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Binding of a model regulator of complement activation (RCA) to a biomaterial surface: surface-bound factor H inhibits complement activation

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Abstract

The complement system is an important inflammatory mediator during procedures such as cardiopulmonary bypass and hemodialysis when blood is exposed to large areas of biomaterial surface. This contact between blood and the biomaterials of implants and extracorporeal circuits leads to an inflammatory response mediated by the complement system. The aim of this study was to assess the ability of a complement regulator (factor H) immobilised on a biomaterial surface to inhibit complement cascade mediated inflammatory responses. The cross-linker *N*-succinimidyl 3-(2-pyridyldithio) propionate was used to immobilise factor H on a model biomaterial surface without affecting the biological activity of the inhibitor. Binding of factor H was then characterised using quartz crystal microbalance-dissipation (QCM-D) and enzyme immunoassays for products of complement activation: bound C3 fragments and soluble C3a, sC5b-9, and C1s-C1INA. Immobilised factor H reduced the amount C3 fragments deposited on the biomaterial surface after incubation with serum, plasma, or whole blood. In addition, lower levels of soluble C3a and sC5b-9 were generated after incubation with whole blood. In summary, we have demonstrated that complement activation on a highly activating model surface can be inhibited by immobilised factor H and have defined prerequisites for the preparation of future biomaterial surfaces with immobilised regulators of complement activation.

Keywords: Biocompatibility; Blood; Complement activation; Factor H

1. Introduction

Contact between blood and a biomaterial surface triggers an inflammatory response against the material. Whole blood–biomaterial contact takes place in many different types of devices, including oxygenators, plasmapheresis equipment, hemodialysers, catheters, stents, vascular grafts, miniature pumps, sensors, and heart aids [1,2]. Contact between blood and biomaterial also occurs, at least initially, during the implantation of biomaterials into soft and hard tissues.

Although progress has been made in reducing side effects, many procedures are still associated with undesirable inflammatory responses. In particular, during cardiopulmonary bypass (CPB) and hemodialysis, exposure of large areas of biomaterials to blood gives rise to a systemic inflammatory response. A major challenge is CPB, during which blood comes in contact not only with a biomaterial but also with the gas surface [3]. A second complicating factor is the ischemia/reperfusion damage that occurs during and after the treatment. These factors generate an inflammatory response which may cause hemostatic problems leading to neurological deficiencies, cognitive disturbances and, in the worst cases, organ failure [4,5]. Hemodialysis is also associated with whole body inflammation, which is thought to contribute to the accelerated arteriosclerosis that occurs in these patients [6,7].

Many of these adverse reactions are related to activation of the cascade systems of which the complement

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Abbreviations: SPDP — *N*-succinimidyl 3-(2-pyridyldithio) propionate; RCA — regulator of complement activation; QCM-D — quartz crystal microbalance-dissipation; EIA — enzyme immunoassay; PAV — polyallylamine

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system is an important element [8]. Complement activation causes activation of leukocytes and platelets, and conversely, inhibition of its activity leads to reduced platelet and leukocyte activation [9–12]. Heparin coatings have been used to reduce the activation of the coagulation and the complement systems on biomaterials surfaces in contact with whole blood. However, the extent to which they decrease complement activation is not always satisfactory, particularly with regard to bound complement fragments [13,14]. Conjugation of a surface with biologically active inhibitors of complement activation is a potential approach to lowering the complement activation on the material surfaces.

Factor H is a serum protein of 150 kDa which belongs to a family of soluble and membrane-bound regulators of complement activation (RCA). It has a fibrillic structure consisting of 20 short consensus repeats (SCR) [15]. The size of factor H is 495 Å × 34 Å, but the protein has a high degree of flexibility and by electron microscopy has been shown to be folded back on itself [16]. Factor H inhibits complement activation by two mechanisms: First, by displacing of factor Bb from the alternative pathway convertase (decay accelerating activity) and, second, by serving as a cofactor of factor I, which mediates cleavage of C3b [17–19]. There have also been reports that factor H inhibits the classical pathway by interfering with the C1 complex; however, the importance of this interaction has not been fully investigated [20,21]. The aim of the present study was to covalently bind factor H to a model biomaterial surface in order to evaluate the ability of surface-bound factor H to inhibit complement activation on a biomaterial surface in contact with whole blood.

2. Materials and methods

2.1. Purification of factor H

Following precipitation of euglobulins, factor H was isolated from 2 units of fresh frozen plasma essentially according to the protocol of Hammer et al. The plasma was pooled from two individuals [22,23].

2.2. SPDP conjugation of proteins

Factor H or control bovine serum albumin (BSA; Intergen Company, Oxford, UK), at approximately 0.5 mg/ml, in 10 mM phosphate-buffered saline pH 7.4 (PBS), was mixed with an equal volume of 0.1 M phosphate buffer, pH 7.9. After addition of 2% (v/v) 2 mg/ml of *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP; Pierce Chemical Company, Rockford, IL, USA) in methanol, the mixture was incubated for 30 min at room temperature (RT). The unconjugated SPDP was removed on a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated in PBS.

