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Autoregulation of thromboinflammation on biomaterial surfaces by a multicomponent therapeutic coating

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1. Introduction

In modern medicine, artificial materials frequently come into contact with blood and other tissue fluids (e.g., in extracorporeal circulation devices used for hemodialysis, hemofiltration, cardiopulmonary bypass (CPB), extracorporeal membrane oxygenation (ECMO), and plasmapheresis). This contact induces a sequence of events involving protein adsorption; in inflammatory reactions, these interactions produce other deleterious effects: Vascular stents elicit fibrosis, restenosis, and thrombosis at the implantation site, and cardiac aids and pumps can trigger thrombotic reactions, leading to emboli.

Simultaneous activation of innate immunity and the thrombotic cascade also occurs during the transplantation of cells, such as islets of Langerhans [3,4], mesenchymal stem/stromal cells (MSC), and hepatocytes [5]. Graft loss results in part from a thromboinflammatory instant blood-mediated inflammatory reaction (IBMIR). This reaction consists of an innate immune attack triggered by activation of the complement and coagulation systems, followed by rapid binding of activated platelets and infiltration of polymorphonuclear leukocytes (PMNs) [3,4]. The corresponding reactions in whole-organ transplantation are ischemia-reperfusion injury and xenogenic/allogeneic antibody-mediated rejection, the major mediator of cell damage during transplantation [6], which is
also triggered by complement activation and thrombotic reactions. Thus, it is important to make surfaces of artificial materials and transplanted cells inert against activation of innate immunity and the thrombotic cascade by regulating thromboinflammation.

Our group has studied the regulation of the coagulation/platelet and complement systems on biomaterial surfaces using various approaches. Nilsson et al. successfully immobilized an ADP-degrading apyrase on substrate surfaces that inhibits both platelet activation and platelet-dependent activation of the coagulation system [7]. Recently, we also identified peptides with high affinity for various domains of human factor H, an abundant plasma protein that regulates complement activation both in solution and on self-surfaces [8]. One of these peptides (5C6) recruited factor H without interfering with its regulatory function, since it bound to a region of this regulator that does not interact with the C3 convertase [8].

We now describe the creation of a combined surface coating that is autoregulatory against thromboinflammation. This surface modification with 5C6 and apyrase can be applied onto substrates (artificial materials) and cells. An amphiphilic polymer, poly(ethylene glycol)-conjugated phospholipid (PEG-lipid) co-immobilizes 5C6 and apyrase on the cell surface, as outlined in Fig. 1. The PEG-lipid derivatives can be immobilized to cell membranes by hydrophobic interactions with the lipid bilayer membranes without either cytotoxicity or a volume increase [9,10]. So far we have previously studied the effect of surface modification of living cells and islets with PEG-lipid derivatives on graft survival during cell transplantation [9]. The other end of the PEG derivative facing the fluid phase can be functionalized to allow binding of peptides, proteins, or oligonucleotides [10]. The PEG-lipid derivative itself is able to suppress coagulation and the inflammatory reactions of the IBMIR to a certain extent [11]. In this paper, we performed various assays to evaluate 5C6 and apyrase function: detection of complement-activation markers on the modified surfaces, hemolytic assays of the complement alternative pathway (AP), and xenogeneic biocompatibility assays of adherent porcine aortic endothelial cells (PAECs) in contact with human whole blood.

2. Materials and methods

2.1. Preparation of factor H and peptides (5C6 and Sc)

2.1.1. Biotinylation of factor H

Human factor H was prepared from human serum [12]. Biotinylation of factor H was performed using biotinamidehexanoic acid N-hydroxysuccinimide ester (Sigma–Aldrich, Inc., St. Louis, USA). A solution of factor H (300 μg/mL in PBS) was mixed with biotinamidehexanoic acid N-hydroxysuccinimide ester (1.76 mM in DMSO) for 30 min at room temperature (RT), followed by dialysis against PBS at 4 °C overnight.

