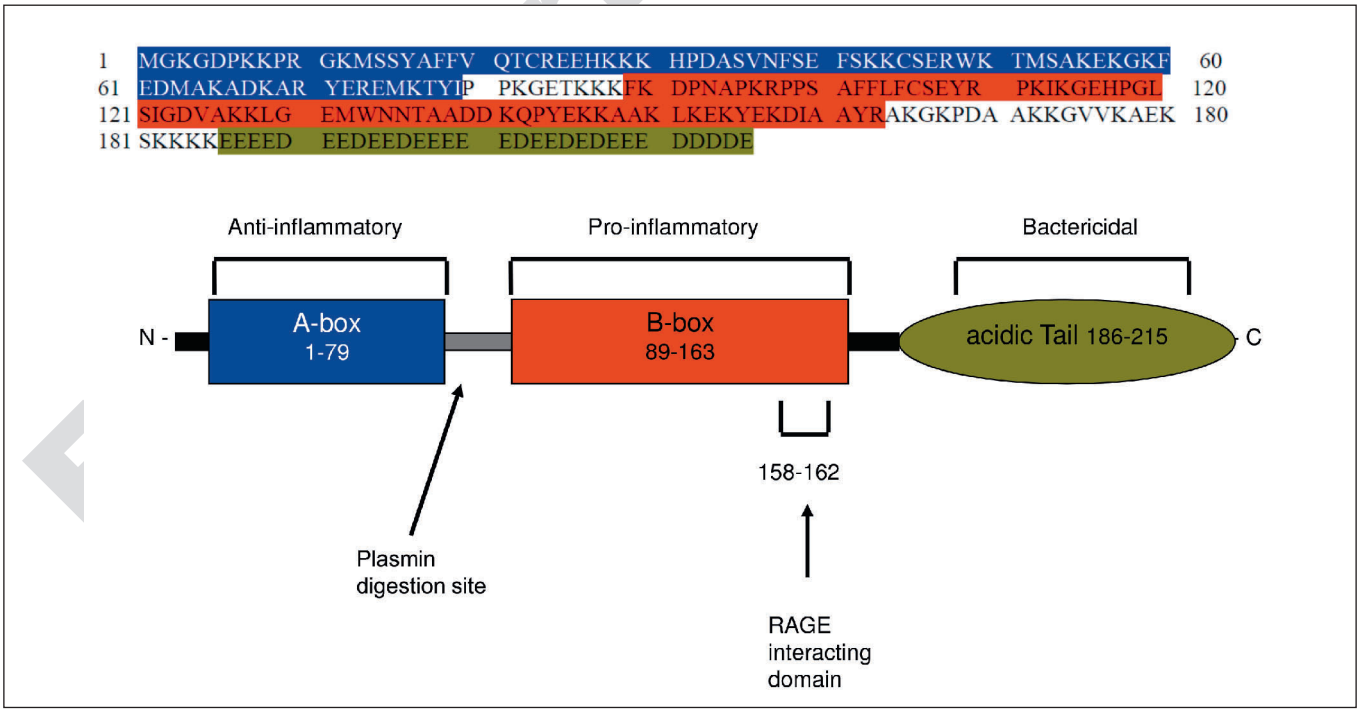

Figure 1: High mobility group box-1 (HMGB1). A schematic drawing of the peptide sequence and organisation of the DNA-binding cytokine high mobility group box-1 (HMGB1).