2.3. Immobilisation of proteins on polystyrene

SPDP was immobilised on the surface of polystyrene Nunc-Immuno F96 MaxiSorp plates (Nunc A/S, Roskilde, Denmark). Unless stated otherwise, all incubations were performed at RT. The plates were washed three times with water from a MilliQ UF plus (Millipore AB, Sundbyberg, Sweden) after each incubation step.

1. To provide primary amines the surface was coated with a high molecular weight polyallylamine, PAV (Corline Systems AB, Uppsala, Sweden) which had been modified to increase its adhesiveness. PAV (0.025 mg/ml in 25 mM borate buffer, pH 9.0) was incubated with the microtitre plates for 15 min.
2. The plates were dried at 50°C for 20 min, and 2 mg/ml SPDP in methanol was bound to the primary amines by incubation for 30 min.
3. Excessive amino groups were blocked by incubation with acetic anhydride (10 μl/ml in 25 mM borate buffer, pH 10.5) for 5 min.
4. The SPDP bound to the surface was reduced by incubation with 10 mg/ml dithiothreitol (DTT) in water for 30 min. The surfaces were washed six times with water to produce the activated surfaces (thereafter referred to as the model surface).
5. Native or SPDP-conjugated factor H or BSA, at the desired concentrations, was incubated in PBS with the plates for 1 h.
6. The plates were washed three times with washing buffer (PBS containing 0.05% (v/v) Tween 20 and 0.02% (v/v) Antifoam (Pharmacia, Uppsala, Sweden)) and three times with PBS. Thereafter, the plates were stored in PBS at 4°C until use.

2.4. Preparation of blood, plasma, and serum for complement assays

Serum was obtained from blood drawn from healthy blood donors which was allowed to clot for 1 h in glass tubes or for 1.5 h in polystyrene tubes and then centrifuged at 2000*g* for 10 min. Serum from 15 different individuals were pooled. Alternatively, whole blood was collected in polystyrene tubes with a Corline heparin surface (Corline AB) and containing 3 IU/ml of soluble heparin (Bio Iberica, Barcelona, Spain) [14,24]. This blood was either incubated in surface-modified plates as described below, or centrifuged at 2000*g* for 10 min to obtain plasma. Plasma and serum were stored at −70°C.

2.5. Complement activation

Samples of plasma or serum (150 μl, diluted in veronal-buffered saline (VBS), containing 0.15 mM Ca^{2+} and

0.5 mM Mg^{2+}) or whole blood (250 μl), were incubated at 37°C for the desired time in polystyrene wells coated with factor H or BSA. After incubation, the samples were transferred to tubes containing EDTA at a final concentration of 10 mM and centrifuged at 2000*g* for 10 min. The samples were frozen at −70°C pending analysis. In some experiments the complement inhibitors EDTA, Mg-EGTA (0.2 M EGTA, 50 mM $MgCl_2$) and Compstatin were added to the serum at a final concentration of 10 mM, 10 mM, and 55 μM, respectively, prior to incubation. Compstatin was a kind gift from Dr J.D. Lambris, Dept. of Pathology, University of Pennsylvania, Philadelphia, USA. It is a cyclic synthetic peptide which is known to preferably inhibit the alternative pathway of complement at this concentration [12,25].

2.6. Enzyme immunoassays (EIA) for the detection of factor H and C3 fragments bound to a surface and fluid-phase C3a, C1s-C1INA, and sC5b-9 complexes

In each EIA washing buffer and working buffer (i.e. washing buffer containing 1% (w/v) BSA, were used. The antibodies used for detection were biotinylated using biotin-amidocaproate *N*-hydroxysuccimide ester (Sigma Chemical Co, St Louis, MO, USA) as described previously [26]. For all assays the plates were stained with colour solution consisting of 10 mg phenylendiamine-dihydrochloride (Sigma) in 40 ml 0.1 M citrate–phosphate buffer (pH 5.0) with 10 μl 30% H_2O_2, for approximately 5 min and the absorbance at 492 nm was measured. Details of each EIA are given below:

2.6.1. Surface-bound factor H

After conjugation with factor H the plates were washed three times with washing buffer. The wells were saturated with 300 μl of working buffer at 37°C for 30 min, and then 200 μl of anti-factor H diluted 1/250 (The Binding Site, Birmingham, UK) followed by biotinylated anti-factor H diluted 1/250 in working buffer were added to the plates and incubated for 30 min at 37°C, respectively. Antibody was detected with 100 μl HRP-conjugated streptavidin (Amersham, Buckinghamshire, UK) diluted 1/500 and incubated for 30 min at 37°C, followed by staining.