2.1.2. Synthesis of factor H-binding peptide or control peptide

Analogs of the factor H-binding peptide 5C6 (ASSRCTYSHWCSH) were prepared using solid phase peptide synthesis (SPPS) and cyclized via oxidation of its cysteine residues as described previously [8]. For surface attachment, the sequence was expanded at the C-terminus by a short spacer group ([PEG3]2) followed by either

Fig. 1. Surface modification of substrate and cell surfaces with 5C6 and apyrase. (a) Immobilization of biotinylated peptide onto polystyrene surfaces via avidin. (b) Immobilization of thiolated peptide and apyrase onto glass surfaces via maleimide-conjugated PEG. Thiolated factor H-binding peptide 5C6 and apyrase are conjugated to the end of PEG chains via a thiol–maleimide reaction. (c) Chemical structure of 5C6-conjugated PEG-lipid for surface modification of cells (erythrocytes, endothelial cells). (d) Schematic representation of a cell surface modified with 5C6 and apyrase, which are co-immobilized on cell surfaces by incorporation into the lipid bilayer membrane. Factor H is recruited to the surface by 5C6 from human blood to impair complement activation, and apyrase degrades ADP to suppress platelet and coagulation activation.
a free cysteine (SCG-Cys) or a lysine residue, to which a biotin moiety was attached by the addition of EDTA (10 mM, pH 7.5) on ice then centrifuged at 10 000 rpm for 5 min. After re-washing with PBS, the erythrocytes were examined using confocal laser scanning microscopy (LSM510 META, Carl Zeiss, Jena, Germany).

### 2.4. Functional evaluation of SCG-immobilized surfaces

#### 2.4.1. Preparation of SCG-conjugated PEG-lipid and PEG-lipid derivatives

Methoxy-PEG-conjugated phospholipid (ME0-PEG-lipid), Mal-PEG-conjugated phospholipid (Mal-PEG-lipid) and biotin-PEG-conjugated phospholipid (biotin-PEG-lipid) were synthesized as previously described [9]. A solution of SCG or Sc (40 µL, 10 mg/mL in PBS) was mixed with Mal-PEG-lipid (360 µL, 28 mg/mL in PBS) to conjugate the peptide at the end of the PEG chain (to yield SCG-PEG-lipid and SC-PEG-lipid, respectively). Biotin-PEG-lipid and FITC-labeled streptavidin (GE Healthcare) were used in order to confirm incorporation of PEG-lipid derivatives into rabbit erythrocyte surfaces. Rabbit erythrocytes (Hautanaholaboratories, Sweden), 1% in PBS, were mixed with biotin-PEG-lipid (500 µg/mL in PBS) for 30 min at RT, FITC-streptavidin (1:10 diluted in PBS) was added and incubated for 5 min. After re-washing with PBS, the erythrocytes were examined using confocal laser scanning microscopy (LSM510 META, Carl Zeiss, Jena, Germany).

#### 2.4.2. Surface modification of rabbit erythrocytes with SCG-conjugated PEG-lipid

Hemolytic assays of the AP were performed using rabbit erythrocytes [11, 14], which were modified with SCG-PEG-lipid or SC-PEG-lipid to examine the effect of peptide incorporation into cell membranes. Mixtures of ME0-PEG-lipid and SCG-PEG-lipid or Me0-PEG-lipid and Sc-PEG-lipid (molar ratios of 100:0, 99:1, 98:2, 95:5, 90:10, 80:20, 50:50, 20:80, 0:100) were used for the surface modification of rabbit erythrocytes. The total concentrations of PEG-lipid were 0.01, 0.1, 0.5, 2.5, and 12.5 mg/mL, respectively. First, rabbit erythrocytes were washed with PBS until the supernatant was clear. A solution of PEG-lipid mixture was added to the erythrocytes and incubated for 30 min at RT with gentle mixing, followed by washing with MgCl2/EGTA buffer (8 mM MgCl2, 2 mM MgSO4, 1 g/L gelatin in veronal-buffered saline). Then, the suspension containing 0.5% of erythrocytes was mixed with 1/8-diluted human serum in MgCl2/EGTA buffer (100 µL) and shaken for 1 h at 37 °C. The supernatant was then collected by centrifugation, and the absorbance of the supernatants at 405 nm was measured to calculate the percentage of erythrocytes lysis.