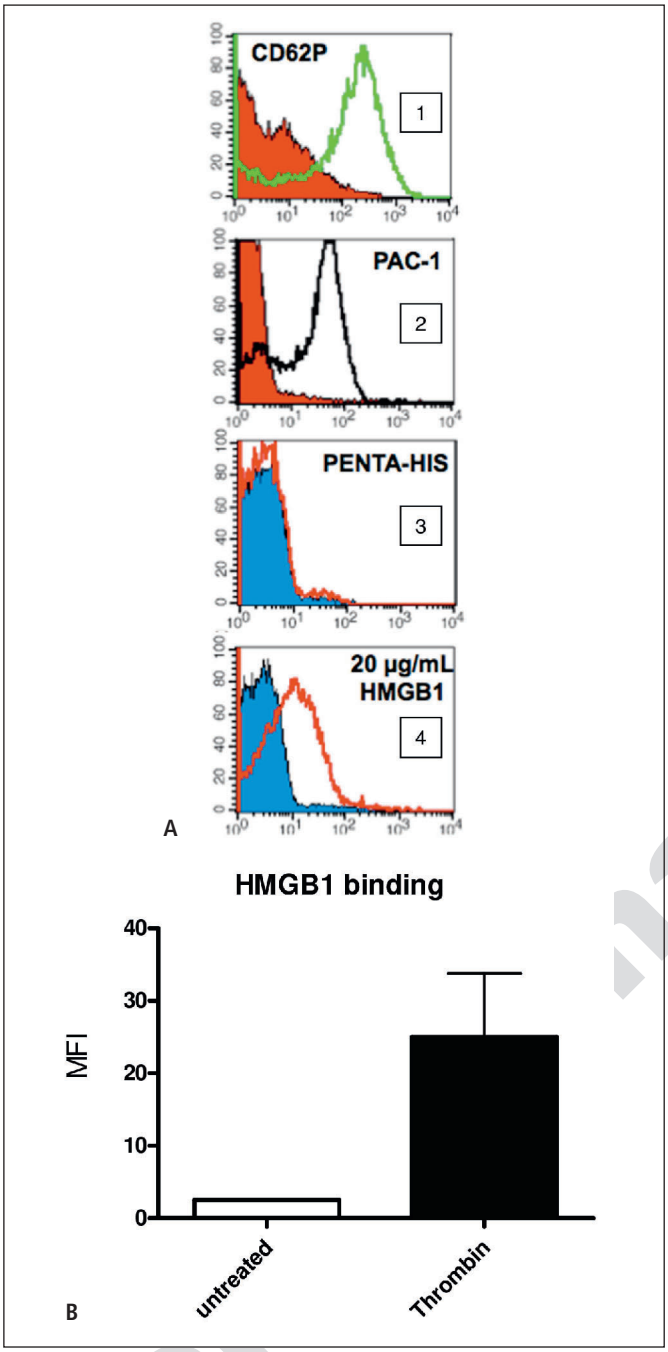

Figure 2: HMGB1 binds to thrombin-activated human platelets. A) Histograms of a representative flow cytometry assay to assess platelet activation via extent of CD62P-expression (panel 1), binding of the GP IIb/IIIa activation specific antibody PAC-1 (panel 2), binding of the secondary anti-His tag antibody in the absence of HMGB1 (panel 3), and binding of 6xhis tagged recombinant HMGB1 detected via the secondary anti-His tag antibody (panel 4). Filled histograms represent resting platelets and open histograms depict activated platelets. B) HMGB1 binding assessed by mean fluorescence intensity (MFI) of fluorescently labelled anti-penta-His antibody following incubation of recombinant 6xhis tagged HMGB1 with resting (white bar) and thrombin (black bar) stimulated human platelets (MFI 2.49 vs 25.01 for resting and thrombin stimulated platelets, respectively, p=0.0079). Data in A are representative of five independent experiments, and data in B are combined from five independent experiments.

0.1% SDS, 0.5% deoxycholic acid, 2 µg/ml aprotinin, 5 µg/ml leupeptin, 0.7 µg/ml pepstatin A, pH 7.4). Lysis was carried out on ice for 45 min. Thereafter lysates were precleared by centrifugation for 15 min at 12,000 rpm at 4 °C and then boiled for 15 min at 60 °C in reducing SDS buffer followed by separation with a 12% polyacrylamide gel. After separation proteins were transferred to PVDF membranes (Immobilon-P, Merck Millipore). RAGE was detected with the polyclonal anti-RAGE antibody N16 (sc-8230) and visualised with the secondary donkey-anti-goat-IgG-HRP (sc-2020).

Detection of RAGE on platelets by immunofluorescence microscopy

Human platelets derived from gel filtered PRP were diluted 1/5 in modified Tyrode's buffer and incubated for 30 min at 37 °C on fibrinogen coated coverslips. Non-adhering platelets were removed by repeated washing with Tyrode's buffer. Platelets were permeabilised with 0.2% triton X-100 for 10 min at RT. Thereafter incubation with the primary antibody (anti-RAGE, N16, 1/25 dilution) was carried out for 30 min at 37 °C followed by two washes with Tyrode's buffer. Incubation with the secondary goat-anti-mouse IgG+M (Jackson ImmunoResearch, West Grove, PA, USA, 1/400 dilution) was carried out for a further 30 min at 37 °C. Cells were washed twice with Tyrode's buffer and fixed with CellFix (BD Biosciences). For subsequent imaging, coverslips were covered with VectoShield mounting medium (Vector Laboratories, Burlingame, CA, USA) and placed upside down onto a coverslide. Imaging was done on an Axioplan 2 microscope with a plan Neo-Fluar 100× oil immersion objective (Zeiss, Oberkochen, Germany).

Immunohistochemistry and immunofluorescence for HMGB, platelets and RAGE in coronary artery thrombi

Sampling of coronary thrombus material was performed in patients, who were admitted with acute myocardial infarction and subjected to routine angioplasty. The Export® catheter system (Medtronic, Sydney, NSW, Australia) was used in cases with obstructive plaque/thrombus material within large proximal coronary arteries and the aspirated otherwise discarded material was used for immunohistology. This procedure was approved by the Alfred Hospital Ethics Committee (EC 25/07). Samples were embedded in Optimal Cutting Temperature compound (OCT) and cutted by Cyrostat (Zeiss MICROM HM 550) in 6 µm thickness. Frozen sections were thawed and incubated with primary antibodies for 1 h at RT. For immunohistochemistry, the primary antibodies used were anti-HMGB1 (clone 10829–1-AP; 1:100 dilution, Proteintech, Chicago, IL, USA); anti-P2Y12 (clone P-14; 1:100 dilution, Santa Cruz); or control antibody (KLH, 1:100), followed by biotinylated secondary antibody (1:200). Detection was achieved by Vectastain ABC kit and DAB substrate following the manufacturer's recommendation. For immunofluorescence, the antibodies used were anti-RAGE-cy5.5 conjugated (bs-0177R-Cy5.5, Bioss antibodies, Woburn, MA, USA); anti-HMGB1-FITC

(036636-FITC, US Biological, Salem, MA, USA) and photos were taken by fluorescence microscope (Olympus BX-61)

Statistical analysis

Statistical analysis were performed using GraphPad Prism (Version 5.01), mean and SEM were used for descriptive statistics. Differences between means were assessed by t-test and one-way ANOVA. P-values less than 0.05 were considered statistically significant.

Results

Production and purification of recombinant HMGB1 in HEK293 cells

The identity of recombinant HMGB1, purified from the supernatant of transiently transfected HEK293 cells, was confirmed using SDS-PAGE; HMGB1 migrated as a single band with the expected molecular weight of approximately 30 kDa. Western blotting using a horseradish peroxidase coupled anti-His(6)-tag monoclonal antibody also resulted in detection of a single band, further confirming that the protein visualised was recombinant HMGB1 (see Suppl. Figure 1, available online at www.thrombosis-online.com).