2.6.2. Surface-bound C3 fragments

After incubation with whole blood, serum or plasma as described above, the plates were washed three times with washing buffer. The wells were saturated with 300 μl of working buffer at 37°C for 15 min, then incubated with 120 μl of anti-C3c diluted 1/200 (Dako AS, Glostrup, Denmark) and horseradish peroxidase (HRP)-conjugated anti-C3c diluted 1/400 (Dako) in working buffer for 30 min at 37°C, followed by staining as described above.

2.6.3. C3a

Plasma was diluted 1/1000 in working buffer and analysed as described previously [27]. The mAb 4SD17.3 was used as the capture antibody. Bound C3a was detected with biotinylated rabbit anti-C3a diluted 1/150, followed by HRP-conjugated streptavidin 1/500. Zymosan-activated serum, calibrated against a solution of purified C3a, served as standard, and the values are given as ng/ml.

2.6.4. C1s-C1INA

Microtitre plates were coated overnight with anti-C1s antibodies (INCSTAR, Stillwater, MN, USA) diluted 1/200 at 4°C. The plates were then saturated with 300 μl working buffer for 30 min at 37°C, and 100 μl of sample, diluted to a final serum concentration of 1/200 in working buffer containing 10 mM EDTA, was incubated for 1 h at RT. Zymosan-activated serum served as a positive control. For the detection of C1s-C1INA complexes, 100 μl biotinylated anti-C1INA (Dako) diluted 1/200 and incubated for 1 h at RT, and 100 μl HRP-conjugated streptavidin (Amersham) diluted 1/500 was then added and incubated with the plates for 1 h at RT, followed by staining.

2.6.5. sC5b-9

Plasma was analysed for sC5b-9 using a modification of the EIA described by Mollnes and coworkers [24,28]. Plasma diluted 1/5 was added to microtitre plates coated with anti-neoC9 mAb MCaE11. sC5b-9 was detected by a polyclonal anti-C5 antibody diluted 1/500 (Dako), followed by HRP-conjugated anti-rabbit immunoglobulin diluted 1/500 (Dako). Zymosan-activated serum defined to contain 40 000 arbitrary units was used as a standard.

2.7. Quartz crystal microbalance-dissipation (QCM-D) analysis

The QCM-D technique relies on the fact that a mass adsorbed onto the sensor surface of a shear mode oscillating quartz crystal causes a proportional change in its resonance frequency. When the adsorbed material is non-rigid, an additional energy dissipation (viscous loss) is also induced.

Analysis of adsorption kinetics, by simultaneous measurement of both the frequency, f, and the energy dissipation, D, was performed on a QCM-D instrument (Q-Sense AB, Gothenburg, Sweden) which is described in detail elsewhere [29]. 5 MHz sensor crystals, spin-coated with hydrophobic polystyrene, were used. Changes in D and f were measured on both the fundamental frequency ($n = 1$, i.e. $f \approx 5$ MHz) and the first overtone ($n = 3$, i.e. $f \approx 15$ MHz).

Changes in the frequency, f, reflect the amount of mass coupled to the surface of the crystal. For thin, evenly

distributed and rigid films, an adsorption-induced frequency shift (Δf) is related to mass uptake (Δm) via the Sauerbrey equation [30], $\Delta f = -(n/C)\Delta m$, where C (equivalent to 17.7 ng cm^{-2} Hz^{-1}) is the mass sensitivity constant and n ($= 1, 3, \ldots$) is the overtone number. However, for proteins adsorbed from the aqueous phase, one must also be aware that water hydrodynamically coupled to the adlayer is included in the measured mass uptake [31]. The dissipation factor (D) reflects frictional (viscous) losses induced by deposited materials such as proteins adsorbed on the surface of the crystal. Hence, changes in the viscoelastic properties of adlayers (e.g. induced by conformational changes) as well as differences between various protein–surface interactions can be monitored [32,33].

In cases in which the adsorption induces a significant energy dissipation, a quantitative analysis of the data is desirable [34]. In the present work we have applied a viscoelastic model developed by Voinova et al. [35]. In brief, the model describes the response of the QCM-D sensor crystal to a viscoelastic layer covered by a Newtonian liquid under no-slip conditions. In this model, the adlayer is represented by a film defined by a uniform thickness, δ, density, ρ, shear viscosity, η, and shear elastic modulus, μ. Using the approach described by Höök et al. [36], the properties of the added layer can be obtained from the measured changes in frequency and dissipation if both the fundamental frequency (5 MHz) and the overtone (15 MHz) are measured. One must be aware, however, that the representation of the protein film with uniform thickness, density, shear elastic modules and shear viscosity to some extent is a simplification of the true film. Accordingly, the estimated values must be regarded as effective values describing the average properties of the adlayer.