After incubation with human serum, the erythrocytes were collected and incubated with 1/5 diluted anti-human factor H antibody for 30 min at RT. After washing with PBS, they were incubated with 1/1000 diluted Alexa488-labeled anti-sheep IgG (Invitrogen, Carlsbad, CA, USA) for analysis by confocal laser scanning microscopy and FACS (BD LSRII, FACSCalibur, BD Biosciences, San Jose, CA, USA).

#### 2.5. Immobilization of apyrase on erythrocytes

##### 2.5.1. Immobilization of apyrase on erythrocytes with Mal-PEG-lipid

Apyrase was immobilized on the surface of erythrocytes (human and rabbit) using Mal-PEG-lipid as previously reported [15]. Thiol groups were introduced into the apyrase by thiolation using Traut’s reagent (Thermo Fisher Scientific, Waltham, MA, USA): Apyrase solution (10 mg/mL, 400 µL from potato, Sigma–Aldrich Inc.) was mixed with Traut’s reagent (10 mg/mL, 66 µL). The solution was incubated with gentle mixing at RT for 1 h. Thiolated apyrase (apyrase-SH) was purified using a spin column (Thermo Fisher Scientific). In order to visually examine the immobilization of apyrase on the erythrocyte surface, we used Alexa 488-labeled apyrase-SH. Prior to thiolation with Traut’s reagent, apyrase was labeled with Alexa Fluor® 488 by using a labeling kit (Invitrogen) according to the manufacturer’s protocol.

Erythrocytes (5 × 1012–5 × 1013 cells) were incubated in solution of Mal-PEG-lipid (100 µL, 10 mg/mL in PBS) for 30 min at RT and then washed three times with cold PBS to obtain erythrocytes modified with Mal-PEG-lipid (Mal-PEG-erythrocyte). To obtain apyrase-erythrocytes, Mal-PEG-erythrocytes were mixed with 50 µL of apyrase-SH solution (600 µg/mL in PBS) and left for 30 min at RT, then washed with cold PBS three times.

##### 2.5.2. Functional evaluation of apyrase on erythrocytes

ATP assay: Rabbit apyrase-erythrocytes (1 × 105, 1 × 107 and 5 × 109 cells/mL) were suspended in PBS containing 2 µM ATP and incubated for 10, 30, or 60 min, then centrifuged. The ATP concentration in the supernatant was determined with a luciferase-based ATP kit (BioThema, Handen, Sweden) according to the manufacturer’s protocol.

Platelet aggregation testing by aggregometry

Blood was drawn into Vacutainer®-evacuated collection tubes containing citrate (Greiner Bio-One GmbH, Kremsmunster, Austria) and centrifuged at 180 g for 20 min at 37 °C.
10 min at 22 °C to prepare platelet-rich plasma (PRP). Apyrase-erythrocytes or erythrocytes (5 × 10^7 cells in 225 µL PRP) were mixed with PRP at 37 °C, and a solution of ADP (5 µM) was added to the PRP and the light transmittance monitored by a Multiplate® aggregometer (Dynabyte Medical, Munich, Germany).

2.5.4. Slide-chamber experiments using whole blood

The slide-chamber model developed by our group [16] was used to evaluate the function of the peptide on substrate and cell surfaces in human whole blood. The chambers and blood-collection materials were coated with heparin according to the Corline method (Corline System AB, Uppsala, Sweden). Whole blood from seven healthy donors was collected into heparin-coated tubes containing soluble heparin (Leo Pharma A/S, Ballerup, Denmark) at a final concentration of 0.05 IU heparin/mL. Human erythrocytes were mixed with the whole blood to a final concentration of 10%. Blood (1.45 mL) was added to each well of the slide chambers, which were covered by poly(vinyl chloride) plates. In addition, blood mixed with PEG-erythrocytes (10% final concentration), with apyrase alone (250 µg/mL), and without additives were used as controls. The chamber was rotated vertically at 22 rpm for 60 min at 37 °C water bath, followed by platelet counting and collection of plasma as described above.

