Mannose-binding lectin binds IgM to activate the lectin complement pathway in vitro and in vivo

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Abstract

Recent evidence has implicated a role for the MBL-dependent lectin pathway in gastrointestinal and myocardial ischemia/reperfusion (I/R)-induced injury. However, previous studies have implicated IgM and the classical pathway as initiators of complement activation following I/R. Thus, we investigated the potential interaction between MBL and IgM leading to complement activation. Using surface plasmon resonance, we demonstrate that MBL does bind human IgM. Subsequently, functional complement activation was demonstrated in vitro following sensitization of human RBCs with mouse anti-human CD59 IgM and more lysis was observed with MBL sufficient sera compared to MBL deficient (KO) sera. Similarly, treatment of human endothelial cells with mouse anti-human CD59 IgM, MBL and MASP-2 activated and deposited C4. These data suggest that the presence of both IgM and MBL can activate the lectin pathway in vitro. Serum ALT levels increased significantly in sIgM/MBL-A/C KO mice reconstituted with WT plasma compared to sIgM/MBL-A/C KO mice treated with MBL-A/C KO plasma following gastrointestinal (G) I/R. Similarly, intestinal C3 deposition was greater in sIgM/MBL-A/C KO mice reconstituted with WT plasma compared to sIgM/MBL-A/C KO mice treated with MBL-A/C KO plasma. These data indicate for the first time that both IgM and MBL-A/C are required for GI/R-induced complement activation and subsequent injury.

Keywords: Antibody; Classical pathway; Ischemia–reperfusion; Lectin pathway and surface plasmon resonance

Introduction

Ischemia–reperfusion (I/R) injury is associated with multiorgan dysfunction syndrome, sepsis, hemorrhagic shock, transplantation and myocardial infarction (Eltzschig and Collard, 2004). Numerous studies have implicated complement in gastrointestinal ischemia–reperfusion (GI/R)-induced tissue injury by...
injury using CR2
complement in infarcted human myocardium, while shown that IgM depositions were colocalized with IgM in I/R-induced injury. Krijnen et al. (2005) have 1999). Similarly, other studies have implicated a role for and GI/R injury (Weiser et al., 1996; Williams et al., 2002). The classical, lectin or alternative pathways can activate complement, a major component of the innate immune system. C1q and mannose-binding lectin (MBL) with their associated serine proteases activate the classical and MBL-dependent lectin complement pathways, respectively. The subsequent cleavage of C3 and C5 can lead to anaphylatoxin production and the formation of the terminal complement complex (C5b-9), which may lyse pathogens and induce inflammation and tissue injury (Riedemann and Ward, 2003; Sim and Laich, 2000; Walport, 2001).

Recent studies from our laboratory have shown that the MBL-dependent lectin pathway initiates complement activation following myocardial I/R and GI/R (Hart et al., 2005; Walsh et al., 2005). Importantly, these studies also demonstrated that I/R-induced injury occurs independent of C1q (e.g., the classical pathway) (Hart et al., 2005; Walsh et al., 2005). Other studies have implicated that MBL plays a role in renal I/R-induced tissue injury (de Vries et al., 2004; Moller-Kristensen et al., 2005). These findings appear to contradict studies by others that have implicated immunoglobulin (Ig)M binding to neoepitopes on hypoxic tissue and classical complement pathway activation as initiators of skeletal and GI/R injury (Weiser et al., 1996; Williams et al., 1999). Similarly, other studies have implicated a role for IgM in I/R-induced injury. Krijnen et al. (2005) have shown that IgM deposits were colocalized with complement in infarcted human myocardium, while another study has implicated a role for IgM in I/R injury using CR2−/− mice, which lack a subset of natural antibodies (Fleming et al., 2002). The moderating effect on inflammatory responses observed following the application of C1 inhibitor led other groups to implicate a role of the classical pathway in I/R-induced injury (Buerke et al., 1998; Horstick et al., 1997). Thus, the role of IgM, MBL, classical and lectin complement pathway activation in these studies appears to contradict our laboratory’s recent findings.