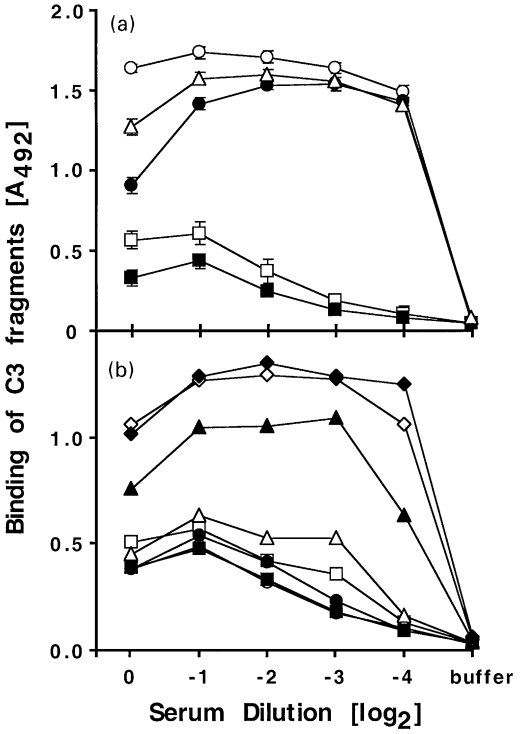

Fig. 1. Effect of immobilised factor H and BSA on the binding of C3 fragments to a model surface. Serial two-fold dilutions of pooled human serum were incubated for 30 min at 37°C in wells bearing immobilised native or SPDP-conjugated factor H or BSA as described in Materials and Methods. Binding of C3 fragments was measured by EIA with absorbance being read at 492 nm. In panel a each point represents duplicates and in panel b single samples. Panel a, binding to immobilised factor H or BSA (220 pmol/well): native non-conjugated factor H (–■–); SPDP-conjugated factor H (–□–); no protein (–△–); native BSA (–●–); SPDP-conjugated BSA (–○–). Panel b, effect of adding varying amounts of immobilised native factor H per well: 100 pmol (–□–); 110 pmol (–■–); 55 pmol (–○–); 28 pmol (–●–); 14 pmol (–△–); 7 pmol (–▲–); 3 pmol (–◇–); no factor H (–♦–).

3. Results

3.1. Effect of various forms of immobilised factor H on the binding of C3 fragments

To assess the binding of C3-fragments to immobilised factor H, model surfaces (polystyrene microtitre plates) bearing immobilised SPDP and treated with DTT were reacted with factor H, SPDP-factor H, BSA and SPDP-BSA; a non-protein treated surface served as a further control. Incubation of the plates with serial dilutions of pooled human serum, followed by EIA measurements of the amount of bound C3 fragments was assessed revealed that both native and SPDP-conjugated factor H efficiently inhibited binding of C3 fragments to the surface at all serum concentrations tested (Fig. 1a). In contrast, neither native nor SPDP-conjugated BSA had any effect on the binding; similarly no inhibitory effect was seen for the control surface bearing immobilised SPDP alone.

We then identified the optimal concentration of factor H for inhibiting the binding of C3 fragments by varying the amount of factor H (0–220 pmol per well): The concentration of factor H could be lowered to 14 pmol per well before the level of C3 fragment binding began to increase (Fig. 1b). Similar experiments using SPDP-treated factor H showed that the dose requirements were similar or higher (data not shown). Because of its efficient inhibition of the binding of C3 fragments, non-SPDP-treated (native) factor H (at 100 pmol per well) was therefore used for the rest of this study.

3.2. Characterisation of surface-bound factor H

Having established that surface-bound factor H could inhibit the binding of C3 fragments to a model surface, we next characterised the immobilisation of factor H in greater detail. Surfaces were prepared with or without SPDP (Section 2.3, step 2, in Materials and methods) and

were then incubated with or without DTT (step 4), resulting in two variants each of the acetylated amine surface and the SPDP-conjugated acetylated amine surface, respectively. We then used an EIA for factor H and the QCM-D technique to analyse the binding of factor H to these surfaces.

The results of four EIA analysis indicated that when diluted in PBS, factor H bound to all four types of surfaces (Fig. 2b); however, if 0.05% Tween (washing buffer) was added to the factor H preparation and all the washes, there was no binding to the acetylated amine surfaces without SPDP (Fig. 2a). Furthermore, the binding in Tween was greater to the non-DTT-treated SPDP surface than to the DTT-treated one. Thus the most effective binding of factor H was to the non-DTT-treated SPDP-conjugated surface, in the presence and absence of Tween.

The binding characteristics of factor H to the two SPDP conjugated surfaces were then investigated in detail with the QCM-D technique. The binding kinetics as reflected by both the frequency change, Δf, and the dissipation change, ΔD, versus time are shown in Fig. 3.