2.6. Co-immobilization of apyrase and SC6 on glass surfaces

2.6.1. Immobilization of apyrase and SC6 onto substrates

A mixed solution of SC6-Cys or SC6-Cys (125 µg/mL) and apyrase-SH (250 µg/mL) was incubated on Mal-PEG-modified substrates for 12 h at 4 °C to immobilize both the peptide and apyrase. The treated surface was used for further experiments after washing with PBS. The apyrase activity on the surface was monitored by the ATP assay described above.

2.6.2. Slide chamber experiments using whole blood

Whole blood from seven healthy donors was collected into heparin-coated tubes without any soluble heparin and then incubated in the slide chambers as described above.

2.7. Co-immobilization of apyrase and SC6 on PAECs (fenogeneric setting)

2.7.1. Surface modification of PAEC cells with SC6-PEG-lipid and MeO-PEG-lipid

Complement activation was measured after exposure of PAECs to human whole blood in the presence and absence of SC6-PEG-lipid in order to assess the function of the peptides. Here a mixture of MeO-PEG-lipid and SC6-PEG-lipid (molar ratios 100:0, 98:2, 95:5, 90:10, and 80:20, total concentrations 0.5 and 5 mg/mL) was used for the surface modification of PAECs. PAECs (a kind gift from Prof Lena Claesson-Welsh) were cultured in F-12 GlutaMAXmedium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in 5% CO2. PAECs (3 × 10^6 cells) were centrifuged at 1000 g for 5 min. After incubation with trypsin/EDTA (Invitrogen) for 3 min at 37 °C, cells were seeded onto APTES-coated glass slides and cultured in the same medium for 2 days. In order to examine the incorporation of PEG-lipid derivatives onto the surface of adherent PAECs, we used biotin-PEG-lipid and FITC-labeled streptavidin as described above. After PAECs were exposed to biotin-PEG-lipid (0.5 mg/mL in medium) for 30 min at RT, FITC-streptavidin (1:10 diluted in PBS) was added and incubated for 5 min. After washing, the PAECs were analyzed by confocal laser scanning microscopy. Adherent PAECs were exposed to the mixture of MeO-PEG-lipid and SC6-PEG-lipid for 20 min at RT, followed by washing with medium, then used for slide chamber experiments. The viability assay was performed by trypan blue exclusion test (viability >95% for PAECs after surface modification).

2.7.2. Surface modification of PAEC cells with peptide and apyrase

Cells were seeded onto APTES-coated glass slides and cultured in culture medium for 2 days. The PAECs were then incubated in peptide-PEG-lipid and Mal-PEG-lipid mixtures (total PEG-lipid: 5 mg/mL; peptide-PEG-lipid: 10 mol%) for 20 min at RT, followed by washing with medium and incubation in a solution of apyrase-SH (250 µg/mL) for 20 min at RT. These PAECs were used for the slide-chamber experiments after washing with medium. To prepare apyrase-immobilized PAECs, PAECs were incubated in a solution of apyrase-SH (250 µg/mL) after being treated with Mal-PEG-lipid (5 mg/mL) for 20 min at RT.

2.7.3. Slide-chamber experiments using whole blood

Whole blood from six healthy donors was subjected into heparin-coated tubes containing soluble heparin to final concentrations of 0.2 or 1.0 IU heparin/mL. After the wells of the chambers were filled with blood (1.65 mL), they were covered by glass slides whose surfaces had been covered with PAECs prepared as described above. The chamber was rotated vertically at 22 rpm for 30 min in a 37 °C water bath, followed by platelet counting and collection of plasma as described above.

