C1q induces a rapid up-regulation of P-selectin and modulates collagen- and collagen-related peptide-triggered activation in human platelets

Caroline Skoglund, Jonas Wetterö, Pentti Tengvall and Torbjörn Bengtsson

Original Publication:

Caroline Skoglund, Jonas Wetterö, Pentti Tengvall and Torbjörn Bengtsson, C1q induces a rapid up-regulation of P-selectin and modulates collagen- and collagen-related peptide-triggered activation in human platelets, 2010, Immunobiology, (215), 12, 987-995.
http://dx.doi.org/10.1016/j.imbio.2009.11.004

C1q induces a rapid up-regulation of P-selectin and modulates collagen- and collagen-related peptide-triggered activation in human platelets

Caroline Skoglund[1*], Jonas Wetterö[2], Pentti Tengvall[3] and Torbjörn Bengtsson [1,4]

[1] Division of Drug Research, Department of Medical and Health Sciences, Linköping University, SE-581 85 Linköping, Sweden

[2] Rheumatology/Autoimmunity and Immune Regulation unit (AIR), Department of Clinical and Experimental Medicine, Linköping University, SE-581 85 Linköping, Sweden

[3] Institute of Clinical Sciences, Department of Biomaterials, Sahlgrenska Academy, University of Gothenburg, SE-405 30 Gothenburg, Sweden

[4] Department of Biomedicine, School of Health and Medical Sciences, Örebro University, SE-701 82 Örebro, Sweden

* Corresponding author:

e-mail: caroline.skoglund@liu.se

Fax: +46 13 149106, phone: +46 13 224767

Running title: Effects of C1q on platelet activation

Keywords: alfaIIbetaI integrin (αIIβI, GpIa/IIa), blood platelet, C1q, C1qR, complement, platelet-neutophil aggregates, P-selectin (CD62P)

List of abbreviations: ADP, adenosine diphosphate; αIIβI/GpIa/IIa, glycoprotein Ia/IIa; CD62P, P-selectin; C1q, complement protein 1q; CRP, C-reactive protein; FITC, Fluorescein; Fura-2AM, Fura-2-acetoxymethylester; GPVI, glycoprotein VI; IgG, immunoglobulin G; IgM, immunoglobulin M; IP_3, inositol(1,4,5)triphosphate; LPS, lipopolysacchride; MBL, mannose-binding lectin; PKC, protein kinase C; PRP, platelet rich plasma; PSGL-1/CD162, P-selectin glycoprotein ligand-1; TRAP, SFLLRN peptide.

Abstract

Blood platelets are emerging as important immunomodulatory cells, but complement interaction with platelets is not well understood. Several platelet structures have been described as complement protein 1q (C1q) binding receptors, such as C1qRp/CD93 and gC1qR. However, there are conflicting results whether these receptors are C1q binding structures, or even at all expressed on the cell surface. Recently, the collagen binding integrin αIIβI was reported to bind C1q on mast cells, and this receptor is also present on platelets. The aim of this study was to further characterize the effects of C1q on platelets, by quantifying the platelet surface expression of P-selectin (CD62P) and monitoring the formation of platelet-neutrophil aggregates. Using flow cytometry, we found that C1q dose-dependently triggered a rapid but moderate and transient up-regulation of P-selectin already within 5 seconds of C1q exposure. Pre-incubation with an antibody directed against gC1qR significantly inhibited (with 57 % compared to control) the up-regulation, whereas an antibody towards the αIIβI-integrin showed no effect. Stimulation with C1q did not change the cytosolic calcium-levels, as measured with the fluorescent ratiometric probe Fura-2, however, a protein kinase C inhibitor (GF109203x) blocked the C1q-induced P-selectin expression. Furthermore, pre-incubation of platelets with C1q diminished both the collagen as well as the collagen-related peptide induced up-regulation of P-selectin, most evident after 90 seconds of stimulation. This indicates that C1q may regulate platelet activation via the GPVI receptor, which is a novel finding. Moreover, C1q antagonized the collagen-induced formation of platelet-neutrophil aggregates, indicating a reduced interaction between platelet P-selectin and neutrophil P-selectin glycoprotein ligand-1(PSGL-1/CD162). In summary, C1q induces a moderate rapid platelet P-selectin expression, modulates subsequent collagen and collagen-related peptide stimulation of platelets, and inhibits the formation of platelet-

neutrophil aggregates. These immuno-regulatory effects of C1q may have a crucial role in innate immunity and inflammation.

Introduction

The complement system is an important part of the innate immunity and is recognized as a modulator of inflammatory processes, for example in atherosclerosis (recently reviewed by Haskard et al., 2008), and at material-tissue interfaces, where complement activation could occur on top of adsorbed plasma proteins (Andersson et al., 2005; Gorbet and Sefton, 2004; Wetterö et al., 2002). The complement system is comprised of about 35 circulating and/or membrane bound proteins and there are three pathways of activation, i.e. the alternative pathway, the lectin pathway, and the classical pathway (Gasque, 2004; Haskard et al., 2008). Activation of the classical pathway is initiated via binding of immunoglobulins G and M (IgG and IgM) to complement protein 1q (C1q). Moreover, C1q may also bind to structures such as C-reactive protein (CRP) and lipopolysaccharides (LPS), thereby leading to activation of the cascade (Gasque, 2004; Sjöwall et al., 2007).

C1q is a 462 kDa molecule with 18 polypeptide chains (A-, B- and C-chains, six of each chain) and it is often described as a "bundle of tulips" with an amino-terminal collagen-like region and a carboxyl rich terminal globular region (Nicholson-Weller and Klickstein, 1999; Reid and Porter, 1976; Wallis et al., 2010). In contrast to other complement proteins which are synthesized by hepatocytes, C1q is produced and secreted by macrophages and dendritic cells (Muller et al., 1978; Schwaeble et al., 1995).

In the last decades, several publications attribute various cellular effects to C1q. Monocytes and granulocytes, B lymphocytes, dendritic cells, mast cells, fibroblasts, smooth muscle and epithelial cells, endothelial cells and platelets are all reported to bind C1q (Andrews et al., 1981; Bobak et al., 1986; Bordin et al., 1983; Bordin et al., 1992; Edelson et al., 2006; Peerschke and Ghebrehiwet, 1987; Vegh et al., 2006; Young et al., 1991). Cellular responses upon C1q binding include for example chemotaxis of dendritic cells (Vegh et al., 2006),

cytokine release from mast cells (Edelson et al., 2006), and enhancement of Fcγ-receptor mediated phagocytocis by macrophages and monocytes (Bobak et al., 1987).

Hence, there has been an extensive search for candidate receptors responsible for the C1q-mediated effects and several C1q binding structures have been described, e.g. cC1qR (calreticulin) and gC1qR reported to bind the collagenous and the globular parts of the C1q molecule, respectively (reviewed in Ghebrehiwet and Peerschke, 2004). However, there are conflicting results whether gC1qR is located on the cell surface or not (van den Berg et al., 1997). Other structures that are suggested to bind C1q are CD91 (low-density lipoprotein receptor-related protein 1 or alpha-2-macroglobulin receptor) (Ogden et al., 2001), CD35 (complement receptor 1) (Klickstein et al., 1997) and CD93 (C1qRp) (Steinberger et al., 2002). In contradiction, recent reports have demonstrated that CD93 does not bind to C1q (McGreal et al., 2002). Furthermore, Edelson and co-workers recently demonstrated C1q binding to the collagen-binding integrin αIIβI (GPIa/IIa) on mast cells (Edelson et al., 2006). The latter is particularly interesting since blood platelets are becoming recognized as important immunomodulatory cells, apart from their established role in haemostasis (as reviewed in von Hundelshausen and Weber, 2007). C1q has previously been shown to inhibit collagen-induced platelet activation and the αIIβI-integrin is one of several platelet collagen binding receptors (Cazenave et al., 1976; Surin et al., 2008). Platelet activation usually involves the release of a wide range of inflammatory mediators, including growth factors, cytokines and thromboxanes (von Hundelshausen and Weber, 2007) . Excessive and/or misdirected platelet activation may thus contribute to the inflammatory reaction. Upon platelet activation, P-selectin (CD62P) is translocated from the α-granules to the platelet surface. This up-regulation of P-selectin is proven to play an important role in the aggregate formation between platelets and PSGL-1 (CD162) bearing cells, such as neutrophil granulocytes (May et al., 2007; Wetterö et al., 2003).