A potential explanation for these experimental differences is that MBL may bind IgM and initiate complement activation. The activation of complement through binding of C1q to immune complexes via the classical pathway is well established (Sim and Laich, 2000; Walport, 2001). In addition, there is mounting evidence that MBL may also bind immune-complexes and activate complement via the lectin pathway (Arnold et al., 2005; Nevens et al., 1992; Roos et al., 2001). An interaction between IgM and MBL has been shown in the purification of murine monoclonal IgM using immobilized rabbit MBL and by direct binding of human serum IgM to MBL in an ELISA (Arnold et al., 2005; Nevens et al., 1992). In addition, MBL binding to polymeric IgA induces complement activation (Roos et al., 2001). These studies suggest that MBL may also activate complement in an antibody-dependent fashion. Our present study strongly underlines this assumption and demonstrates the interactions between IgM and MBL leading to complement activation in experimental vivo and in vitro models.

Materials and methods

Surface plasmon resonance (SPR)

Experiments were performed on a BIAcore 3000 (BIAcore AB, Uppsala, Sweden) as described (Montalto et al., 2001). The BIAcore reagents for the interaction analysis including CM5 biosensors, N-hydroxysuccinimide (NHS), N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), 1 mM ethanolamine, pH 8.5, were obtained from Pharmacia Biosensor AB (Uppsala, Sweden). SPR was used to detect the interaction of IgM with full-length recombinant human MBL (rhMBL). rhMBL [~4000 Resonance Units (RU)] was immobilized by amine coupling (EDC/NHS) according to the manufacturer’s instructions (BIAcore, Uppsala, Sweden) and the remaining sites blocked with ethanolamine. The control flow cell was activated with EDC/NHS and blocked with ethanolamine. The running buffer consisted of 10 mM HEPES, 143 mM NaCl, 10 mM CaCl2 and 10 mM MgCl2, pH 7.4.

Human IgM (Sigma) was buffer exchanged with running buffer using Vivaspin 30 kD concentrators (Vivascience Limited, Lincoln, UK). Human IgM was injected at 20 μl/min for 2 min at various concentrations. Each analyte concentration was separated by regeneration of the CM5 biosensor using 350 mM EDTA. Each sensorgram represents the relative response after subtraction of the reference (control) flow cell.

Cell culture

Human umbilical vein endothelial cells (HUVECs) from pooled donors were obtained from VEC Technologies (Troy, NY). The cells were maintained in M199 media (Gibco) supplemented with 20% FBS, 1% penicillin/streptomycin, 10 μg/mL heparin and 100 μg/mL of endothelial cell growth supplement (BD Bioscience). The cells were cultured in 75 cm2 flasks coated with 0.1% gelatin.

IgM-mediated hemolysis

Human blood was drawn from normal volunteers into Alsever’s solution, centrifuged and the red blood cell (RBC) layer removed. The isolated human RBCs were washed 4 × with GVB+ (150 mM NaCl, 7.2 mM...
sodium barbital, 400 μM MgCl₂, 60 μM CaCl₂ and 0.1% gelatin), counted and resuspended in GVB⁺⁺ at 5 × 10⁶ cells/mL. Subsequently, cells were sensitized with mouse anti-human CD59 IgM antibody (clone C2G4; gift from Dr. Lloyd Klickstein: Brigham and Women’s Hospital) followed by two washes with GVB⁺⁺. Serum from C1qa⁻/- (Botto et al., 1998) or MBL-A/C⁻⁻ mice was diluted with GVB⁺⁺ as indicated and added to round bottom 96-well plates followed by the addition of 3 × 10⁶ RBCs. After incubation for 30 min at 37°C, the samples were centrifuged, the supernatant transferred to a flat bottom 96-well plate and read at 405 nm (SpectraMax Plus, Molecular Devices).