After exposure of the PAEC-coated glass slides to whole blood, they were washed with PBS and then immersed in 4% paraformaldehyde (Apoteket) and incubated with anti-human factor H antibody (diluted 1:10) for 30 min at RT. After a PBS wash, they were incubated with Alexa488-labeled anti-sheep IgG, then examined by confocal laser scanning microscopy.

2.7.4. Measurement of C3a, C5a, and TAT in plasma

C3a, C5a, sC5b-9, and TAT in plasma were measured by conventional sandwich EIA. For soluble C3a, plasma was diluted 1:500 to 1:6000 in working buffer (PBS containing 0.05% Tween 20, 10 mg/mL BSA, and 10 mM EDTA). As previously reported [17], C3a was captured by anti-human C3a mAb 4SD17.3 and detected by biotinylated polyclonal rabbit anti-C3a antibody and HRP-conjugated streptavidin. Zymosan-activated serum, calibrated against purified C3a, served as a standard. Values were expressed as ng/mL. C5a was analyzed with a commercial kit (HyCult Biotechnology, Uden, The Netherlands) according to their protocol. Samples were diluted 1:5–1:25, and values were expressed as ng/mL.

2.7.5. Statistical analysis

Results are presented as means ± SEM. Data plotting and statistical analysis were performed using Prism version 5.04 for Macintosh software (Graphpad, San Diego, CA, USA). Differences between means of two groups were statistically evaluated using the paired Student’s t-test or repeated measures one-way ANOVA with Dunnett’s post hoc test when more than two groups were being compared to a control. Data were assumed to be normally distributed.

3. Results

3.1. Properties of SC6-immobilized surfaces in lepirudin-plasma

Factor H-specific (SC6) and scrambled control (Sc) peptides were immobilized onto polystyrene and glass surfaces through biotin/avidin reactions and covalent thiol–maleimide bonding via the PEG chain, respectively (Fig. 1a,b). The modified surfaces were compared regarding factor H binding, both from lepirudin plasma and in purified form. SC6 coated surfaces bound significantly higher amounts compared to Sc, and the binding was dependent on peptide density (Supplemental Fig. 1a–e). The modified surfaces were incubated with lepirudin-anticoagulated whole blood and complement activation (generation of C3a, C5a, sC5b-9) and granulocyte activation (expression of CD11b) was monitored. Coating with SC6 significantly reduced all complement activation makers when compared to the control and lowered granulocyte activation via the AP (Supplemental Fig. 2a–e). Taken together, these results indicate that factor H, captured by SC6 immobilized on surfaces, can suppress complement activation in human whole blood.

3.1.1. Effect of erythrocyte bound SC6 on hemolytic assays

Next, SC6 was incorporated onto the erythrocyte surface by using a PEG-lipid derivative that could be anchored to the cell membrane through hydrophobic interactions (Fig. 1c,d) [9,10]. The PEG-lipid derivative on erythrocytes was detected using fluorescent streptavidin and biotin-PEG-lipid (Supplemental Fig. 3a). The modified erythrocytes were then incubated with human serum and subjected to immunostaining for factor H, followed by FACS analysis (Fig. 2a, Supplemental Fig. 3b). Confocal microscopy revealed fluorescent staining on the erythrocyte surfaces modified with SC6-PEG-lipid/MeO-PEG-lipid but not on those treated with SC-PEG-lipid/MeO-PEG-lipid; and FACS analysis yielded similar results. Next, we performed hemolytic assays to examine the effect of SC6 on complement activation via the AP, by incubating rabbit erythrocytes with human serum [13,14]. The optimal concentration of SC6 to coat the
erythrocyte surfaces was determined by mixing inert MeO-PEG-lipid with 5C6-PEG-lipid at different proportions. Under uninhibited conditions, approximately 99% of the rabbit erythrocytes were hemolyzed after incubation with human serum. However, the lysis (calculated as \( y/(1-y) \), where \( y \) is the fraction lysed [18]) was suppressed by surface modification with PEG-lipid and was further reduced in the presence of 5C6, as compared to Sc-modified groups (Fig. 2b, Supplemental Fig. 3c). As the PEG-lipid concentration increased, the difference between 5C6-immobilized erythrocyte and Sc-immobilized erythrocyte became smaller as seen in Fig. 2b. The effect of PEG can be also seen clearly as the surface density of PEG is larger. Under optimal conditions, 5C6 was able to specifically recruit functional factor H from human serum and suppress AP-mediated complement activation on the erythrocyte surface.