Complement activation is described to occur on the membrane of the platelet (Del Conde et al., 2005; Peerschke et al., 2008; Peerschke et al., 2006) and is enhanced upon platelet activation (Del Conde et al., 2005). In addition, we recently showed a regulatory effect of C1q and C-reactive protein on platelet adhesion and activation at plasma protein-coated surfaces (Skoglund et al., 2008). Since conflicting results regarding the role of C1q receptors and C1q-mediated effects on various types of cells are found in the literature, mechanistic studies on C1q-mediated cell regulation are warranted. Hence, the present study was undertaken to further clarify the cellular effects of C1q and its interaction with platelet surface receptors. More specifically we have studied effect of C1q on platelet P-selectin expression and the regulation of collagen stimulation and the role of the gC1qR and the collagen receptors αIIβI and glycoprotein VI. Our paper also addresses the intracellular signaling events in platelets induced by C1q binding.

Materials and methods

Isolation of platelets and neutrophil granulocytes

Peripheral blood was drawn from apparently healthy, non-medicated donors at the Linköping University Hospital. Platelets were isolated as described previously (Bengtsson and Grenegård, 1994). In short, five parts of heparinized (10 IU/mL) whole blood mixed with one part of an acid-citrate-dextran solution (85 mM sodium citrate, 71 mM citric acid and 111mM glucose) was centrifuged at 220 x g for 20 minutes at room temperature. Platelet rich plasma (PRP) was collected and platelets were pelleted by centrifugation at 480 x g for 20 minutes at room temperature. The plasma was discarded and the platelets washed and resuspended in calcium-free Krebs-Ringer phosphate buffer, pH 7.4, supplemented with 10 mM glucose and 1.5 mM magnesium sulphate (KRG). Cell density was assessed using a Bürker chamber and light microscopy, which also confirmed that isolated platelets showed no signs of activation and that the contamination of other blood cells was negligible. Before experiments, the extracellular concentration of Ca^{2+} was set to 1 mM, ($CaCl_2$).

Neutrophils were isolated according to Böyum and others (Boyum, 1968; Ferrante and Thong, 1980). Whole blood was layered onto Lymphoprep and Polymorphprep (Axis-Shield AS, Oslo, Norway) and centrifuged at 480 x g for 40 minutes at room temperature. The fraction containing neutrophils was harvested and washed in PBS (10 mM sodium hydrogen phosphate, 10 mM potassium dihydrogen phosphate and 0.15 M sodium chloride, pH 7.4) at 480 x g for 10 minutes. Remaining red blood cells were removed by brief hypotonic lysis at 4° C followed by washing in KRG at 200 x g at 4° C. Isolated neutrophils were counted in a Coulter counter ZM Channelyser 256 (Coulter-Electronics Ltd., Luton, UK) and kept on ice until experiments.

Platelet P-selectin expression

Isolated platelets (2 x 10^8 /mL) were incubated in a 24-well plate (Sarstedt AG & Co, Nümbrecht, Germany) at 37 °C for 5 minutes. C1q, 8-80 μg/mL (Quidel Corp., San Diego, CA) or as positive control 10 μg/mL TRAP (SFLLRN, Biotechnology Centre of Oslo, Oslo University, Norway) was added under shaking conditions, and samples were taken after 5, 15, 45 and 120 seconds. To investigate the role of the gC1qR, monoclonal antibodies were used. Clone 60.11 is directed against the amino-terminal part of the receptor also described as the C1q binding epitope and clone 74.5.2 is directed towards the carboxyl-terminal (Abcam, Cambridge, UK). The role of the αIIβI integrin was evaluated by pre-incubation of platelets with clone AK7, directed against the alfa2 subunit of the integrin (AbD Serotec, Oxford, UK). GF109203X (Tocris, Ellisville, MO) was used to elucidate the role of protein kinase C (PKC) in the signaling following C1q stimulation. Since the C1q preparations contain glycerol as a stabilizer, buffer with a corresponding concentration of glycerol (Sigma Chemical Co, St Louis, MO) was added in some samples, as control. As further controls, we treated C1q with the intention of modifying the protein structure using heat-inactivation (56 º C, 30 minutes), 5 cycles of thawing and freezeing (-70 ° C) or addition of deionized water to the C1q suspension (1:1).

To investigate the effect of C1q on collagen-, collagen-related peptide- or TRAP-induced P-selectin expression, platelets were pre-incubated with C1q (80μg/mL) before addition of collagen (3 μg/mL, equine type 1 collagen, Chrono-log, Haverston, PA), collagen-related peptide (1 μg/mL, Gly-Cys-Hyp-$(Gly\text{-}Pro\text{-}Hyp)_{10}$-Gly-Cys-Hyp-Gly-$NH_2$, kindly provided by Dr. Richard W. Farndale and Dr. Graham Knight, Cambridge, UK) or TRAP (10 μg/mL). Samples were taken after 15, 45, 90 and 120 seconds. In control experiments, human serum albumin (HSA, Albuminativ®, Octapharma AB, Stockholm, Sweden) was used instead of C1q. Direct immunofluorescence staining of platelets was performed by incubation with

saturating concentrations of monoclonal (clone AK-4) fluorescein isothiocyanate (FITC)-conjugated anti P-selectin (CD62P; BD Biosciences, NJ, USA) for 10 minutes at room temperature in the dark. To account for non-specific staining, FITC-labeled irrelevant isotype-matched monoclonal (clone DAK-GO1) antibodies (DAKO, Glostrup, Denmark) were used. Samples were then fixed in 1% ice-cold paraformaldyhyde (Fluka, Sigma) for 15 minutes and diluted in PBS prior to flow cytometry analysis. Control experiments with non-fixed cells confirmed that this fixation protocol did not influence the P-selectin expression. Immediately after staining, the samples were analyzed with flow cytometry in a FACS Calibur equipped with an argon 488 laser (Becton Dickinson, San Jose, Ca, USA). Platelets were identified by their specific light scattering characteristics, and in some samples confirmed by positive staining for phycoerythrin-labelled anti-GPIb (CD42b, clone AN51) (DAKO, Glostrup, Denmark). A total of 10,000 events were collected in the platelet gate and data was analyzed using the CellQuestPro software (Becton Dickinson).

Cytosolic calcium

Platelet cytosolic Ca^{2+} levels were investigated using the fluorescent ratiometric probe Fura-2. Briefly, platelets were loaded with Fura-2 by incubating PRP with 4 μM Fura-2-acetoxymethylester (Fura-2-AM, Sigma) in the presence of 0.5 U/mL apyrase (Sigma), for 40 min at room temperature and under gentle shaking. Platelets were then washed as described above. Upon addition of C1q (5-50 μg/mL) and thrombin 0.1 U/mL (Sigma) to 1.5 ml aliquots of a platelet suspension, measurements of cytosolic Ca^{2+} were performed in a F-2000 spectrofluorometer (Hitachi Ltd., Tokyo, Japan) under constant stirring (300 rpm) and at 37 °C. Fluorescence emission was registered at 510 nm during simultaneous excitation at 340 nm and 380 nm.

Platelet adhesion to collagen coated surfaces

Platelet adhesion to collagen coated surfaces was performed as previously described (Eriksson and Whiss, 2005). In short, microplates were coated with 0.1 mg/mL bovine collagen (RnDSystems, Abingdon, UK) or 2 mg/mL fibrinogen (American Diagnostica Inc., Greenwich, CN). Unattached proteins were removed by washing in 0.9 % NaCl before addition of 50 µl diluted PRP (some samples were pre-incubated with 80 µg/mL C1q). Cells were allowed to adhere for 1 hour at room temperature followed by washing in 0.9% NaCl to remove non-adhering platelets. Enzymatic detection of adhering platelets was then performed by adding *P*-nitrophenyl-phosphate (Sigma) and 0.1% Triton X- 100 (Sigma) in a citrate/citric acid buffer (0.1 M, pH 5.4). The reaction was stopped after one hour by addition of 2M NaOH, and absorbance measured at 405 nm (Spectramax microplate reader, Molecular Devices, Sunnyvale, CA) and percentage platelet adhesion was calculated.