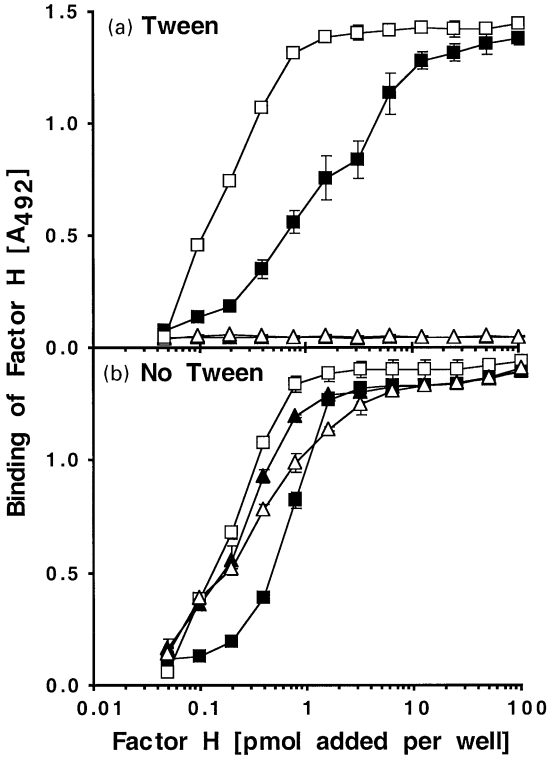

Fig. 2. Immobilisation of factor H to various surfaces as detected by EIA. Factor H was immobilised at varying concentrations (from 0 to 100 pmol factor H per well) to surfaces prepared with or without SPDP, in the presence or absence of DTT. Factor H was reacted in the presence of DTT with the amine surface prepared without SPDP (–▲–) or conjugated with SPDP (–■–). Factor H was also allowed to interact with these same surfaces without DTT treatment (–□–, with SPDP; –△–, without SPDP). In panel a, factor H was in PBS containing 0.05% Tween; in panel b, factor H was in PBS.

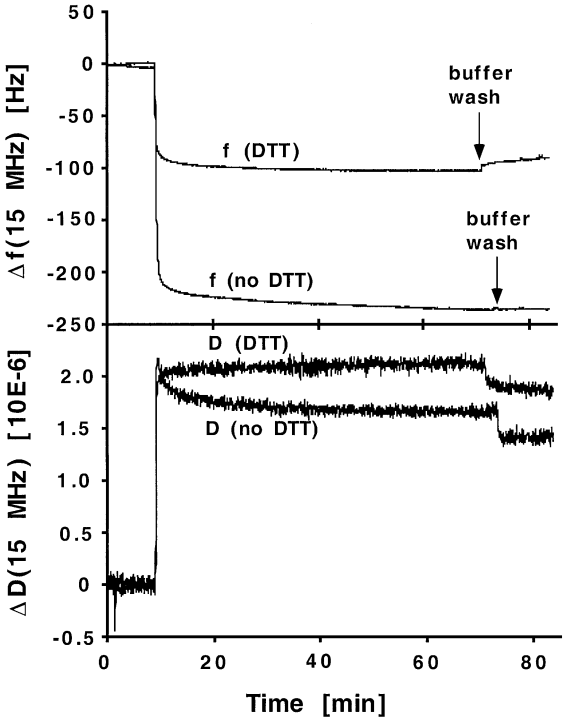

Fig. 3. Binding of factor H (1100 pmol/ml) to a SPDP-conjugated polystyrene surface detected by QCM-D. Upper panel: monitoring of factor H binding (changes in frequency, Δf) to a surface treated with DTT and to one which was not treated. Lower panel: the dissipation changes, ΔD, for the binding of factor H to the surfaces shown in the upper panel. Only the data for the overtone (15 MHz) are shown.

The analyses confirmed that the non-DTT-treated surface-bound more factor H, Δf (15 MHz) = −234 Hz (1381 ng/cm^2), than did the DTT-treated one, Δf (15 MHz) = −93 Hz (549 ng/cm^2). Despite binding more factor H, the change in D was smaller (ΔD (15 MHz) = 1.4×10^{-6} for the non-DTT treated surface than for the DTT-treated surface (1.9×10^{-6}), indicating that the layer formed by factor H was less elastic and viscous, and thus was more rigid, on the non-DTT-treated surface (Fig. 3). The differences in the properties of the complete factor H layers (after the buffer wash), quantified according to the viscoelastic model, are shown in Table 1.

3.3. *Effect of immobilised factor H on classical and alternative pathway activation*

In order to investigate which activation pathway was affected by immobilised factor H, experiments were performed with pooled human serum and specific complement inhibitors. The generated activation fragments were monitored using EIAs.

Incubation of human serum in SPDP-treated wells bearing immobilised factor H or BSA showed the deposition of C3 on immobilised factor H was delayed for about 15 min when compared to SPDP-conjugated BSA.