3.2. Effect of 5C6 on complement activation on the surface of PAECs

PAECs were treated with a mixture of 5C6-PEG-lipid and MeO-PEG-lipid (total PEG-lipid concentrations: 0.5 or 5 mg/mL) and exposed to human whole blood. After exposure to blood, the PAECs were subjected to immunostaining for factor H (Supplemental Fig. 4b), which could be detected on the PAEC surfaces modified with 5C6-PEG-lipid/MeO-PEG-lipid. The generation of C3a and sC5b-9 by PAEC surfaces modified with MeO-PEG-lipid was generally lower when compared to the control PAECs, indicating that the PEG coating by itself protected the cell surfaces from complement activation. However, a significantly greater reduction of C3a and sC5b-9 markers was seen in the presence of 5C6 on the PAEC surfaces, with an optimal effect at peptide concentrations of 2e10% (Supplemental Fig. 4c,d). In contrast, the platelet count did not decrease in either control PAECs or PAECs modified with PEG-lipid, and no differences were seen in TAT due to the presence of heparin, which can suppress coagulation (Supplemental Fig. 4e,f). Collectively, these results indicate that 5C6 was able to specifically recruit factor H from human blood, thereby suppressing complement activation on the PAEC surface.

3.3. Effect of apyrase on coagulation on the surface of erythrocytes

Next we immobilized apyrase on erythrocytes by using Mal-PEG-lipid and Alexa 488-apyrase-SH, as shown in Fig. 1d. Fluorescence
was clearly observed at the periphery of erythrocytes treated with Mal-PEG-lipid and Alexa 488-apyrase-SH, but not those treated with Alexa 488-apyrase-SH alone (Fig. 3a). No morphological changes were observed after immobilization of apyrase.

The effect of the erythrocyte-bound apyrase on ATP decay and ADP-induced platelet aggregation was then tested. Apyrase-immobilized erythrocytes degraded exogenously added ATP in a dose-dependent manner (Fig. 3b). In addition, ADP-induced platelet aggregation was effectively suppressed in the presence of apyrase-erythrocytes, whereas no suppression was seen in non-modified erythrocytes (Fig. 3c). These results indicate that apyrase had been immobilized with retained activity.

When human whole blood was rotated in the slide chamber, the platelet count gradually decreased with time, reflecting coagulation and platelet activation. There was less decrease in platelet count in whole blood mixed with apyrase-erythrocytes and apyrase, whereas PEG-erythrocytes showed a reduction similar to that of whole blood (Supplemental Fig. 5a) with TAT generation following a similar trend (Supplemental Fig. 5b). In contrast, there were no differences in complement activation markers among the groups

![Fig. 3. Activity of apyrase immobilized to erythrocyte surfaces. (a) Immobilization of Alexa 488-labeled apyrase on rabbit erythrocytes using surface modification with PEG-lipid (left). The control consisted of erythrocytes mixed with Alexa 488-labeled apyrase (right). Representative pictures taken by confocal laser-scanning microscopy. (b) ATPase activity of apyrase immobilized on erythrocytes (n = 3). Data shown are means ± SEM. Repeated measures one-way ANOVA with Dunnett’s post hoc-test was used to compare all groups with apyrase-negative erythrocytes. *** p < 0.001. (c) ADP-induced platelet aggregation in PRP in the presence of apyrase-immobilized human erythrocytes, as monitored by aggregometry.](image)
Supplemental Fig. 5c,d), indicating that the immobilized apyrase did not affect complement. These results demonstrate that in whole blood, apyrase immobilized on cell surfaces is able to inhibit platelet activation as well as suppressing coagulation activation.