Formation of platelet-neutrophil aggregates

Platelets and neutrophils isolated from the same donor were pre-warmed at 37 °C for 5 minutes prior to mixing. In some experiments, platelets were pre-incubated with C1q (80 µg/mL) before mixing with neutrophils. The cells were co-incubated during shaking conditions at 37 °C, unstimulated or stimulated with collagen (3 µg/mL) for 30, 60, 90 and 150 seconds and fixed in 4% ice-cold paraformaldehyde for 30 minutes. Platelet-neutrophil aggregates were visualized in an Axiovert 200 microscope with an oil immersion 63x/1.4NA objective (Carl Zeiss GmbH, Göttingen, Germany) and digital images were obtained using a Axiocam MRm camera and the AxioVision software (version 4.6.3.0)

Statistics

Data are presented as arithmetic averages ± standard error of the means (SEM). The number of individual experiments (individual blood donors) is indicated by n. Statistical significance was evaluated using one-way analysis of variance combined with Bonferroni´s or Dunnett´s post hoc test. Significance levels of * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), or ns (not significant) were used. All statistical analyses were performed with GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA).

Results

C1q induces P-selectin expression

By using flow cytometry, we found that C1q (80 μg/mL) induced a moderate, but rapid and highly significant expression of P-selectin on the platelet surface, compared to non-stimulated controls ($p<0.001$, Fig. 1A-B). The expression was most evident after 15 seconds, was still elevated after 45 seconds, and returned to resting control levels within 3-5 minutes. Further experiments targeting the temporal resolution indicated that the expression of P-selectin was elevated already after 5 seconds (Fig 1A. insert). In comparison and as expected, stimulation with TRAP induced a massive increase in platelet P-selectin (Fig. 1A). Upon addition of C1q, the geometric mean fluorescence value after 15 seconds was 36.7 ± 2.5 compared to 22.4 ± 1.2 in the unstimulated control, and 245.5 ± 17.5 in the TRAP-stimulated positive control. The up-regulation of P-selectin induced by C1q was concentration dependent as shown in Fig. 1C. Since the commercial C1q preparations typically contain a high concentration of glycerol, experiments with a glycerol control confirmed that the C1q-induced stimulation was not due to unspecific effects e.g. viscosity changes. Heat-inactivation, addition of deionized water or repeated cycles of thawing and freezing of the C1q preparation before addition to the platelets, did not induce further P-selectin expression (not shown).

Role of the C1q-receptors and integrin αIIβI

To investigate the involvement of gC1qR and the αIIβI integrin in C1q-induced up-regulation of P-selectin, antibodies directed against these receptors were used. Unfortunately, clones 60.11 and 74.5.2, both described to block the gC1qR, induced P-selectin expression and gave raise to an intracellular Ca^{2+} elevation when used at concentrations higher than 3 μg/mL. It is thus possible that the antibodies are receptor agonists, which call for some caution. Nevertheless, as shown in Fig. 2A, pre-incubation with 3 μg/mL of antibody 60.11

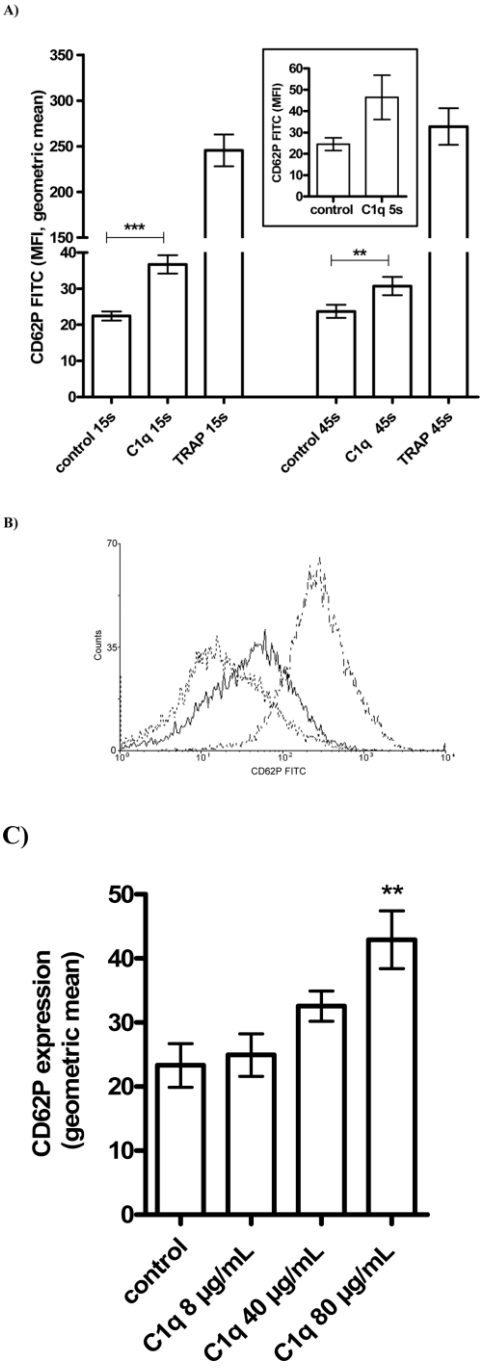


Figure 1. C1q induces a moderate but rapid and concentration dependent platelet P-selectin (CD62P) expression.

A) Washed platelets (2 x 10^8 /mL) were pre-incubated for 3 minutes in the presence of 1 mM Ca^{2+} at 37 °C, followed by stimulation under shaking conditions with C1q (80 µg/mL) or TRAP-6 (10 µg/mL). Samples were taken in duplicates after 15 and 45 seconds. Before fixation in 1 % paraformaldehyde, platelets were stained for CD62P using a FITC-conjugated
monoclonal antibody. CD62P-FITC fluorescence was analyzed in the platelet population using flow cytometry. The data is presented as geometric mean fluorescence intensity values (MFI) from 10,000 collected events in the platelet gate ± SEM , n=17-23. Statistical significance was evaluated using repeated measures one-way analysis of variance combined with Bonferroni´s post hoc test, ** ($p \leq 0.01$) and *** ($p \leq 0.001$). The insert shows that P-selectin was present on the platelet surface even within 5 seconds after addition of C1q (80 µg/mL), n=3

B) Representative histogram showing the changes in CD62P-FITC fluorescence upon C1q (solid line) or TRAP (broken line) treatment for 15 seconds. (Dotted line: un-stimulated control).

C) C1q (8-80 µg/mL) induces a concentration dependent P-selectin expression. n=3

significantly reduced the C1q-induced P-selectin up-regulation (-57%). 3µg/mL of 74.5.2 decreased the C1q-induced expression by 52 %, although not statistically significant. Pre-incubation of platelets with a blocking antibody towards the αIIβI integrin did not significantly affect the C1q-induced P-selectin expression.

Involvement of protein kinase C (PKC)

Pre-incubation of platelets with the protein kinase C inhibitor GF109203X at 5 or 50 µM completely inhibited the C1q-induced expression of P-selectin (Fig 2B). Addition of C1q in the presence of DMSO (dimethylsulfoxide), confirmed that the effects were not influenced by the solvent.

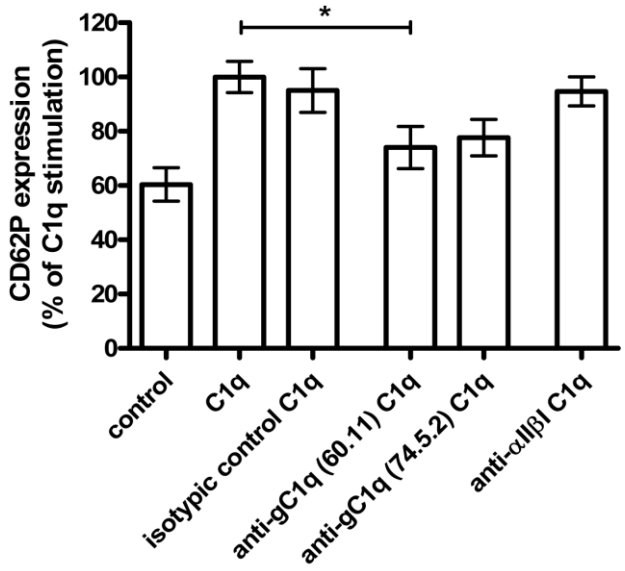
C1q does not elevate intracellular Ca^{2+}

Using the fluorescent probe Fura-2, we investigated whether the platelet P-selectin response was accompanied by a change in cytosolic calcium-levels. None of the C1q concentrations tested, 5, 20 (Fig. 3) or 50 µg/mL (not shown), yielded any intracellular Ca^{2+} elevations. The cytosolic calcium transient triggered by thrombin remained unaffected by the presence of C1q.

