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

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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 s 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 s 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

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

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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. 2006, 2008) 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 the 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 111 mM glucose) was centrifuged at 220*g* for 20 min at room temperature. Platelet rich plasma (PRP) was collected and platelets were pelleted by centrifugation at 480*g* for 20 min 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*g* for 40 min 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*g* for 10 min. Remaining red blood cells were removed by brief hypotonic lysis at 4 °C followed by washing in KRG at 200*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 (2×10^8/mL) were incubated in a 24-well plate (Sarstedt AG & Co., Nümbrecht, Germany) at 37 °C for 5 min. 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 s. 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 °C, 30 min), 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 (80 μg/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-Pro-Hyp)$_{10}$-Gly-Cys-Hyp-Gly-NH_2, kindly provided by Dr. Richard W. Farndale and Dr. Graham Knight, Cambridge, UK) or TRAP