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Classical pathway complement activation on human endothelial cells

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Abstract

Endothelial cells regulate vascular integrity and express complement binding proteins including gC1qR/p33 (gC1qR), which recognize C1q, a subunit of the first component of the classical complement pathway. Experiments were performed to investigate classical complement pathway activation on resting endothelial cells and endothelial cells exposed to shear stress. C1q deposition and C4 activation (C4d) were demonstrated by solid phase ELISA and flow cytometry on human microvascular and umbilical vein endothelial cells after exposure to serum or plasma. C4d deposition was accompanied by downstream complement activation including C3b and C5b-9 deposition. C4 activation failed to occur in C1q depleted serum, but was not affected by Factor B depleted serum, confirming classical complement pathway activation. Moreover, C4 activation occurred following exposure of endothelial cells to purified C1 and C4, in the absence of other plasma proteins, and in the absence of detectable cell surface IgG and IgM. Shear stress (18 dynes/cm^2) increased C1q ($n=9$, $p<0.05$) and C4d ($n=9$, $p<0.05$) deposition approximately two-fold, and enhanced endothelial cell gC1qR expression ($n=7$, $p<0.05$). Treatment of endothelial cells with anti gC1qR monoclonal antibody F(ab′)$_2$ fragments reduced C4d deposition by approximately 20% ($n=5$, $p<0.05$). These data demonstrate direct classical complement pathway activation on endothelial cells. gC1qR appears to play a minor but definable role, whereas cell surface IgG or IgM are not required.

Keywords: Complement; gC1qR; Classical pathway; Shear stress

1. Introduction

Atherosclerosis and its related thrombotic complications are leading causes of cardiac death and stroke in developed countries. Recent studies have shown that the development of atherosclerotic lesions is associated with inflammation (Peerschke et al., 2004; Acosta et al., 2004; Ross, 1999; Tiong and Brieger, 2005) and related thrombosis (Libby et al., 2002). Complement activation has been demonstrated in atherosclerotic lesions, and is considered an important contributor in the development of pathological plaques (Bhakdi, 1998; Niculescu et al., 2004; Torzewski et al., 1997). A number of complement proteins have been identified in atherosclerotic lesions, including C1q (Niculescu and Rus, 1999; Yasojima et al., 2001), the recognition unit of the classical complement pathway.

Vascular endothelial cells express several C1q binding sites, including cC1qR/calreticulin and gC1qR/p33 (gC1qR) (Eggleton et al., 2000; Ghebrehiwet and Peerschke, 2004). Among these, gC1qR is the most versatile. gC1qR binds C1q via its globular head domain, and also recognizes high molecular weight kininogen and factor XII, with the capacity to activate the kinin system (Joseph et al., 2004; Reddigari et al., 1993) and coagulation cascade (Peerschke and Ghebrehiwet, 1998). Recently purified gC1qR was found to directly activate the classical complement pathway (Ghebrehiwet et al., 2006).

Vascular endothelial cells (EC) play an important role in regulating local hemostasis and thrombosis (Saadi et al., 2000). Given the ability of gC1qR to interact with components of complement, coagulation, and kinin systems, endothelial cell gC1qR may play a crucial role in local inflammatory reactions and thrombotic complications related to atherosclerosis (Ghebrehiwet and Peerschke, 2004; Guo et al., 1999).

Complement activation and endothelial cell damage are involved in the pathogenesis of atherosclerosis. However, the mechanism of complement activation in atherosclerotic lesions,

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the effect of blood flow/shear stress on this process, and the participation of gC1qR have not been investigated. The present study was designed to evaluate direct classical complement pathway activation on endothelial cells and access the role of gC1qR.

2. Methods

2.1. Cell culture

Bone marrow (BMEC) (Schweitzer et al., 1997) and brain (Weksler et al., 2005) microvascular endothelial cell lines were used between passages 14 and 30. BMEC were grown to confluence on 0.2% gelatin (Sigma–Aldrich Corp., St. Louis, MO), and maintained in Dulbecco's Modified Eagle Media (DMEM), supplemented with 5% fetal bovine serum (FBS), 10 mM HEPES (hydroxyethyl piperazine ethanesulfonic acid), and 1:100 penicillin/streptomycin (10,000 unit penicillin and 10,000 μg/ml streptomycin) (Invitrogen Corp., Carlsbad, CA). Human brain microvascular endothelial cells were grown to confluence on 1:20 collagen type I (Rat Tail collagen I, Becton Dickinson, Lincoln Park, NJ), and maintained in endothelial cell basal medium-2 (EBM-2) (Cambrex Corporation, NJ) supplemented with 5% FBS, 1:1000 human fibroblast growth factor (hFGF), 1:1000 vascular endothelial cell growth factor (VEGF), 1:1000 ascorbic acid, 1:1000 h-epidermal growth factor and 50 μg/ml gentamicin sulfate (Cambrex Corporation, NJ). Primary human umbilical vein endothelial cells (HUVEC) (ScienCell Research Laboratories, San Diego, CA) (passages 2 and 3) were grown to confluence on 1% gelatin, and maintained in endothelial cell medium (ECM) (ScienCell Research Laboratory) supplemented with 5% FBS, 1:5 penicillin/streptomycin (ScienCell Research Laboratory) and 1:5 endothelial cell growth supplement (ECGS) (ScienCell Research Laboratory).