C1q inhibits collagen- and collagen- related peptide-induced P-selectin expression

Collagen or collagen-related peptide (which activates via GPVI, Morton et al., 1995) activation of washed platelets induced a substantial increase in P-selectin expression, although not as potent as upon addition of TRAP (MFI values of 60.0±11.0, 90.5±25 and 193.0±8.4 after collagen, collagen-related peptide and TRAP stimulation for 45 seconds, respectively). Pre-incubation of platelets with C1q (80 µg/mL) for 5 minutes, markedly inhibited the collagen (3 µg/mL) and collagen-related peptide (1 µg/mL) induced up-regulation of P-

A)



B)

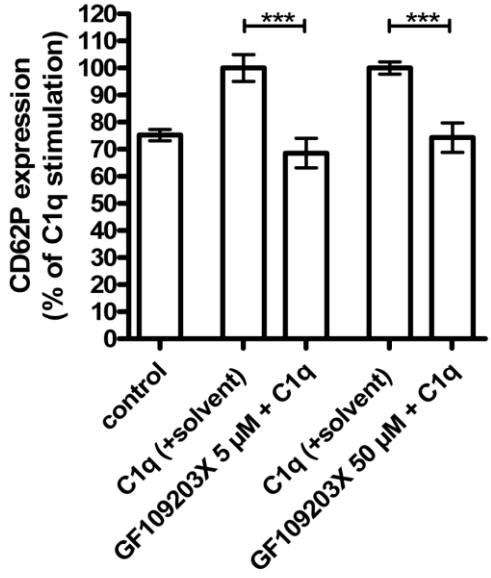


Figure 2. The C1q-induced up-regulation of P-selectin is inhibited by a gC1qR-antibody and by inhibition of PKC, but remains unaffected by blockade of the αIIβI-integrin.
A) Washed platelets (2 x 10^8 /mL) were pre-incubated at 37 °C with antibodies directed against two different epitopes of the gC1q-receptor (60.11 or 74.5.2, 3 µg/mL), a blocking antibody directed against the αIIβI-integrin (AK7, 3 µg/mL) or an isotypic control antibody for 5 minutes before stimulation with C1q (80 µg/mL) under shaking conditions. Samples were taken in duplicates after 15 seconds. Before fixation in 1 % paraformaldehyde, platelets were stained for CD62P using a FITC-conjugated monoclonal antibody. The FITC-fluorescence was collected from 10,000 events in the platelet gate, and the CD62P expression is presented as % induced by C1q stimulated cells ± SEM, n=4-5. Statistical significance was evaluated with one-way analysis of variance combined with Dunnett´s post hoc test, comparing antibody treated with untreated cells stimulated by C1q ,* ($p \leq 0.05$).
B) Washed platelets (2 x 10^8 /mL) were pre-incubated for 5 minutes at 37 °C with the PKC inhibitor GF109203X at 5 and 50 µM before stimulation with C1q (80 µg/mL) and flow cytometry analysis as described above. The CD62P expression is presented as % induced by C1q stimulated cells ± SEM , n=3-4. One-way analysis of variance combined with Bonferonni´s post hoc test was used to test statistical significance between PKC inhibited and untreated cells stimulated by C1q, *** ($p \leq 0.001$).

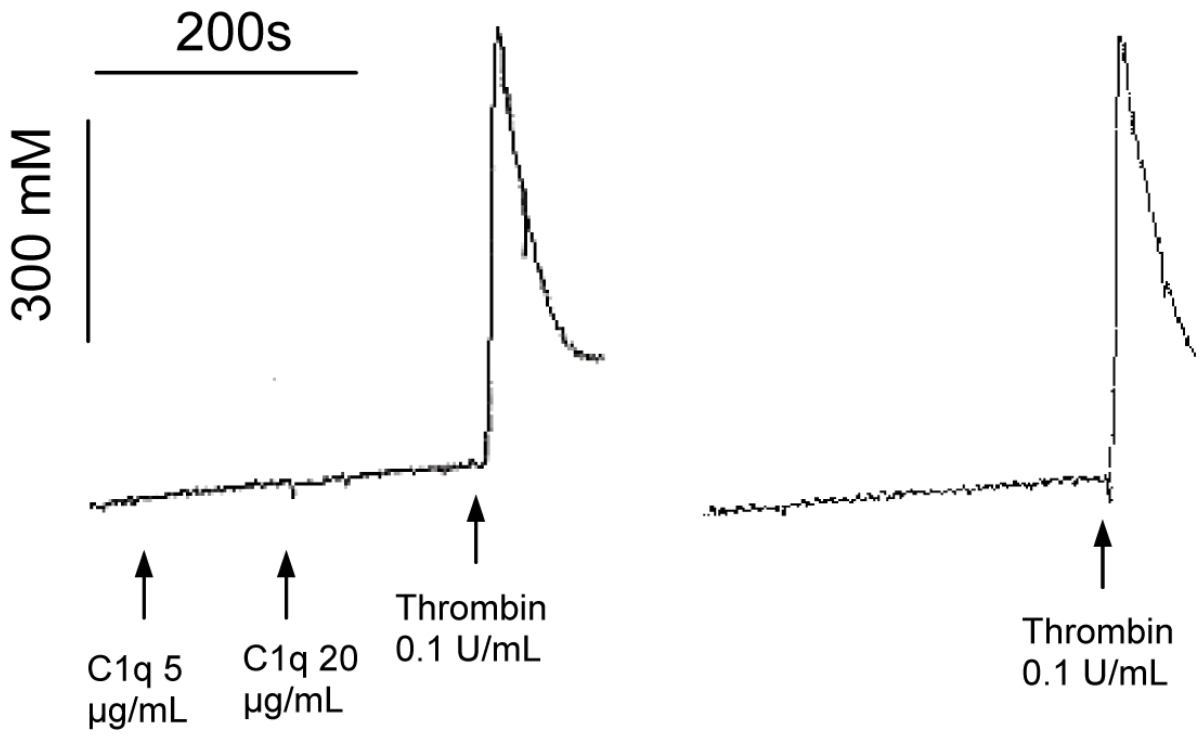
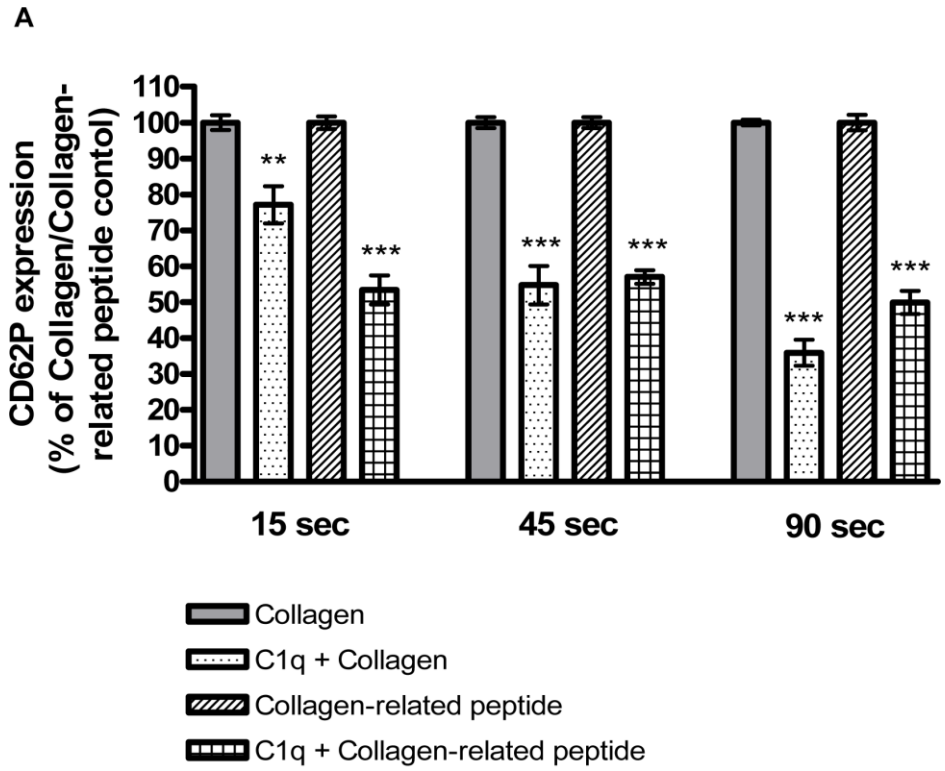