3.4. Effect of co-immobilized 5C6/apyrase on the activation of complement and coagulation in whole blood

5C6 and apyrase were co-immobilized onto the substrate surfaces to investigate their combined effect. We initially examined the ATPase activity of 5C6/apyrase surfaces to ensure that the apyrase was not inhibited by the presence of 5C6 (Supplemental Fig. 6). When the co-immobilized substrate surfaces were exposed to blood in the slide chamber, the platelet consumption was significantly lower for 5C6/apyrase-, Sc/apyrase-, and apyrase-immobilized surfaces than for the control PEG surface (Fig. 4a). TAT levels showed similar trends, indicating that co-immobilized apyrase can suppress activation of platelets and coagulation (Fig. 4b). Markers of complement activation (C3a, sC5b-9) were significantly reduced on the 5C6/apyrase surface when compared to the Sc/apyrase, apyrase, and PEG surfaces (Fig. 4c, d), indicating that complement activation was suppressed on the co-immobilized 5C6 surfaces.

Finally, the cell surfaces of PAECs were modified with both peptide and apyrase using Mal-PEG-lipid (Fig. 1d), and the adherent PAECs were tested in the slide-chamber model. Both platelet consumption and TAT levels were significantly lower for 5C6/apyrase-, Sc/apyrase- and apyrase-modified cell surfaces than for the control PAEC surfaces (Fig. 5a,b). In addition, C3a and sC5b-9 were significantly reduced on the 5C6/apyrase modified cell surfaces when compared to the other groups (Fig. 5c,d), and factor H could be detected by immunostaining on the PAEC surfaces modified with 5C6 and apyrase (Fig. 5e). Collectively, these results show that factor H is recruited to the surfaces of substrates, and that PAEC surfaces, modified with 5C6/apyrase, can suppress both coagulation and complement activation in whole blood.

4. Discussion

In this paper, we have demonstrated in two human whole-blood models that activation of platelets, coagulation and complement systems can be controlled simultaneously by co-immobilizing the
factor H-binding peptide 5C6 and apyrase on both biomaterial and cell surfaces.

The complement system is controlled by regulators of complement activation (RCA), which mainly regulate classical pathway (CP) and AP C3 convertases. In addition to plasma membrane regulators (MCP, DAF, and CD59), the plasma proteins, C4-binding protein (C4BP) and factor H also regulate surface-bound CP and the AP convertases, respectively. Both these fluid-phase regulators are recruited to host cells by binding to cell-surface glycosaminoglycans [19,20] to regulate complement activation through the CP and AP, respectively. The concept of exploiting those complement-regulatory effects on foreign surfaces has been demonstrated by our group [21]: Engberg et al. have used streptococcal M protein-derived peptides to specifically recruit human C4BP to substrate surfaces, thereby reducing complement activation via the CP and suggesting RCA-binding peptides as promising.

**Fig. 5.** Attenuation of platelet, coagulation, and complement activation on 5C6/apyrase-co-immobilized PAECs in whole blood. Whole blood (0.2 IU/mL heparin) was incubated on control PAECs, or apyrase-immobilized, 5C6/apyrase, or Sc/apyrase-co-immobilized PAECs for 30 min at 37°C (n = 6). The figures show (a) platelet consumption and generation of TAT, (b) C3a, and (d) sC5b-9. (e) Immunofluorescence staining of factor H on PAEC surfaces modified with 5C6/apyrase (left), Sc/apyrase (middle), or apyrase (right) after incubation in whole blood. Representative pictures taken by confocal laser-scanning microscopy. Repeated measures one-way ANOVA with Dunnett’s post hoc test was used to compare all groups versus control (a–b) and apyrase/5C6 (c–d), **, p < 0.01, ***, p < 0.001.
regulators of complement activation both on cell and material surfaces. Several studies have indicated that complement activation on material surfaces is initiated through the CP [22,23]. However, the AP C3 convertase is of major importance because of its ability to amplify the reaction on the surface [24–26], thereby triggering various inflammatory reactions. Therefore, it seems of major importance to regulate the AP C3 convertase in order to avoid complement attack.