Table 1
Parameters describing the factor H layer on the DTT-treated and the non-DTT-treated SPDP-conjugated surfaces (after buffer wash), calculated according to the viscoelastic model. The uncertainty in the fit between the model and the measured data is estimated to be less than 10%

	SPDP-conjugated surface		
	DTT-treated	Non-DTT-treated	
Thickness, δ_{fH}	5.6	10.4	nm
Density, ρ_{fH}	1.20	1.38	kg m^{-3}
Viscosity, η_{fH}	3.4×10^{-3}	10.2×10^{-3}	N s m^{-2}
Elasticity, μ_{fH}	1.8×10^{5}	6.8×10^{5}	N m^{-2}
Characteristic relaxation time, $\tau_{fH}=\eta_{fH}/\mu_{fH}$	1.9×10^{-8}	1.5×10^{-8}	s

With the addition of Compstatin (at a 55 μM concentration which inhibits alternative pathway activation) there was only minor C3 deposition in the presence of immobilised factor H after 20 min (Fig. 4a). In the presence of 10 mM EGTA and 2.5 mM Mg^{2+}, which inhibit classical pathway activation, the deposition of C3 fragments occurred much more slowly onto immobilised factor H than onto SPDP-conjugated BSA (Fig. 4b). SPDP-conjugated BSA, rather than native BSA, was used to make sure that there would be sufficient binding to the surface. 10 mM EDTA inhibited C3 deposition to both surfaces (Fig. 4b).

We then used an EIA to compare the generation of soluble C1s-C1INA complexes in serum in the presence of immobilised factor H, immobilised SPDP-conjugated BSA (200 pmol per well), or no ligand. After incubation of pooled human serum with immobilised factor H for 30 min a considerable reduction in the formation of C1s-C1INA complexes in serum diluted 2^{-2}–2^{-4} was observed (Fig. 5).

3.4. Inhibition of complement activation in plasma and whole blood by immobilised factor H

To illustrate the ability of immobilised factor H to inhibit the complement activation in whole blood, heparinised blood and plasma were obtained from one donor (Fig. 6). Heparin plasma was incubated in wells for 20 min at 37°C. The amount of generated bound C3 fragments, and fluid-phase C3a and sC5b-9 complexes were monitored by EIA. In the control wells bearing BSA, the binding of C3 fragments from heparin plasma increased immediately and reached a maximum at 20 min (Fig. 6, panel c). Both C3a (Fig. 6, panel b), and sC5b-9 (Fig. 6, panel b), increased linearly during the whole time interval. In the factor H-containing wells both the binding of C3 fragments and the generation of C3a and sC5b-9 complexes were reduced to almost baseline levels.

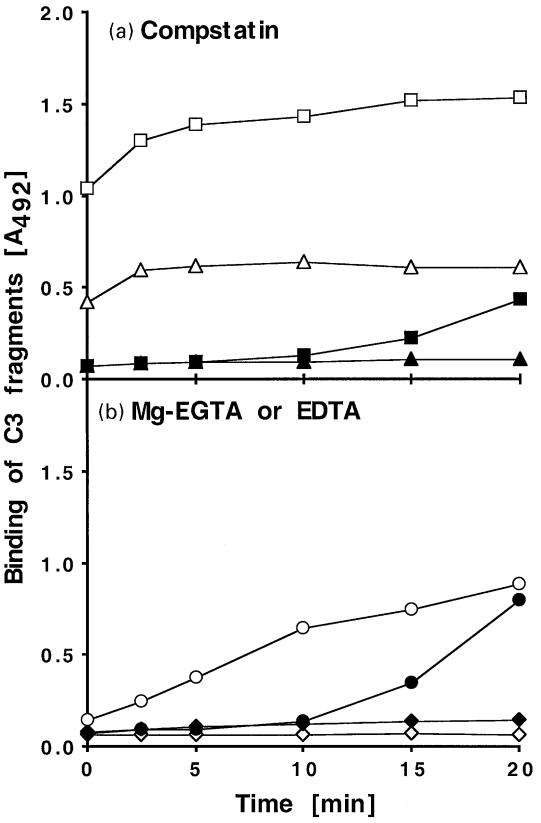

Fig. 4. Time-dependent effect of immobilised factor H on the binding of C3 fragments as detected by EIA. Pooled serum (diluted 1/2) was incubated as single samples at 37°C in micro-titre wells bearing native factor H or SPDP-conjugated BSA. In order to study the initial effects on the classical and the alternative pathway activation several different complement inhibitors were added. Panel a, effect of the inhibition of the alternative pathway by 55 μM Compstatin. Human serum (diluted 1/2) was incubated with surfaces bearing immobilised native factor H in the; presence (–▲–) or absence (–■–) of 55 μM Compstatin or with control surfaces bearing SPDP-conjugated BSA in the presence (–△–) or absence (–□–) of 55 μM Compstatin. Panel b, effect of the inhibition of the classical pathway by 10 mM Mg-EGTA and the inhibition of both pathways by 10 mM EDTA. Human serum (diluted 1/2) was incubated with surfaces bearing immobilised native factor H in the presence of 10 mM Mg-EGTA (–●–) or 10 mM EDTA (–♦–) or with control surfaces bearing SPDP-conjugated BSA in the presence of 10 mM Mg-EGTA (–○–) or 10 mM EDTA (–◇–).