Figure 3. C1q does not induce Ca^{2+}-signaling in platelets
Platelet rich plasma was incubated with the fluorescent probe Fura-2-AM (4µM) at room temperature and gentle shaking for 40 minutes before isolation of platelets. The effect of 5-50 µg/mL C1q on the cytosolic Ca^{2+} levels was analyzed in a Hitachi F-2000 spectrofluorometer where the fluorescence signal from 1.5 ml aliquots of the platelet suspension was detected under constant stirring (300rpm) and at 37 °C. Fluorescence emission was registered at 510 nm during simultaneous excitation at 340 nm and 380 nm. The figure shows representative original traces from one of totally 3 experiments.

selectin. Compared to controls, pre-incubation with C1q significantly inhibited P-selectin expression with 22.9 ± 5.1 % ($p \leq 0{,}01$) at 15 seconds, with 45.3 ± 5.3 % ($p \leq 0.001$) at 45 seconds, and with 64.1 ± 3.6 % ($p \leq 0.001$) at 90 seconds of collagen stimulation, and with 46.6 ± 4.0 % ($p \leq 0{,}001$) at 15 seconds, with 42.7 ± 1.8 % ($p \leq 0.001$) at 45 seconds, and with 50.1 ± 3.2 % ($p \leq 0.001$) at 90 seconds of stimulation with collagen-related peptide, (Fig. 4A-B). Inhibition by C1q was still detectable after 120 seconds of collagen stimulation, although not as pronounced as after 90 seconds. C1q did not significantly inhibit the TRAP stimulated up-regulation of P-selectin. Introduction of HSA instead of C1q did not yield such effects, excluding a general protein effect.



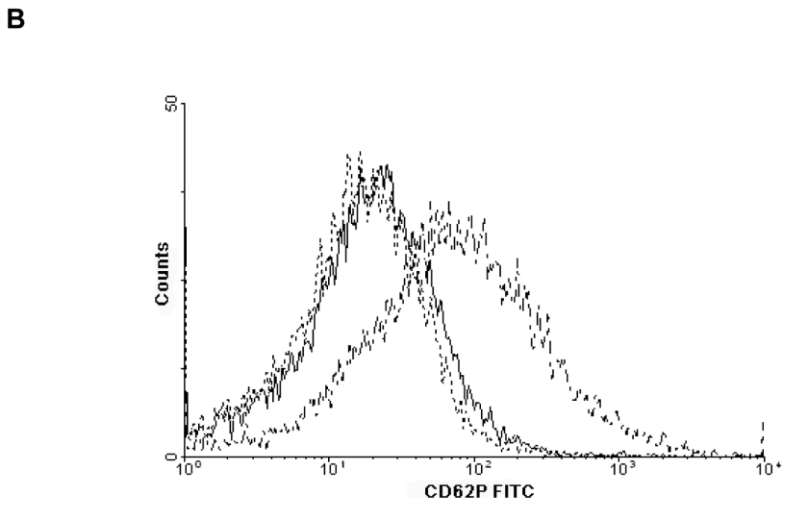


Figure 4. C1q inhibits further collagen or collagen-related peptide-induced platelet activation.
A) Washed platelets (2 x 10^8 /mL) were pre-incubated with C1q (80 µg/mL) at 37 °C under shaking conditions for 5 minutes followed by collagen (3µg/mL), collagen-related peptide (1 µg/mL) or TRAP (10 µg/mL) stimulation. Samples were taken in duplicates after 15, 45 and 90 seconds. Before fixation in 1 % paraformaldehyde, platelets were stained for CD62P using a FITC-conjugated monoclonal antibody. CD62P-FITC fluorescence was analyzed in the platelet population using flow cytometry. Statistical significance was evaluated with a one-way analysis of variance combined with Bonferroni post hoc test comparing C1q treatments with collagen/collagen-related peptide controls, ** ($p<0.01$), *** ($p<0.001$). The data is expressed as % of collagen or collagen-related peptide control, calculated from geometric mean fluorescence values from 10,000 collected events in the platelet gate ± SEM , n=6-11 (collagen), n=3 (collagen-related peptide)
B) Representative histogram showing the changes in CD62P FITC fluorescence upon addition of collagen for 90 seconds to platelets with or without pre-incubation with C1q (80 µg/mL). Un-stimulated control (dotted line), collagen (broken line) and C1q + collagen (solid line).

Platelet adhesion

Pre-incubation of platelets with C1q did not affect platelet adhesion to a collagen coated surface, whereas the antibody directed against the αIIβI integrin inhibited the adhesion with 66.0 ± 3.6 % (Fig. 5)

C1q regulates platelet-neutrophil aggregate formation

Since P-selectin plays an important role in the adhesion between platelets and neutrophils (May et al., 2007; Wetterö et al., 2003), we investigated whether the observed C1q-mediated down-regulation of P-selectin in collagen-stimulated platelets affected the formation of platelet-neutrophil aggregates. When a mixture of platelets and neutrophils were challenged with collagen (3 μg/mL) the formation of platelet-platelet aggregates as well as platelet-neutrophil aggregates was markedly increased compared to the unstimulated control (Fig. 6). Pre-treatment of platelets with C1q (80 μg/mL), prior to mixing with neutrophils and subsequent collagen stimulation, considerably reduced the formation of platelet-neutrophil aggregates, compared to the collagen control.

Discussion

Increasing amounts of evidence during recent years recognize blood platelets as important players in innate immunity e.g. due to their expression of pathogen recognition receptors, adhesion molecules and release of inflammatory mediators (von Hundelshausen and Weber, 2007). Complement activation is a crucial part of innate immunity and inflammation and during the past decade receptors for complement proteins have been identified on various cell types, including platelets (Edelson et al., 2006; Peerschke and Ghebrehiwet, 1994; Steinberger et al., 2002; Vegh et al., 2006). In the present study, we found that C1q induces a rapid platelet P-selectin expression, modulates subsequent collagen and collagen-related peptide stimulation of platelets, and inhibits the formation of platelet-neutrophil aggregates. When we

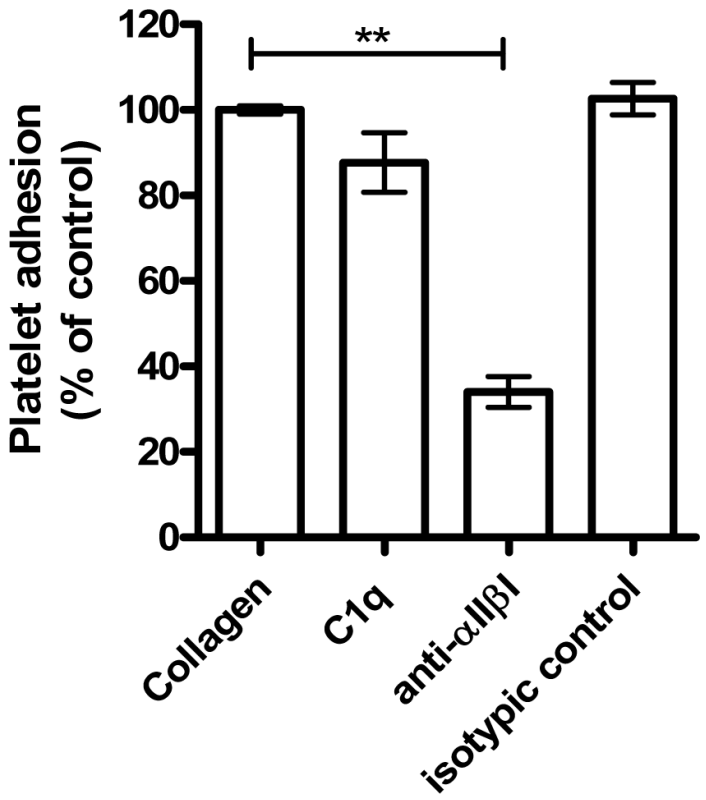


Figure 5. C1q does not affect platelet adhesion to collagen.
Microplates were coated with 0.1 mg/mL bovine collagen and unattached proteins were removed by washing in 0.9 % NaCl before addition of 50 µl diluted platelet rich plasma (PRP). Some samples were pre-incubated with 80 µg/mL C1q or 10 µg/mL anti-αIIβI antibody. Cells were allowed to adhere for 1 hour at room temperature followed by washing in 0.9% NaCl to remove non-adhering platelets. Enzymatic detection of adhering platelets was performed by adding *P*-nitrophenyl-phosphate and 0.1% Triton X-100 in a citrate/citric acid buffer (0.1 M, pH 5.4). The reaction was stopped after one hour by addition of 2M NaOH, absorbance was measured at 405 nm and percentage platelet adhesion was calculated. Statistical significance was evaluated with one-way analysis of variance combined with Bonferonni´s post hoc test comparing treatments with collagen control,*** ($p \leq 0.001$), n=4