We earlier examined the possibility to suppress complement activation through the AP by covalent immobilization of factor H on substrate surfaces [12,27]. Although this approach is promising, it has limitations: First, the protein binds randomly to the surface, so that only a fraction of the protein maintains an active conformation. Second, large amounts of factor H are needed to coat the surface. In our approach, 5C6-coated cells and materials specifically recruit factor H from host blood, thereby mimicking the host surface and protecting themselves from attack by complement [28,29]. Coating with a small peptide also allows us to avoid the costly and cumbersome preparation and immobilization of recombinant proteins. By using 5C6, we can avoid random binding of the protein and instead obtain a consistent orientation and configuration of the proteins: The optimal surface density of 5C6 for hemolytic assays was found to be at a maximum of 105, including spatial requirements of the interaction between factor H and C3b. Presumably, since factor H has a functional domain regions at each of its termini, access to C3b may be limited by steric hindrance at higher densities.

Platelets are intimately involved in the incompatibility processes that occur on foreign surfaces and are directly activated by surfaces [30]. Not only are platelets closely connected to the coagulation cascade, but they can also interact with leukocytes and trigger activation of complement, thereby acting as an important hub that mediates crosstalk between these components [30,31]. When platelets are activated, a multi-step process begins that involves adhesion, aggregation, contraction, and secretion. Released ADP is essential for recruiting and aggregating platelets; therefore, it is an obvious target for platelet inhibition [32]. In order to regulate platelet activation, vascular ADP is continuously degraded under homeostatic conditions by CD39 (NTPDase-1, EC 3.6.1.5), an enzyme present on human endothelial cells [33]. In the present study, we used the homologous potato apyrase as a prototype enzyme to obtain proof-of-concept that this group of enzymes can be a valuable tool for attenuating thrombotic reactions on material and cell surfaces.

Recent studies have shown that NTPDase-1 (CD39) is both anti-inflammatory and immunosuppressive, and that extracellular adenosine signaling modulates neutrophil accumulation, particularly during conditions of acute injury (e.g., ambient hypoxia) [34–37]. In such conditions, extracellular adenosine is mainly derived from the enzymatic phosphohydrolysis of precursor nucleotides to adenosine [38,39]. This two-step enzymatic process involves the ecto-apyrase CD39 (converting ATP/ADP to AMP) and the ecto-5 nucleotidase CD73 (converting AMP to adenosine). Both enzymes have been implicated in attenuating acute injury and inflammation in models of ambient hypoxia [34], cyclic mechanical stretch [40], and bleomycin-induced lung injury [41]. Studies of NTPDase1-deficient mice have identified an important role for this enzyme in controlling acute immune responses [42]. NTPDase1-KO mice demonstrate exacerbated inflammation in experimental models of hypoxia, endotoxin-induced lung inflammation, and colitis [34,40]. Similarly, humans carrying a single specific point mutation in the NTPDase1 gene have recently been shown to be more susceptible to inflammatory bowel disease [40]. These studies suggest that the use of CD39 would also have an anti-inflammatory and immunosuppressive effect in cell and whole-organ transplantation.

5. Conclusions

In summary, our combined coating consists of three major components for protection of the underlying surface: 1) the PEG linker, 2) the factor H-binding peptide 5C6, and 3) the ADP-degrading apyrase. Factor H was specifically recruited to the 5C6-modified surfaces and inhibited complement attack when they were exposed to human whole blood. In addition, activation of platelets and coagulation was efficiently attenuated, by degrading ADP. Thus, by inhibiting thromboinflammation using a multicomponent approach, we have created a hybrid surface with the potential to greatly reduce incompatibility reactions involving biomaterials and therapeutic cells.

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J.D.L. is the inventor of the factor H-binding peptide 5C6 and is the founder of Amyndas Biotherapeutics, which performs clinical development of 5C6 for various indications. None of the other authors have conflicts of interest.

Appendix A. Supplementary data

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