Recombinant HMGB1 binds to activated human platelets

Whole blood samples derived from healthy human donors were treated with thrombin (0.1 U/ml) or vehicle and thereafter incubated with recombinant HMGB1 (20 µg/ml). Platelet activation in whole blood samples was assessed by flow cytometry. Upon stimulation, platelets expressed P-selectin (CD62P) and showed binding of the GP IIb/IIIa activation-specific antibody PAC-1 (►Figure 2A). HMGB1 binding to both resting and activated human platelets was assessed over the concentration range 5 µg/ml – 40 µg/ml, and an HMGB1 concentration of 20 µg/ml was then chosen for further experiments. There was significant binding of recombinant HMGB1 to activated human platelets compared to resting platelets (n=5, p=0.0079, ►Figure 2B). HMGB1 did not cause activation of resting platelets (see Suppl. Figure 2, available online at www.thrombosis-online.com) and did not induce platelet aggregation (see Suppl. Figure 3, available online at www.thrombosis-online.com). Cytokine release by platelets as assessed by sCD40L ELISA in supernatants of highly purified platelets treated with platelet agonists was not increased by HMGB1 alone or the addition of HMGB1 to the platelet agonists (data not shown).

HMGB1 does not bind to activated platelets from RAGE$^{-/-}$ mice

To confirm that HMGB1 was binding specifically to RAGE and not other receptors such as members of the toll-like receptor family (e.g. TLR4), we next examined binding to platelets deficient in either RAGE or TLR4. Blood samples from wild-type C57Bl6, RAGE$^{-/-}$, and TLR4$^{-/-}$ mice were stimulated with vehicle or thrombin (0.1 U/ml), before incubating with HMGB1 (20 µg/ml). Binding of HMGB1 was assessed by flow cytometry in the CD41-positive platelet gate. As with human platelets, HMGB1 did not bind to resting mouse platelets. There was a significant increase in binding of HMGB1 to thrombin-activated wild-type platelets (n=5, * $p<0.05$, ►Figure 3) and thrombin-activated TLR4$^{-/-}$ platelets (n=5, ** $p<0.05$, ►Figure 3). Thrombin-activated platelets from RAGE$^{-/-}$ mice did not bind HMGB1 n=5, p=ns, ►Figure 3).

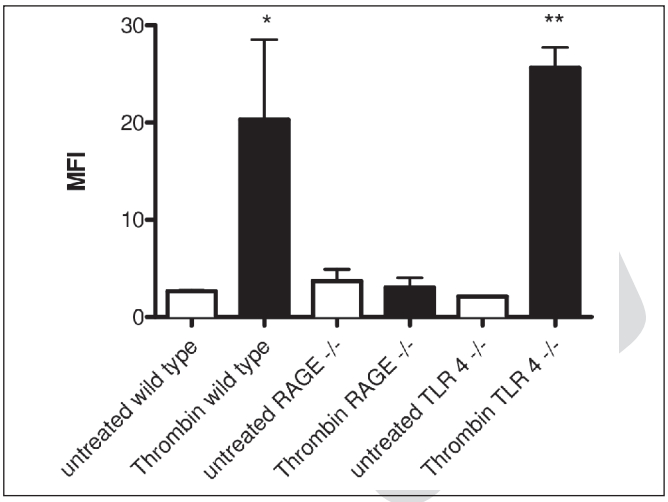

Figure 3: HMGB1 binding to thrombin-activated murine platelets is RAGE-dependent. Binding of recombinant HMGB1 to murine platelets from wild-type (C57Bl6), RAGE$^{-/-}$, and TLR 4$^{-/-}$ mice assessed by flow cytometry. High MFI values depict strong binding of HMGB1. White bars represent resting platelets und black bars thrombin-activated platelets. Data are combined from five independent experiments.

Human platelets also express the receptor for advanced glycation end products (RAGE)

Platelet RAGE expression was detected by RT-PCR using mRNA isolated from highly purified platelets. Peripheral blood mononuclear cells were used as a positive control for RAGE mRNA-expression. Highly purified platelets contained RAGE mRNA and were negative for the CD45 leukocyte mRNA (►Figure 4A). Western blot analysis using highly purified platelets confirmed RAGE protein expression in lysates from resting, ADP- and thrombin stimulated platelets (►Figure 4B). In addition, RAGE expression concentrated around the plasma membrane of adhering platelets was observed by immunofluorescence microscopy (►Figure 4C).

Platelet RAGE expression increases upon platelet activation

Surface expression of RAGE on human platelets was assessed by flow cytometry following stimulation with thrombin (0.1 U/ml) or PMA (100 nmol/l). RAGE expression was detected on the surface

of resting platelets (n=6, p=0.0009, ▶Figure 5), but was significantly increased upon stimulation with thrombin (n=6, p=0.0026, ▶Figure 5) or PMA (n=6, p=0.019, ▶Figure 5). Platelet RAGE expression was significantly increased in patients with acute ST-elevation myocardial infarction (STEMI) compared to patients with stable coronary artery disease (CAD), (n=5 in each group, p=0.039, see Suppl. Figure 4 and Suppl. Table 1, available online at www.thrombosis-online.com).

HMGB1 is highly expressed in coronary artery thrombi in patients with ACS

Coronary artery thrombi were extracted via aspiration thrombectomy in patients with acute myocardial infarction. Thrombi were embedded in OCT and sectioned by Cryostat, staining was carried out with a monoclonal anti-HMGB1 antibody. Immunohistochemical analysis of platelet-rich human coronary artery thrombi from a total of six individual patients revealed high levels of expression of HMGB1 (▶Figure 6A and B). Colocalisation of HMGB1 with platelets is demonstrated by additional staining of the platelet P2Y12 ADP receptor with an anti-P2Y12 antibody (▶Figure 6B).