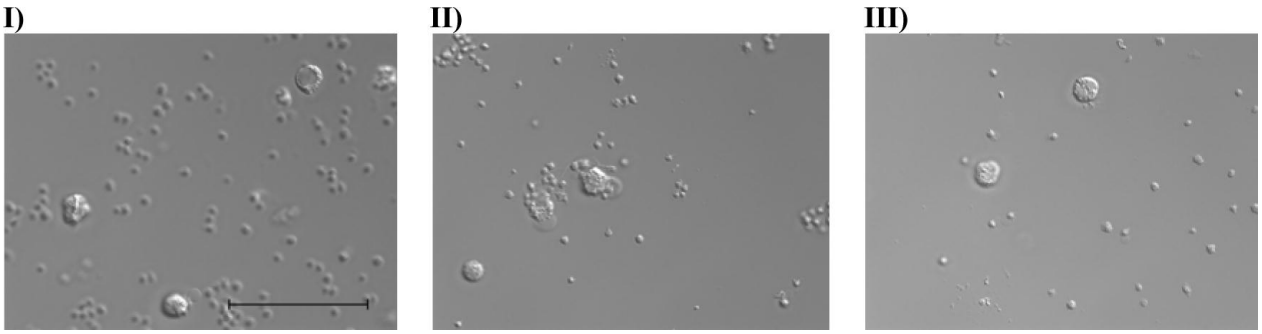


Figure 6. C1q regulates the formation of platelet-neutrophil aggregates
Washed platelets (1 x 10^8 /mL) were pre-incubated with C1q (80 µg/mL) at 37 °C for 5 minutes. Isolated neutrophils (2 x 10^6 /mL) from the same blood-donor were simultaneously pre-warmed and then mixed with the platelets. The mixture of platelets and neutrophils was challenged with collagen (3 µg/mL) and samples were taken after 30-150 seconds. Samples were fixed in 4% ice-cold paraformaldehyde for 30 minutes, washed in PBS and visualized using microscopy. Representative images from samples taken after 120 seconds, in I) untreated control II) collagen III) C1q + collagen. Bars: 20 µm. n=3

investigated the effects of C1q on blood platelets and by using flow cytometry we found that monomeric C1q induces a moderate but rapid, within 5 seconds, up-regulation of P-selectin on the platelet surface. In correlation, Peerschke et al. have previously reported, by using fluorescence microscopy, that *aggregated* C1q induces P-selectin expression. However, the up-regulation of P-selectin found in the present study was not as potent as the one presented in the study by Peerschke et al, where the observed P-selectin expression was in the same range as that provoked by thrombin (Peerschke et al., 1993). Moreover, we also found that the up-regulation of P-selectin upon C1q stimulation was transient, and the expression returned to control levels within 3-4 minutes. This is interesting since P-selectin is known to be shedded or possible actively cleaved off from platelets and can be found in the circulation in vivo, as soluble sP-selectin (sCD62P). The level of sP-selectin is increased during a variety of cardiovascular diseases, which may reflect an increased platelet activation (Blann et al., 2003).

Complement activation may occur on the platelet membrane (Peerschke et al., 2008). P-selectin on the platelet surface is described to function as a ligand for C3b, and thereby proposed to facilitate further activation of the complement cascade (Del Conde et al., 2005). It is thus possible that the moderate increase in P-selectin expression upon C1q stimulation as observed in the present study is sufficient to trigger further activation of complement, without leading to massive platelet activation. Consequently, a rapid C1q-induced up-regulation of platelet P-selectin may play an important role at sites of inflammation, e.g. in an atherosclerotic vessel, or at IgG-coated surfaces where C1q is transiently detectable (Tengvall et al., 1996; Wetterö et al., 2001).

In search of the receptor(s) responsible for the C1q-induced P-selectin expression, we used monoclonal antibodies directed towards the gC1qR. Furthermore, since Edelson and co-workers (Edelson et al., 2006) recently identified the αIIβI integrin as a C1q binding structure,

we investigated if the C1q-induced P-selectin expression was dependent upon this receptor, using an anti-αIIβI antibody. Pre-incubation of platelets with 3 μg/mL of antibody against gC1qR (clone 60.11) inhibited C1q-induced P-selectin up-regulation, whereas no inhibition was observed when blocking the αIIβI integrin. We therefore conclude that the initial and rapid effects of C1q are mediated, at least in part, via the gC1qR and not via binding of C1q to the αIIβI integrin. However, the identification of C1q binding structures has not been straightforward and there are some controversies in previously published reports. C1qRp is reported to bind C1q and elicit various cellular responses (Steinberger et al., 2002; Tenner, 1998). Contradictory, C1qRp was later identified to be identical to CD93 and in a study by McGreal et al, no binding between CD93 and C1q was detected (McGreal et al., 2002). Moreover, conflicting results regarding the presence on the cell surface of the receptor with affinity for the globular heads of the C1q molecule (gC1qR) have been revealed. Several studies suggest that gC1qR is present on the surface of e.g. platelets and neutrophils (Eggleton et al., 1995; Peerschke et al., 1994). On the other hand, gC1qR is also shown to be primarily an intracellular protein and in a study by van den Berg et al, gC1qR could not be detected by flow cytometry on the surfaces of monocytes and Raji cells unless the cells where first treated with saponin. However, in the same study, gC1qR was detected on the surface of neutrophils using digoxigenin-conjugated antibodies (van den Berg et al., 1997). Furthermore, gC1qR is shown to be identical to a kininogen-binding protein, the p33, that is involved in the kallikrein-kinin system (Herwald et al., 1996). It is evident that more studies are needed to elucidate the effects of C1q and the role of the proposed C1q receptors on different types of cells. Despite the previous report indicating that gC1qR is not located on the surface of various cells, our results indicate that gC1qR may be present on the surface of platelets. However, previous studies have shown that the expression of gC1q receptors on platelets is activation dependent (Peerschke et al., 2003). Consequently, it is possible that the isolation

procedure leads to priming of platelets and an up-regulation of gC1qR. Such a scenario is relevant as it reflects an *in vivo* situation where platelets in circulation may become primed by a vide range of substances (Gresele et al., 2008). Furthermore, as the antibodies directed towards gC1qR are frequently used in different studies it is valuable information that we in the present study found that both of the gC1qR antibodies (60.11 and 74.5.2) proved to be somewhat blunt tools as they upon pre-incubation provoked an intracellular Ca^{2+} increase and induction of P-selectin at concentrations above 3 µg/mL.

Peerschke et al. reported that C1q induces platelet activation accompanied by an increase in inositol-1,4,5-triphosphate (IP_3) production (Peerschke et al., 1993). IP_3 releases Ca^{2+} from intracellular calcium stores through binding to specific receptors (Brass et al., 1987). In our experiments, however, the addition of C1q to platelets loaded with the ratiometric fluorescent calcium probe Fura-2, did not elicit any detectable changes in the cytosolic calcium levels. Furthermore, presence of C1q in the suspensions did not affect a subsequent Ca^{2+} mobilization triggered by thrombin. This suggests that C1q induces P-selectin expression through a Ca^{2+} independent pathway.

It has become evident that platelet alfa-granule secretion is a far more complicated process then has been appreciated (Blair and Flaumenhaft, 2009) and does not appear to be an "all or nothing" phenomenon, but instead a highly specific process where granules may be released differently depending on the stimulus (Italiano et al., 2008).

Protein kinase C enzymes are important mediators in the intracellular signaling triggered by various platelet agonists (Cohen et al., 2009). Thrombin-induced up-regulation of P-selectin is shown to be dependent upon the novel PKC isoforms ε and η as well as the atypical PKCζ, whereas inhibitors towards the conventional isoforms α and β and the novel δ were without effect (Libersan and Merhi, 2003). The PKC inhibitor GF109203X inhibits all of the PKC-isoforms mentioned above, although with different potency (Martiny-Baron et al., 1993).

Thus, the concentrations of GF109203X used in the present study, 5 and 50 μM, are likely to inhibit several isoforms of the enzyme. Since the addition of C1q to washed platelets in this study did not provoke an intracellular elevation in Ca^{2+}, and both the novel and the atypic PKC isoforms are Ca^{2+} independent (Libersan and Merhi, 2003), we speculate that one of these isoforms of PKC is involved in the C1q-induced up-regulation of P-selectin. In the case of thrombin, it was recently shown that mice lacking the PKCθ isoform displayed a reduced expression of P-selectin upon stimulation, another indication that the novel isoforms are involved in alfa-graule secretion (Cohen et al., 2009).