Whole human blood containing 3 IU/ml of heparin was also incubated in the wells. The effect with whole blood was even more pronounced than in the plasma experiment, with a considerable inhibition of both the binding of C3 fragments and the generation of C3a and sC5b-9 complexes in the factor H bearing wells.

4. Discussion

The ability of the complement system to distinguish between self and non-self is to a large extent coupled to the function of the regulators of complement activation

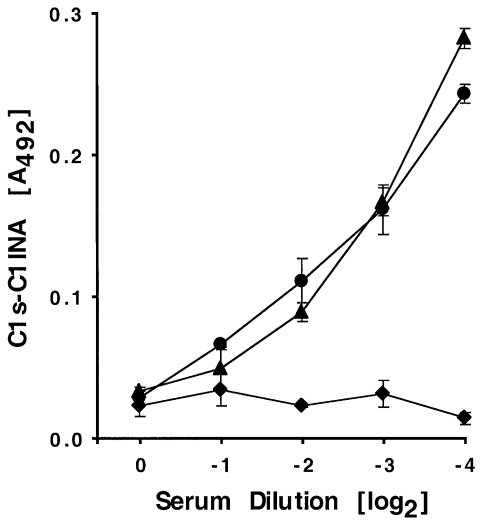

Fig. 5. Generation of soluble C1s-C1INA complexes in serially diluted serum incubated at 37°C for 30 min in wells with immobilised factor H (–♦–), SPDP-conjugated BSA (–▲–) or no ligand (–●–).

(RCA), e.g. DAF, MCP, C4BP, CR1 and factor H [37–41]. These proteins are encoded by a cluster of genes on chromosome 1. Although the RCA family member factor H is a plasma protein, most of its functions are exerted at biological and artificial surfaces. Consequently, it has been suggested that the binding of factor H to a biomaterial surface defines the blood biocompatibility of a biomaterial with respect to the complement system [42]. Furthermore, although many RCA proteins and variants thereof are available in recombinant form, naturally occurring human factor H can be obtained in large quantities. This protein was therefore a particularly suitable candidate of the RCA proteins for immobilisation on a biomaterial surface.

As a model surface we used polystyrene which was coated with a polyamine, followed by the binding of the cross-linker SPDP to the free amines. Remaining free amines were acetylated by acetic anhydride. Our initial experiments showed that SPDP-conjugated factor H could be covalently bound to this surface if the surface-bound SPDP was reduced by DTT. The biological effect of the coupling was monitored by its ability to reduce the binding of C3 fragments to the surface after incubation in human serum. The corresponding surface without SPDP was much less biologically active (not shown) despite the fact it also bound appreciable quantity of factor H (Fig. 2b). However, in the presence of the detergent Tween 20, the binding of factor H was negligible (Fig. 2a), indicating that this binding to surfaces without SPDP was non-covalent.

Early in our study we observed that native factor H also bound to the surface-bound and reduced SPDP with the same or even better biological activity than SPDP-conjugated factor H. We assume that the free thiol