Discussion

HMGB1 is emerging as a key molecule of the innate immune system implicated in both infectious and sterile inflammation (2), and in atherosclerosis, a sterile chronic vascular inflammatory disease (19). The discovery of high expression of HMGB1 in athero-

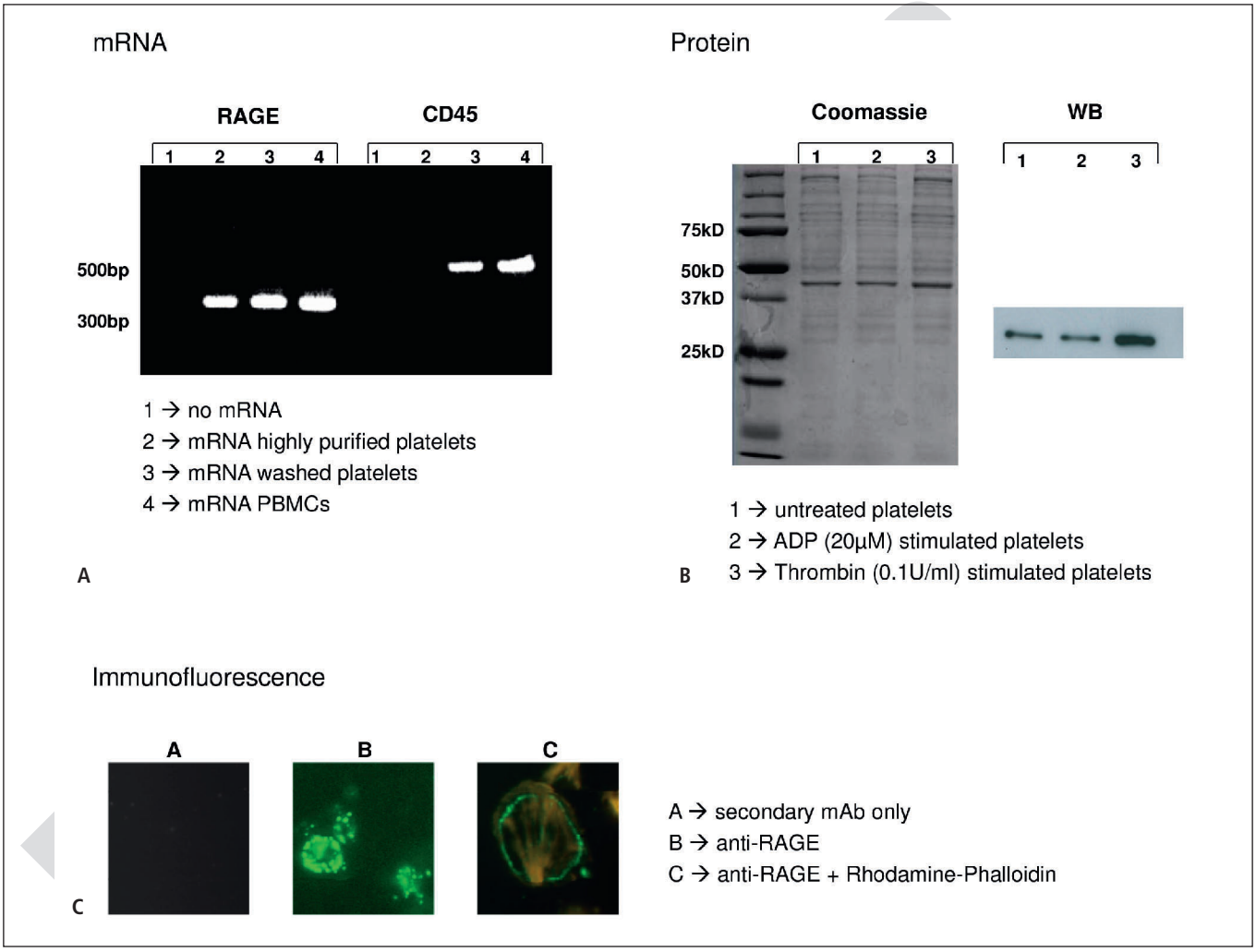

Figure 4: RAGE protein and mRNA are expressed in human platelets. A) Representative agarose gel showing RT-PCR results with RAGE- and CD45-specific primers. RT-PCR was carried out with mRNA from highly purified (2) and washed platelets (3), with mRNA from peripheral blood mononuclear cells (4) used as a positive control for CD45 mRNA. B) Western blot analysis of RAGE expression in lysates of highly purified resting platelets (1), highly purified ADP stimulated platelets (2), and highly purified thrombin stimulated platelets (3). C) Representative immunofluorescence microscopy pictures of platelets adhering on fibrinogen coated coverslips. Green fluorescently labelled secondary antibody was used to detect binding of the RAGE-specific antibody N16. Rhodamine-phalloidin (red fluorescence) was used to visualise the platelet actin cytoskeleton. Data in A, B and C are representative of 5, 4 and 6 independent experiments, respectively.

sclerotic plaques (8) and the marked reduction of atherogenesis by specific blockade of HMGB1 in animal models (9), strongly suggest a functional role for this molecule in the inflammatory process driving this disease. Activated platelets foster early stages of atherosclerosis (10), accompany the chronic process of atherosclerosis (12, 20), and ultimately cause deadly consequences of advanced atherosclerosis plaque rupture- coronary artery thrombosis leading to myocardial infarction (13). Furthermore, there is growing evidence and recognition of immunological functions of platelets (21, 22) and indeed we found specific binding of recombinant HMGB1 to activated platelets.