All endothelial cells were detached with 1X trypsin/EDTA (Invitrogen Corp., Carlsbad, CA). Trypsin was neutralized with excess culture medium. Cells were washed by centrifugation at $150 \times g$ for 5 min, and the cell pellet was suspended in HEPES buffered modified Tyrode's solution (HMBT) containing 1 mM $CaCl_2$ and 2 mM $MgCl_2$ (Peerschke et al., 1996), or gelatin containing Veronal buffer with magnesium and calcium (GVB^{2+}) (Boston BioProducts Inc., Worcester, MA).

2.2. Plasma/serum

Platelet poor plasma (PPP), anticoagulated with 0.32% sodium citrate and normal human serum (NHS) were prepared as described previously (Peerschke et al., 2006). Since activation of complement by thrombin and plasmin, two enzymes present in serum, has been described (Schaiff and Eisenberg, 1997; Huber-Lang et al., 2006), preliminary studies compared complement activation on endothelial cells following incubation with plasma or serum. Dilution (1/10) of anticoagulated plasma in divalent cation containing buffers (HBMT or GVB^{2+}) assured the presence of sufficient calcium and magnesium to support complement activation.

2.3. Antibodies

A murine monoclonal antibody, designated 74.5.2 (Ghebrehiwet et al., 1996; Peerschke et al., 2006), against gC1qR was used at 5–100 μg/ml throughout the study. Biotinylated monoclonal anti-human C1q, and C4d antibodies, as well as monoclonal anti-human iC3b and scC5b-9 were obtained from Quidel Corporation (San Diego, CA), and used at dilutions of 1:200 (~1 to 5 μg/ml) in HBMT. Polyclonal anti-human C1q and C4 antibodies were raised in our laboratory. These antibodies were biotinylated (Nguyen et al., 2000), and used at dilutions of 1:200 (about 1–2 μg/ml) in GVB^{2+}.

2.4. Classical pathway complement activation on endothelial cells

Deposition of C1q and C4d, a degradation product of C4b, on endothelial cells was measured by solid phase ELISA or flow cytometry. For ELISA, endothelial cell suspensions were immobilized on poly-L-lysine (Sigma–Aldrich Corp., St. Louis, MO) (10 μg/ml) coated 96-well microtiter plates (Becton Dickinson, Lincoln Park, NJ) by centrifugation ($1000 \times g$, 5 min) (~2×10^4 cells/well). Adherent cells were fixed with 0.5% glutaraldehyde, followed by neutralization with 100 mM glycine–0.1% bovine serum albumin (BSA, 99%, fatty acid free, Sigma–Aldrich). After washing, endothelial cells were exposed (1 h, 37 °C) to PPP, NHS, or C1q- (classical pathway) or Factor B- (alternative pathway) depleted serum (Quidel Corporation, San Diego, CA) diluted (1:10) in HBMT. In separate experiments, BMEC were exposed to the combination of purified C1 (5 μg/ml) (The Binding Site, Inc.) and C4 (5 μg/ml) (Quidel Corporation) in GVB^{2+} for 60 min at 37 °C. Bound complement components were detected by addition of biotinylated anti-C1q, biotinylated anti C4d, anti iC3b, or anti scC5b-9 antibodies, as needed. Primary antibody binding was detected using alkaline phosphatase conjugated streptavidin (Immunopure Streptavidin, Pierce Biotechnology Inc.) or an alkaline phosphatase conjugated goat anti mouse antibody, followed by addition of 1 mg/ml *p*-nitrophenyl phosphate substrate (pNPP) (Pierce Biotechnology, Inc.). Color development was quantified at 405 nm (reference at 490 nm) using a Thermomax microplate reader (Molecular Devices Corp., Palo, Alto, CA). Background complement deposition was measured on endothelial cells exposed to HBMT instead of plasma or serum.

To rule out artifacts of endothelial cell fixation, complement activation was evaluated by flow cytometry of endothelial cell suspensions in HBMT incubated with PPP (1:10) at 37 °C for 45 min. After washing, classical pathway complement activation was measured using biotinylated anti-human C4d (30 min at 25 °C), detected with Delight 647 conjugated streptavidin (Pierce Biotechnology Inc.). MOPC 21 (IgG1k) (Sigma–Aldrich) was used as a non-immune primary antibody control. Background C4d deposition was assessed on endothelial cells exposed to HMBT instead of plasma.