In our initial studies, using an anti-αIIβI antibody, we found that the rapid and transient P-selectin up-regulation induced by C1q was not mediated via the αIIβI-integrin. However, upon a longer pre-incubation of platelets with C1q, at a physiological concentration, the following collagen and collagen-related peptide-induced expression of P-selectin was diminished. In agreement with this, monomeric C1q has been shown to inhibit collagen-induced aggregation in both isolated platelets and in PRP, as well as reducing the serotonin release from isolated platelets (Cazenave et al., 1976; Csako and Suba, 1981). On the other hand, we did not observe any effect of C1q on the adhesion of platelets to collagen coated surfaces, in correlation with a previous study (Peerschke and Ghebrehiwet, 1990). This is a very interesting finding considering that we in the present study, apart from collagen also used a specific GPVI-activating peptide, i.e. the collagen-related peptide (Morton et al., 1995). The GPVI receptor is regarded as the collagen receptor responsible for activation of non-adherent cells, whereas the αIIβI-integrin is considered to be responsible for adhesion to collagen (reviewed by Surin et al., 2008). It is therefore plausible that previously described C1q-mediated inhibitory effects on platelet activation by collagen and the effects observed in the present study are actually mediated via C1q binding to GPVI, thus explaining the lack of inhibition of platelet adhesion to collagen. However, Jung and Moroi have suggested that the

αIIβI-integrin may also bind soluble collagen and that this requires inside-out activation of the receptor. They have proposed a model of αIIβI activation where the receptor is converted from its resting state via ADP receptor signalling and, depending on agonist-concentration, the receptor binds soluble collagen with low or high affinity (Jung and Moroi, 2000).

The binding of C1q via the collagenous part to any of the collagen receptors on the platelet would expose its globular heads, thereby hypothetically facilitating further immunoglobulin and subsequent complement deposition on the platelet membrane, without provoking an extensive "true" collagen-induced activation. Vice versa, exposure of the collagenous part of the C1q molecule upon binding of C1q to the gC1qR offers the possibility of interaction with other receptors/cells.

We and others have reported that P-selectin binding to its ligand PSGL-1 is crucial in the formation of platelet-neutrophil aggregates (May et al., 2007; Wetterö et al., 2003), which are formed during different inflammatory conditions e.g. cardiovascular disease. In the present study, we investigated if the regulatory effects of C1q on collagen-induced P-selectin expression affected the formation of platelet-neutrophil aggregates. By microscopic analysis, we found that C1q inhibited the collagen-induced formation of platelet-neutrophil aggregates, indicating that the number of sites for P-selectin/PSGL-1 interaction is significantly reduced. These results further strengthen the role of C1q as an important modulator of inflammatory processes.

In summary, the present study demonstrates that C1q causes a moderate up-regulation of P-selectin on the platelet surface. This effect is PKC dependent, but Ca^{2+} independent, and seems to be initiated by C1q binding to the gC1qR, whereas the αIIβI-integrin is not involved. C1q also regulates, through GPVI, collagen- and collagen-related peptide-induced P-selectin expression. Moreover, C1q treatment reduces the collagen-triggered formation of P-selectin dependent platelet-neutrophil aggregates. Future studies intend to target the receptors and

intracellular signaling pathways that may be involved in C1q-mediated modulation of platelet function.

Acknowledgements. The authors wish to thank Dr. Andreas Eriksson for assistance with the platelet adhesion experiments. This study was supported by the strategic research area "Materials in Medicine", the County Council of Östergötland, the Swedish Research Council, the Heart and Lung Foundation, Swedish Society of Medicine, the Swedish Fund for Research Without Animal Experiments, the and the Nanna Svartz, Magn. Bergvall, Lars Hiertas and Goljes research foundations.

References

Andersson, J., Ekdahl, K. N., Lambris, J. D., Nilsson, B., 2005. Binding of C3 fragments on top of adsorbed plasma proteins during complement activation on a model biomaterial surface. Biomaterials. 26**,** 1477-1485.

Andrews, B. S., Shadforth, M., Cunningham, P., Davis, J. S. t., 1981. Demonstration of a C1q receptor on the surface of human endothelial cells. J Immunol. 127**,** 1075-1080.

Bengtsson, T., Grenegård, M., 1994. Platelets amplify chemotactic peptide-induced changes in F-actin and calcium in human neutrophils. Eur J Cell Biol. 63**,** 345-349.

Blair, P., Flaumenhaft, R., 2009. Platelet alpha-granules: basic biology and clinical correlates. Blood Rev. 23**,** 177-189.

Blann, A. D., Nadar, S. K., Lip, G. Y., 2003. The adhesion molecule P-selectin and cardiovascular disease. Eur Heart J. 24**,** 2166-2179.

Bobak, D. A., Frank, M. M., Tenner, A. J., 1986. Characterization of C1q receptor expression on human phagocytic cells: effects of PDBu and fMLP. J Immunol. 136**,** 4604-4610.

Bobak, D. A., Gaither, T. A., Frank, M. M., Tenner, A. J., 1987. Modulation of FcR function by complement: subcomponent C1q enhances the phagocytosis of IgG-opsonized targets by human monocytes and culture-derived macrophages. J Immunol. 138**,** 1150-1156.

Bordin, S., Kolb, W. P., Page, R. C., 1983. C1Q receptors on cultured human gingival fibroblasts: analysis of binding properties. J Immunol. 130**,** 1871-1875.

Bordin, S., Smith, M., Ghebrehiwet, B., Oda, D., Page, R. C., 1992. Smooth muscle and epithelial cells express specific binding sites for the C1q component of complement. Clin Immunol Immunopathol. 63**,** 51-57.

Boyum, A., 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest Suppl. 97**,** 77-89.

Brass, L. F., Shaller, C. C., Belmonte, E. J., 1987. Inositol 1,4,5-triphosphate-induced granule secretion in platelets. Evidence that the activation of phospholipase C mediated by platelet thromboxane receptors involves a guanine nucleotide binding protein-dependent mechanism distinct from that of thrombin. J Clin Invest. 79**,** 1269-1275.

Cazenave, J. P., Assimeh, S. N., Painter, R. H., Pachham, M. A., Mustard, J. F., 1976. Clq inhibition of the interaction of collagen with human platelets. J Immunol. 116**,** 162-163.

Cohen, S., Braiman, A., Shubinsky, G., Ohayon, A., Altman, A., Isakov, N., 2009. PKCtheta is required for hemostasis and positive regulation of thrombin-induced platelet aggregation and alpha-granule secretion. Biochem Biophys Res Commun. 385**,** 22-27.

Csako, G., Suba, E. A., 1981. A physiologic regulator of collagen-induced platelet aggregation: inhibition by Clq. Thromb Haemost. 45**,** 110-115.

Del Conde, I., Cruz, M. A., Zhang, H., Lopez, J. A., Afshar-Kharghan, V., 2005. Platelet activation leads to activation and propagation of the complement system. J Exp Med. 201**,** 871-879.

Edelson, B. T., Stricker, T. P., Li, Z., Dickeson, S. K., Shepherd, V. L., Santoro, S. A., Zutter, M. M., 2006. Novel collectin/C1q receptor mediates mast cell activation and innate immunity. Blood. 107**,** 143-150.

Eggleton, P., Ghebrehiwet, B., Sastry, K. N., Coburn, J. P., Zaner, K. S., Reid, K. B., Tauber, A. I., 1995. Identification of a gC1q-binding protein (gC1q-R) on the surface of human neutrophils. Subcellular localization and binding properties in comparison with the cC1q-R. J Clin Invest. 95**,** 1569-1578.

Eriksson, A. C., Whiss, P. A., 2005. Measurement of adhesion of human platelets in plasma to protein surfaces in microplates. J Pharmacol Toxicol Methods. 52**,** 356-365.

Ferrante, A., Thong, Y. H., 1980. Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leucocytes from human blood by the Hypaque-Ficoll method. J Immunol Methods. 36**,** 109-117.

Gasque, P., 2004. Complement: a unique innate immune sensor for danger signals. Mol Immunol. 41**,** 1089-1098.

Ghebrehiwet, B., Peerschke, E. I., 2004. cC1q-R (calreticulin) and gC1q-R/p33: ubiquitously expressed multi-ligand binding cellular proteins involved in inflammation and infection. Mol Immunol. 41**,** 173-183.

Gorbet, M. B., Sefton, M. V., 2004. Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes. Biomaterials. 25**,** 5681-5703.