Our finding of specific binding of HMGB1 to platelets points towards a novel immunological function, which is especially interesting in the light of a recent report showing that platelet microparticles from patients with systemic sclerosis have increased surface bound HMGB1 (23). This could be due to increased uptake of blood borne HMGB1 thereby reflecting our *in vitro* findings as a relevant phenomenon occurring *in vivo* in patients with immunological disease. However, according to the authors it may also be endogenous HMGB1, which has been described as a platelet protein earlier by others (24). More evidence for a role of endogenous HMGB1 comes from a recent study that elegantly demonstrates induction of neutrophil extracellular traps by platelet derived HMGB1, thereby adding evidence for the immunological functions of platelets (25).To further delineate the platelet receptors involved in HMGB1 binding we set out to characterize the binding of HMGB1 to murine platelets from mice deficient in either the receptor for advanced glycation end products (RAGE) or toll like receptor 4 (TLR 4), both of which have been characterised as HMGB1 receptors (26, 27). Flow cytometry of mouse platelets derived from wild-type (C57BL6), RAGE$^{-/-}$, and TLR4$^{-/-}$ mice conclusively demonstrated that recombinant HMGB1 required RAGE, but not TLR4, to bind to thrombin-activated platelets.

Like its ligand HMGB1, RAGE has also been implicated in the development of atherosclerosis (28, 29). Experimental data from platelet activating effects of traditional RAGE ligands, such as advanced glycation endproducts (AGEs), imply RAGE expression on platelets (30, 31) and pilot experiments with monoclonal antibodies to RAGE in flow cytometry suggest RAGE expression on the platelet surface (31). We have confirmed and extended these findings using various methods such as mRNA analysis of highly purified platelets, Western blot, immunofluorescence and flow cytometry and in particular relate RAGE expression to the platelet activation state. Recent evidence indicates a role for platelet mRNA in de novo protein synthesis in platelets, regulated at the translational level (32, 33). Therefore we used mRNA from highly purified platelets and identified platelet mRNA specifically encoding RAGE. Platelet-specific expression of RAGE was confirmed at the protein level by Western blotting, immunofluorescence microscopy and flow cytometry. Furthermore, the activation-dependent increase in RAGE expression occurred mostly on the platelet membrane of thrombin-activated non-permeabilised human platelets. The increase in platelet RAGE expression upon stimulation with thrombin together with the observation that thrombin stimulated RAGE$^{-/-}$ platelets fail to bind HMGB1 strongly implicates RAGE as the major platelet receptor for HMGB1. Evidence for the pathophysiological relevance of platelet-expressed RAGE in inflammation comes from a recent observation of increased platelet reactivity in obese patients. These patients had significantly reduced levels of soluble RAGE (34), which acts as a decoy receptor for RAGE ligands and has been proposed to reduce levels of circulating HMGB1 *in vivo* (35).

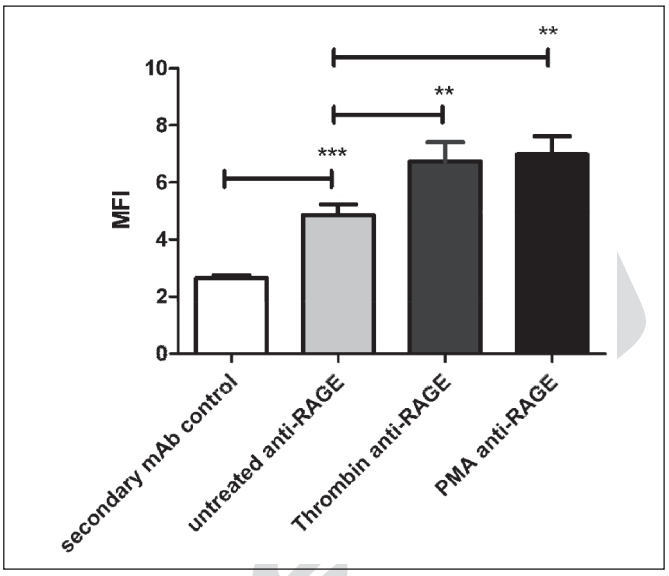

Figure 5: RAGE expression on the membrane of human platelets increases upon platelet activation. Depicted are the mean fluorescence intensity values (MFI) of RAGE expression on the surface of resting, thrombin, and PMA stimulated human platelets (n=6). The white bar represents the background binding of the fluorescently labelled secondary antibody that was used to detect binding of the RAGE-specific antibody N16.

Activated platelets and their surface adhesion receptors are important for progression of atherosclerotic plaques (36) and promote recruitment of monocytes to the vessel wall and plaques (37). Within the plaques, macrophage differentiation from monocytes is crucially involved in the transition from stable to unstable/vulnerable plaques (38, 39). HMGB1 is chemotactic for monocytes *in vivo* (9). We hypothesise, and are investigating this in ongoing studies, that platelet-bound HMGB1 facilitates monocyte recruitment and macrophage activation thereby mediating two different aspects of monocyte biology, migration and activation. This dual role of HMGB1 in the context of monocytes is also supported by a recent study that elegantly demonstrates monocyte recruitment is mediated by HMGB1 in complex with CXCL12 (also known as SDF-1) via a cellular pathway independent of HMGB1–mediated TLR4 monocyte activation, which leads to the release of TNF alpha and other cytokines (40). Whether CXCL12, which has been demonstrated to be bound and presented by platelets (41) is a prerequisite for HMGB1-binding to activated platelets should be addressed in future studies. We did not see binding of activated platelets to immobilised HMGB1 in static adhesion assays (data not shown) further indicating that co-factors may be important for binding of soluble HMGB1 to activated platelets. Interestingly, platelets do express SDF-1 (42) and expression is increased upon pla-