**IgM-dependent C4 deposition**

HUVECs were grown to confluence in 96-well plates coated with 0.1% gelatin. Mouse anti-human CD59 IgM antibody (clone C2G4), rat anti-human CD59 IgG antibody (clone YTH53.1) or rat anti-human CD1d IgM antibody (clone 3C11; gift from Dr. Sean Colgan: Brigham and Women’s Hospital), rhMBL (0.1 μg/mL) and rhMBL-associated serine protease (rhMASP-2) (0.5 μg/mL) in GVB⁺⁺ were added to the cells and incubated at 37°C for 1 h followed by washing with ELISA wash buffer supplemented with or without calcium (45 mM NaCl, 10 mM HEPES, 680 μM CaCl₂ and 490 μM MgCl₂). Human C4 (60 μg/mL) was added for 30 min at 37°C in GVB⁺⁺ followed by washing (e.g., 2 × with ELISA wash buffer). Cells were fixed in 1% paraformaldehyde for 15 min at RT followed by washing (e.g., 3 × with ELISA wash buffer). Goat anti-human C4 (ICN) was diluted 1:1000 in PBS/1% BSA and incubated for 1 h at RT followed by three washes. Subsequently, donkey anti-goat IRDye 800 Ab (Rockland) was diluted 1:1000 in PBS/1% BSA and incubated for 1 h at RT. Following washing (3 ×), the plates were read on the 800 nm channel of the Odyssey Infrared Imaging System (Li-Cor).

**Gastrointestinal I/R**

C57BL/6 (wild type (WT)), MBL-A/C⁻⁻ and slgM/MBL-A/C⁻⁻ (slgM/MBL-A/C KO) mice were heparinized (20 U) and blood was collected by cardiac puncture. MBL-A/C⁻⁻ mice, deficient in MBL A and C, were bred with slgM⁻/-, which lack circulating IgM (Boes et al., 1998), to make a novel mouse line, slgM/MBL-A/C KO, which are MBL null and also lack slgM. Blood was centrifuged at 3000 rpm for 10 min and plasma was collected. WT and slgM/MBL-A/C KO male mice (8–12 weeks) were anesthetized with isoflurane. Freshly harvested plasma (0.5 mL) was administered through penile vein injection just prior to surgery. Following a midline laparotomy, the superior mesenteric artery was clamped for 20 min, released and reperfused for 3 h as described (Stahl et al., 2003; Hart et al., 2005; Zhao et al., 2002). Gastrointestinal tissues were harvested and peripheral circulating blood collected following reperfusion. All animal experiments were conducted under a protocol approved by the HMA standing committee on animals at Brigham and Women’s Hospital.

**Alanine aminotransferase (ALT) serum activity measurements**

Serum ALT levels were measured with a kit purchased from Teco Diagnostics as described (Hart et al., 2005).

**Immunostaining for C3 deposition**

Tissue samples were fixed in 10% formalin, dehydrated and embedded in paraffin. Sections (~7 μm) were cut and tissue stained for C3 deposition with a FITC-conjugated goat anti-mouse complement C3 antibody (MP BioMedicals) as described (Hart et al., 2005). Slides were analyzed with a Zeiss confocal microscope as previously described using the same pinhole, voltage and laser settings (Hart et al., 2005).

**Statistical analysis**

All data are plotted as means ± SEM. The data for the ALT assay were analyzed with a one-way ANOVA followed by the Dunnett’s post hoc test using SigmaStat software (SPSS) with p ≤ 0.05 denoted as significant.

**Results**

SPR was used to characterize human IgM interactions to rhMBL immobilized to the biosensor surface. Representative sensorgrams for the interactions of human IgM at various concentrations to immobilized rhMBL are shown in Fig. 1. We observed a dose-dependent increase in IgM binding to immobilized rhMBL. This binding was calcium dependent, as the biosensor was regenerated successfully using EDTA (data not shown), suggesting that the MBL-IgM interactions are via the carbohydrate recognition domains (CRD).