Gresele, P., Falcinelli, E., Momi, S., 2008. Potentiation and priming of platelet activation: a potential target for antiplatelet therapy. Trends Pharmacol Sci. 29**,** 352-360.

Haskard, D. O., Boyle, J. J., Mason, J. C., 2008. The role of complement in atherosclerosis. Curr Opin Lipidol. 19**,** 478-482.

Herwald, H., Dedio, J., Kellner, R., Loos, M., Muller-Esterl, W., 1996. Isolation and characterization of the kininogen-binding protein p33 from endothelial cells. Identity with the gC1q receptor. J Biol Chem. 271**,** 13040-13047.

Italiano, J. E., Jr., Richardson, J. L., Patel-Hett, S., Battinelli, E., Zaslavsky, A., Short, S., Ryeom, S., Folkman, J., Klement, G. L., 2008. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. Blood. 111**,** 1227-1233.

Jung, S. M., Moroi, M., 2000. Signal-transducing mechanisms involved in activation of the platelet collagen receptor integrin alpha(2)beta(1). J Biol Chem. 275**,** 8016-8026.

Klickstein, L. B., Barbashov, S. F., Liu, T., Jack, R. M., Nicholson-Weller, A., 1997. Complement receptor type 1 (CR1, CD35) is a receptor for C1q. Immunity. 7**,** 345-355.

Libersan, D., Merhi, Y., 2003. Platelet P-selectin expression: requirement for protein kinase C, but not protein tyrosine kinase or phosphoinositide 3-kinase. Thromb Haemost. 89**,** 1016-1023.

Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D., Schachtele, C., 1993. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. J Biol Chem. 268**,** 9194-9197.

May, A. E., Langer, H., Seizer, P., Bigalke, B., Lindemann, S., Gawaz, M., 2007. Platelet-leukocyte interactions in inflammation and atherothrombosis. Semin Thromb Hemost. 33**,** 123-127.

McGreal, E. P., Ikewaki, N., Akatsu, H., Morgan, B. P., Gasque, P., 2002. Human C1qRp is identical with CD93 and the mNI-11 antigen but does not bind C1q. J Immunol. 168**,** 5222-5232.

Morton, L. F., Hargreaves, P. G., Farndale, R. W., Young, R. D., Barnes, M. J., 1995. Integrin alpha 2 beta 1-independent activation of platelets by simple collagen-like peptides: collagen

tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for alpha 2 beta 1-independent platelet reactivity. Biochem J. 306 (Pt 2)**,** 337-344.

Muller, W., Hanauske-Abel, H., Loos, M., 1978. Biosynthesis of the first component of complement by human and guinea pig peritoneal macrophages: evidence for an independent production of the C1 subunits. J Immunol. 121**,** 1578-1584.

Nicholson-Weller, A., Klickstein, L. B., 1999. C1q-binding proteins and C1q receptors. Curr Opin Immunol. 11**,** 42-46.

Ogden, C. A., deCathelineau A., Hoffmann P. R., Bratton D., Ghebrehiwet B., Fadok V. A., Henson P. M., 2001. C1q and Mannose Binding Lectin Engagement of Cell Surface Calreticulin and Cd91 Initiates Macropinocytosis and Uptake of Apoptotic Cells. J Exp Med 194, 781-796.

Peerschke, E. I., Ghebrehiwet, B., 1987. Human blood platelets possess specific binding sites for C1q. J Immunol. 138**,** 1537-1541.

Peerschke, E. I., Ghebrehiwet, B., 1990. Modulation of platelet responses to collagen by Clq receptors. J Immunol. 144**,** 221-225.

Peerschke, E. I., Ghebrehiwet, B., 1994. Platelet membrane receptors for the complement component C1q. Semin Hematol. 31**,** 320-328.

Peerschke, E. I., Murphy, T. K., Ghebrehiwet, B., 2003. Activation-dependent surface expression of gC1qR/p33 on human blood platelets. Thromb Haemost. 89**,** 331-339.

Peerschke, E. I., Reid, K. B., Ghebrehiwet, B., 1993. Platelet activation by C1q results in the induction of alpha IIb/beta 3 integrins (GPIIb-IIIa) and the expression of P-selectin and procoagulant activity. J Exp Med. 178**,** 579-587.

Peerschke, E. I., Reid, K. B., Ghebrehiwet, B., 1994. Identification of a novel 33-kDa C1q-binding site on human blood platelets. J Immunol. 152**,** 5896-5901.

Peerschke, E. I., Yin, W., Ghebrehiwet, B., 2008. Platelet mediated complement activation. Adv Exp Med Biol. 632**,** 81-91.

Peerschke, E. I., Yin, W., Grigg, S. E., Ghebrehiwet, B., 2006. Blood platelets activate the classical pathway of human complement. J Thromb Haemost. 4**,** 2035-2042.

Reid, K. B., Porter, R. R., 1976. Subunit composition and structure of subcomponent C1q of the first component of human complement. Biochem J. 155**,** 19-23.

Schwaeble, W., Schafer, M. K., Petry, F., Fink, T., Knebel, D., Weihe, E., Loos, M., 1995. Follicular dendritic cells, interdigitating cells, and cells of the monocyte-macrophage lineage are the C1q-producing sources in the spleen. Identification of specific cell types by in situ hybridization and immunohistochemical analysis. J Immunol. 155**,** 4971-4978.

Sjöwall, C., Wetterö, J., Bengtsson, T., Askendal, A., Almroth, G., Skogh, T., Tengvall, P., 2007. Solid-phase classical complement activation by C-reactive protein (CRP) is inhibited by fluid-phase CRP-C1q interaction. Biochem Biophys Res Commun. 352**,** 251-258.

Skoglund, C., Wetterö, J., Skogh, T., Sjöwall, C., Tengvall, P., Bengtsson, T., 2008. C-reactive protein and C1q regulate platelet adhesion and activation on adsorbed immunoglobulin G and albumin. Immunol Cell Biol. 86**,** 466-474.

Steinberger, P., Szekeres, A., Wille, S., Stockl, J., Selenko, N., Prager, E., Staffler, G., Madic, O., Stockinger, H., Knapp, W., 2002. Identification of human CD93 as the phagocytic C1q receptor (C1qRp) by expression cloning. J Leukoc Biol. 71**,** 133-140.

Surin, W. R., Barthwal, M. K., Dikshit, M., 2008. Platelet collagen receptors, signaling and antagonism: emerging approaches for the prevention of intravascular thrombosis. Thromb Res. 122**,** 786-803.

Tengvall, P., Askendal, A., Lundström, I., 1996. Complement activation by IgG immobilized on methylated silicon. J Biomed Mater Res. 31**,** 305-312.

Tenner, A. J., 1998. C1q receptors: regulating specific functions of phagocytic cells. Immunobiology. 199**,** 250-264.

van den Berg, R. H., Prins, F., Faber-Krol, M. C., Lynch, N. J., Schwaeble, W., van Es, L. A., Daha, M. R., 1997. Intracellular localization of the human receptor for the globular domains of C1q. J Immunol. 158**,** 3909-3916.

Vegh, Z., Kew, R. R., Gruber, B. L., Ghebrehiwet, B., 2006. Chemotaxis of human monocyte-derived dendritic cells to complement component C1q is mediated by the receptors gC1qR and cC1qR. Mol Immunol. 43**,** 1402-1407.

Wallis R., Mitchell D. A., Schmid R., Schwaeble W. J., Keeble A. H., 2010. Paths reunited: Initiation of the classical and lectin pathways of complement activation. Immunobiology. 215, 1-11

Wetterö, J., Askendal, A., Bengtsson, T., Tengvall, P., 2002. On the binding of complement to solid artificial surfaces in vitro. Biomaterials. 23**,** 981-991.

Wetterö, J., Bengtsson, T., Tengvall, P., 2001. C1q-independent activation of neutrophils by immunoglobulin M-coated surface. J Biomed Mater Res. 57**,** 550-558.

Wetterö, J., Tengvall, P., Bengtsson, T., 2003. Platelets stimulated by IgG-coated surfaces bind and activate neutrophils through a selectin-dependent pathway. Biomaterials. 24**,** 1559-1573.

von Hundelshausen, P., Weber, C., 2007. Platelets as immune cells: bridging inflammation and cardiovascular disease. Circ Res. 100**,** 27-40.

Young, K. R., Jr., Ambrus, J. L., Jr., Malbran, A., Fauci, A. S., Tenner, A. J., 1991. Complement subcomponent C1q stimulates Ig production by human B lymphocytes. J Immunol. 146**,** 3356-3364.