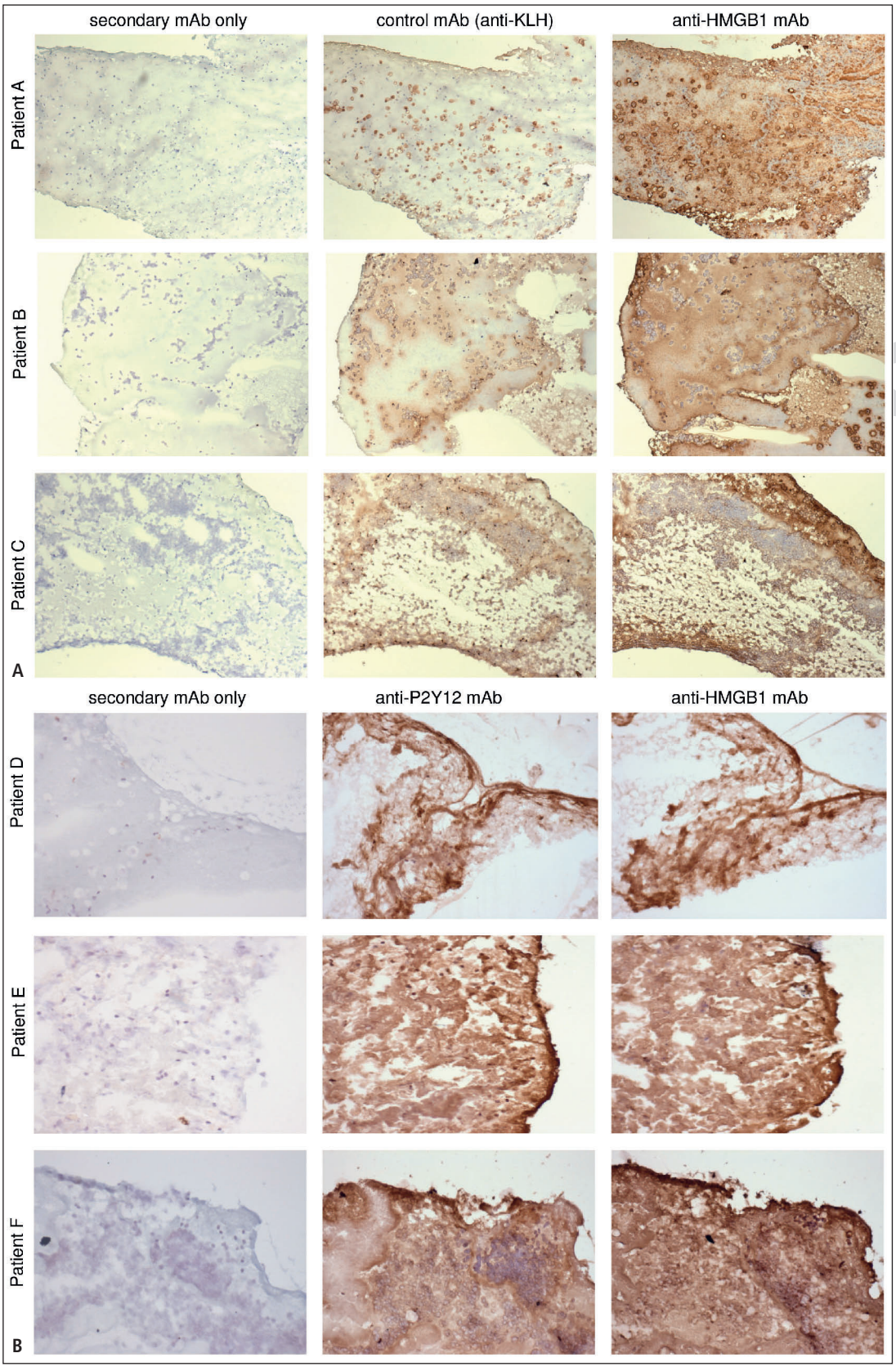

Figure 6: HMGB1 is expressed in platelet rich human coronary artery thrombi. A) Fresh coronary artery thrombi extracted by thrombus aspiration from three consecutive patients (a-c) with acute myocardial infarction were sectioned and stained for HMGB1 with a HMGB1-specific monoclonal antibody. Anti-keyhole limpet hemocyanine (KLH) monoclonal antibody was used to control for unspecific binding. Dark brown colour represents specific staining for endogenous human HMGB1. B) Fresh coronary artery thrombi extracted by thrombus aspiration from three consecutive patients (d-e) with acute myocardial infarction were sectioned and stained for the platelet P2Y12 receptor with a monoclonal anti-P2Y12 or a HMGB1-specific monoclonal antibody. Dark brown colour represents specific staining for the platelet P2Y12 receptor or endogenous human HMGB1, respectively.

What is known about this topic?

- HMGB1 is a DNA binding cytokine that is found abundantly in atherosclerotic lesions. Neutralisation of HMGB1 in animal models reduces atherosclerotic lesions.
- Platelets express endogenous HMGB1 and platelet/neutrophil interactions have partially been attributed to platelet derived HMGB1 in inflammation.

What does this paper add?