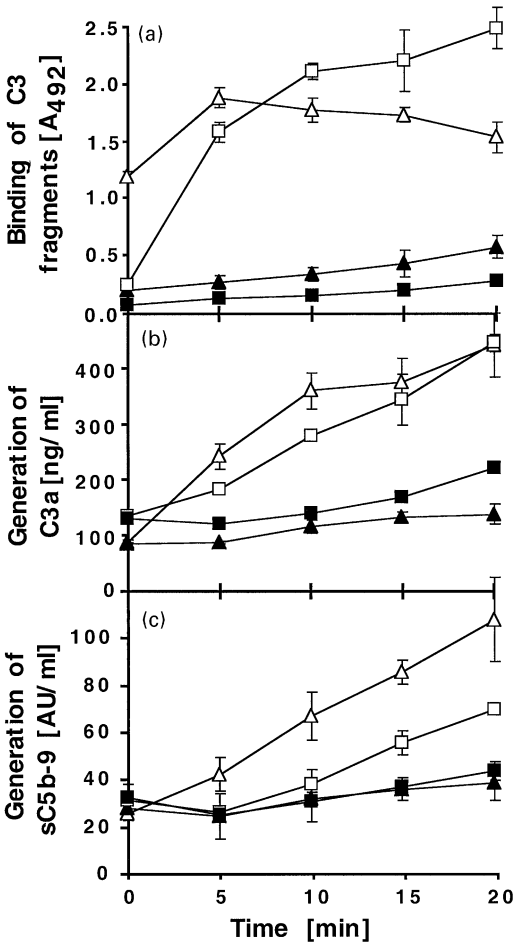

Fig. 6. Time-dependent effect of immobilised factor H on the binding of C3 fragments from heparin plasma and whole blood to the model surface. Immobilised factor H was compared with BSA. Each timepoint represents qaudruplicates in blood and duplicates in plasma. Binding of C3 fragments (panel a), generation of C3a (panel b) and generation of sC5b-9 complexes (panel c) after incubation of heparin plasma with immobilised factor H (–■–) or control BSA (–□–) or of whole blood with immobilised factor H (–▲–) or BSA (–△–).

groups of the reduced SPDP molecules attacked accessible internal disulphide bonds of the SCR domains of factor H. Offering a favoured site at which the free thiol groups could react on the non-conjugated factor H molecules as probably also resulted in a more uniform (and favourable) orientation of the molecule compared to the binding to more randomly dispersed SPDP molecules of the SPDP-conjugated factor H. Because of the relative efficiency of native factor H, it was used in the remaining experiments.

Quantitation by both EIA and QCM-D showed that when the surface-conjugated SPDP was not reduced by DTT, it bound more factor H than did the surface which was subjected to DTT treatment (Figs. 2 and 3). Despite this higher binding, a much lower biological activity was obtained. The relationship between dissipation and frequency for the DTT-treated and the non-DTT-treated

surfaces showed that the conformation of factor H on the two surfaces differed. The dissipation shift was higher on the DTT-treated surface, while the opposite relationship was observed for the frequency shifts. On the surface with reduced SPDP, the viscoelasticity of factor H layer was higher, i.e., the molecules were organised in a more flexible way. In addition to the viscosity and elasticity, the density and the effective (hydrodynamic) thickness of the added layer also varied considerably: The factor H layer on the non-DTT treated surface was almost twice as thick as the layer on the DTT-treated surface (10.4 nm compared to 5.6 nm); the corresponding densities are 1.38 and $1.2\,kg\,m^{-3}$. Both values are within the range of values commonly obtained for the density of water ($1.0\,kg\,m^{-3}$) and proteins ($1.4\,kg\,m^{-3}$). These findings indicate that pure binding of factor H to the surface is not enough to reduce complement activation and that the orientation and conformation of the molecule is crucial for the function of factor H on the material surface. This conclusion is consistent with the three-dimensional structure of factor H, which is an extended fibrilar molecule.

Incubation of serum, plasma or blood with the factor H-covered surfaces showed that the bound factor H abrogated the binding of surface-bound C3 fragments and inhibited the generation of soluble C3a and sC5b-9. This inhibition of complement activation by factor H was mediated by both the alternative and the classical pathways. As expected, when the classical pathway was blocked by EGTA, the deposition of C3 fragments was retarded. Furthermore, under conditions in which only the classical pathway was operative (in the presence of Compstatin or at serum concentrations of 10% (v/v) or less), immobilised factor H totally inhibited the classical pathway activation, as indicated by an abrogation of the generation of C1s-C1INA complexes and the binding of C3 fragments. Although the way in which factor H inhibits the classical pathway is unclear, these observations support previous studies which have identified factor H as a C1q binding protein with the ability to inhibit the hemolytic activity of fluid-phase C1 [20,21].

The degree of complement activation on a biomaterial surface depends upon the properties of the material that is used, e.g. the hydroxyl and amino groups available for C3 binding and the balance between hydrophilic and hydrophobic groups. This study and previous studies from our laboratory using Compstatin to block the alternative pathway activation [12] have shown how swiftly the classical pathway is activated on a material surface. They also show how efficiently the alternative pathway feedback loop amplifies the activation. These observations suggest that activation of complement on a biomaterial surface takes place in two steps: First, complement activation is initiated by either the classical or the alternative pathway, depending on the material involved. Second, once C3b is deposited on the surface, the alternative pathway feedback loop has the potential to drastically amplify the activation. This situation presumably explains why biomaterial surfaces are generally thought to mainly activate the alternative pathway independent of which pathway actually triggered the activation. It also suggests that inhibition of the initial activation of the triggering pathway is important if we are to inhibit the whole complement activation on the model surface. This two-step activation suggests that a complement inhibitor immobilised on a biomaterial surface should, like factor H, have the ability to inhibit both activation pathways.

Taken together, our data demonstrate that an RCA protein can be covalently linked to a biomaterial surface while maintaining its functional properties. However, further efforts need to be made to improve both the inhibitors and the techniques by which they are bonded to the surface.

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