- Binding of HMGB1 to activated platelets via the receptor for advanced glycation end products (RAGE) is demonstrated. RAGE is expressed on mRNA and protein level in human platelets and surface RAGE expression increases upon platelet activation.
- HMGB1 does not directly activate haemostatic functions of platelets but is found in human coronary artery thrombi extracted from patients with acute myocardial infarction, thereby indicating a possible role for platelet/HMGB1 interactions in the pathogenesis of atherothrombosis.

telet activation and highly increased in platelets from patients with acute myocardial infarction (43). A possible interaction between SDF-1 (CXCL12) and platelet bound HMGB1 may facilitate platelet aggregate formation with immune cells as it has been described for platelet derived SDF-1 alone (44). Furthermore, it remains to be assessed whether platelet bound HMGB1 exerts the effects described above. In the future, this may be further studied by injecting activated platelets with HMGB1 bound on their surface in analogy to the elegant study by Huo et al. that demonstrated exacerbation of atherosclerosis by repeated injection of activated platelets in a mouse model of diet-induced atherosclerosis (10). However, activated platelets appear ideal for local targeted therapies as they express the platelet and megakaryocyte exclusive integrin receptor GPIIb/IIIa in an activation-specific conformation. This active conformation is specifically recognised by activation-specific single chain antibodies (scFv) (45). Fusion constructs composed of activation specific scFv and recombinant proteins have already successfully been used by our group for targeted anticoagulation (46) and ultrasound targeted molecular imaging of thrombosis and thrombolysis (47). Whether an HMGB1-blocking peptide targeted to activated platelets could be a novel therapeutic strategy to halt atherosclerotic plaque progression needs to be evaluated in further studies.

In conclusion, the DNA-binding cytokine HMGB1 has been identified as a novel platelet binding protein, which requires platelet specific expression of RAGE. Platelet activation leading to increased RAGE surface expression is the prerequisite for HMGB1 binding. Platelet-rich human coronary artery thrombi strongly express HMGB1 thereby suggesting that platelet-bound HMGB1 has pro-inflammatory functions. Our findings raise the possibility for the development of novel platelet-targeted anti-inflammatory therapies for the treatment of inflammatory and atherothrombotic disease.

Acknowledgements

This work has been supported by the NHMRC of Australia (Project Grant 472632, Bobik A, Peter K, Agrotis A), the Australian Research Council and the German Research Foundation (AH 185/1–1, Ahrens I). We thank Dr Keyue Liu and Dr Masahiro Nishbori for providing the monoclonal anti-KLH antibody for the immunohistochemistry.

Conflicts of interest

None declared.

References

1. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002; 418: 191–195.
2. Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. Annu Rev Immunol 2011;29: 139–162.
3. de Souza AW, Westra J, Limburg PC, et al. HMGB1 in vascular diseases: Its role in vascular inflammation and atherosclerosis. Autoimmun Rev 2012; 11: 909–917.
4. Wang H, Bloom O, Zhang M, et al. HMG-1 as a late mediator of endotoxin lethality in mice. Science 1999; 285: 248–251.
5. Bianchi ME, Manfredi AA. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. Immunol Rev 2007; 220: 35–46.
6. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011; 473: 317–325.
7. Inoue K, Kawahara K, Biswas KK, et al. HMGB1 expression by activated vascular smooth muscle cells in advanced human atherosclerosis plaques. Cardiovasc Pathol 2007; 16: 136–143.
8. Kalinina N, Agrotis A, Antropova Y, et al. Increased expression of the DNA-binding cytokine HMGB1 in human atherosclerotic lesions: role of activated macrophages and cytokines. Arterioscler Thromb Vasc Biol 2004; 24: 2320–2325.
9. Kanellakis P, Agrotis A, Kyaw TS, et al. High-mobility group box protein 1 neutralisation reduces development of diet-induced atherosclerosis in apolipoprotein e-deficient mice. Arterioscler Thromb Vasc Biol 2011; 31: 313–319.
10. Huo Y, Schober A, Forlow SB, et al. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. Nat Med 2003; 9: 61–67.
11. Langer HF, Gawaz M. Platelet-vessel wall interactions in atherosclerotic disease. Thromb Haemost 2008; 99: 480–486.
12. Eisenhardt SU, Habersberger J, Murphy A, et al. Dissociation of pentameric to monomeric C-reactive protein on activated platelets localizes inflammation to atherosclerotic plaques. Circ Res 2009; 105: 128–137.
13. Schulz C, Massberg S. Platelets in atherosclerosis and thrombosis. Handb Exp Pharmacol 2012: 111–133.
14. Duerschmied D, Suidan GL, Demers M, et al. Platelet serotonin promotes the recruitment of neutrophils to sites of acute inflammation in mice. Blood 2013; 121: 1008–1015.
15. Lievens D, Zernecke A, Seijkens T, et al. Platelet CD40L mediates thrombotic and inflammatory processes in atherosclerosis. Blood 2010; 116: 4317–4327.
16. Peter K, Bobik A. HMGB1 signals danger in acute coronary syndrome: emergence of a new risk marker for cardiovascular death? Atherosclerosis 2012; 221: 317–318.
17. Liu K, Mori S, Takahashi HK, et al. Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. FASEB J 2007; 21: 3904–3916.
18. Birschmann I, Mietner S, Dittrich M, et al. Use of functional highly purified human platelets for the identification of new proteins of the IPP signaling pathway. Thromb Res 2008; 122: 59–68.
19. Libby P. Inflammation in atherosclerosis. Arterioscler Thromb Vasc Biol 2012; 32: 2045–2051.
20. Langer HF, Bigalke B, Seizer P, et al. Interaction of platelets and inflammatory endothelium in the development and progression of coronary artery disease. Semin Thromb Hemost 2010; 36: 131–138.

21. Semple JW, Italiano JE, Jr., Freedman J. Platelets and the immune continuum. Nat Rev Immunol 2011; 11: 264–274.
22. Duerschmied D, Bode C, Ahrens I. Immune functions of platelets. Thromb Haemost 2014; 112: 678–691.
23. Maugeri N, Franchini S, Campana L, et al. Circulating platelets as a source of the damage-associated molecular pattern HMGB1 in patients with systemic sclerosis. Autoimmunity 2012; 45: 584–587.
24. Rouhiainen A, Imai S, Rauvala H, et al. Occurrence of amphoterin (HMG1) as an endogenous protein of human platelets that is exported to the cell surface upon platelet activation. Thromb Haemost 2000; 84: 1087–1094.
25. Maugeri N, Campana L, Gavina M, et al. Activated platelets present high mobility group box 1 to neutrophils, inducing autophagy and promoting the extrusion of neutrophil extracellular traps. J Thromb Haemost 2014; 12: 2074–2088.
26. Yan SF, Yan SD, Ramasamy R, et al. Tempering the wrath of RAGE: an emerging therapeutic strategy against diabetic complications, neurodegeneration, and inflammation. Ann Med 2009; 41: 408–422.
27. Park JS, Gamboni-Robertson F, He Q, et al. High mobility group box 1 protein interacts with multiple Toll-like receptors. Am J Physiol Cell Physiol 2006; 290: C917–924.
28. Harja E, Bu DX, Hudson BI, et al. Vascular and inflammatory stresses mediate atherosclerosis via RAGE and its ligands in apoE-/- mice. J Clin Invest 2008; 118: 183–194.
29. Soro-Paavonen A, Watson AM, Li J, et al. Receptor for advanced glycation end products (RAGE) deficiency attenuates the development of atherosclerosis in diabetes. Diabetes 2008; 57: 2461–2469.
30. Varo N, Libby P, Nuzzo R, et al. Elevated release of sCD40L from platelets of diabetic patients by thrombin, glucose and advanced glycation end products. Diab Vasc Dis Res 2005; 2: 81–87.
31. Gawlowski T, Stratmann B, Ruetter R, et al. Advanced glycation end products strongly activate platelets. Eur J Nutr 2009; 48: 475–481.
32. Denis MM, Tolley ND, Bunting M, et al. Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anucleate platelets. Cell 2005; 122: 379–391.
33. Zimmerman GA, Weyrich AS. Signal-dependent protein synthesis by activated platelets: new pathways to altered phenotype and function. Arterioscler Thromb Vasc Biol 2008; 28: s17–24.
34. Vazzana N, Guagnano MT, Cuccurullo C, et al. Endogenous secretory RAGE in obese women: association with platelet activation and oxidative stress. J Clin Endocrinol Metab 2012; 97: E1726–1730.
35. Fukami A, Adachi H, Yamagishi S, et al. Factors associated with serum high mobility group box 1 (HMGB1) levels in a general population. Metabolism 2009; 58: 1688–1693.
36. Schulz C, Penz S, Hoffmann C, et al. Platelet GPVI binds to collagenous structures in the core region of human atheromatous plaque and is critical for atheroprogression in vivo. Basic Res Cardiol 2008; 103: 356–367.
37. Seizer P, Gawaz M, May AE. Platelet-monocyte interactions--a dangerous liaison linking thrombosis, inflammation and atherosclerosis. Curr Med Chem 2008; 15: 1976–1980.
38. Shah PK, Falk E, Badimon JJ, et al. Human monocyte-derived macrophages induce collagen breakdown in fibrous caps of atherosclerotic plaques. Potential role of matrix-degrading metalloproteinases and implications for plaque rupture. Circulation 1995; 92: 1565–1569.
39. Martinet W, Schrijvers DM, De Meyer GR. Molecular and cellular mechanisms of macrophage survival in atherosclerosis. Basic Res Cardiol 2012; 107: 297.
40. Schiraldi M, Raucci A, Munoz LM, et al. HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. J Exp Med 2012; 209: 551–563.
41. Zernecke A, Schober A, Bot I, et al. SDF-1alpha/CXCR4 axis is instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells. Circulation Res 2005; 96: 784–791.
42. Stellos K, Langer H, Daub K, et al. Platelet-derived stromal cell-derived factor-1 regulates adhesion and promotes differentiation of human CD34+ cells to endothelial progenitor cells. Circulation 2008; 117: 206–215.
43. Stellos K, Bigalke B, Langer H, et al. Expression of stromal-cell-derived factor-1 on circulating platelets is increased in patients with acute coronary syndrome and correlates with the number of CD34+ progenitor cells. Eur Heart J 2009; 30: 584–593.
44. Stellos K, Bigalke B, Borst O, et al. Circulating platelet-progenitor cell coaggregate formation is increased in patients with acute coronary syndromes and augments recruitment of CD34+ cells in the ischaemic microcirculation. Eur Heart J 2013; 34: 2548–2556.
45. Schwarz M, Meade G, Stoll P, et al. Conformation-specific blockade of the integrin GPIIb/IIIa: a novel antiplatelet strategy that selectively targets activated platelets. Circ Res 2006; 99: 25–33.
46. Stoll P, Bassler N, Hagemeyer CE, et al. Targeting ligand-induced binding sites on GPIIb/IIIa via single-chain antibody allows effective anticoagulation without bleeding time prolongation. Arterioscler Thromb Vasc Biol 2007; 27: 1206–1212.
47. Wang X, Hagemeyer CE, Hohmann JD, et al. Novel single-chain antibody-targeted microbubbles for molecular ultrasound imaging of thrombosis: validation of a unique noninvasive method for rapid and sensitive detection of thrombi and monitoring of success or failure of thrombolysis in mice. Circulation 2012; 125: 3117–3126.