In order to determine if IgM and MBL interactions result in complement activation, serum from C1q KO and MBL-A/C KO mice was serially diluted and used in a RBC hemolytic assay. Using human RBCs coated with mouse anti-human CD59 IgM mAb, more hemolytic activity was observed in C1q KO serum compared to MBL-A/C KO serum (Fig. 2). These data
demonstrate that in the absence of C1q, mouse IgM interacts with mouse complement components in C1q deficient sera (likely MBL) resulting in more hemolytic activity compared to MBL deficient sera, which contains C1q.

To investigate the specificity of IgM/MBL interactions further, we used HUVECs and mAbs (e.g., IgG or IgM) against CD59, an abundant endothelial cell complement regulatory protein (Brooimans et al., 1992). In order to ascertain whether complement activation via MBL interactions with IgM or IgG took place in this cell-based assay, mouse anti-human CD59 IgM (clone C2G4), MASP-2 and C4 were added to the cells. We observed a dose-dependent increase in C4 deposition on HUVECs in the presence of IgM, but not IgG (Fig. 3). In separate experiments, we observed a 6-fold increase in C4 deposition on HUVECs when a different monoclonal

IgM antibody specific to another endothelial cell surface marker (rat anti-CD1d, clone 3C11) was used and compared to IgM alone. These data suggest that MBL interacts with IgM on human endothelial cells and results in complement activation in the presence of MASP-2.

We next investigated whether IgM interacts with MBL in vivo to induce complement activation and
tissue injury following GI/R. sIgM/MBL-A/C KO mice were injected with plasma (0.5 ml) from WT or MBL-A/C KO mice to restore either sIgM and MBL-A/C or sIgM alone, respectively. As a control for the increase in blood volume and other plasma components, sIgM/MBL-A/C KO plasma was injected into sIgM/MBL-A/C KO mice. As an additional control, WT plasma was injected into WT mice to control for potential exacerbated injury induced by increasing complement components and other unknown factors. Interestingly, sIgM/MBL-A/C KO mice injected with WT plasma had a significant increase in ALT levels following GI/R when compared to sIgM/MBL-A/C KO mice injected with MBL-A/C KO or sIgM/MBL-A/C KO plasma (Fig. 4). There was no significant difference between sIgM/MBL-A/C KO mice injected with WT plasma or WT mice injected with WT plasma (Fig. 4). These data demonstrate that the presence of both sIgM and MBL-A/C in the WT plasma is required for I/R-induced injury since reconstitution of sIgM alone with the MBL-A/C KO plasma does not increase injury.

C3 deposition in the intestine following GI/R is MBL dependent (Hart et al., 2005). No increase in C3 deposition was observed in sIgM/MBL-A/C KO animals following GI/R compared to sham-operated controls (Fig. 5(B) compared to Fig. 5(A), respectively). These data demonstrate that complement activation does not take place following GI/R in the absence of sIgM and MBL-A/C (Fig. 5(B)). Restoration of sIgM and MBL-A/C using WT plasma in the sIgM/MBL-A/C KO mice significantly increased C3 deposition in the intestine (Fig. 5(C)). In contrast, restoration of only sIgM through the addition of MBL-A/C KO plasma to the sIgM/MBL-A/C KO animals did not increase C3 deposition following GI/R (Fig. 5(D)). Collectively, these data suggest that interactions between IgM and MBL-A/C in vivo can induce complement activation and deposition following GI/R.

Fig. 5. C3 deposition in the intestine. Confocal micrographs of intestinal sections of sham-operated sIgM/MBL-A/C KO mice (A) or sIgM/MBL-A/C KO (B), sIgM/MBL-A/C KO+WT plasma (C) and sIgM/MBL-A/C KO+MBL-A/C KO plasma (D) following GI/R. Each micrograph is representative of three mice.
Discussion

The present study suggests that an interaction between IgM and MBL leads to activation of the MBL-dependent portion of the lectin complement pathway. SPR experiments demonstrate in vitro that MBL can bind human IgM. Further, serum from C1q KO mice, which contains MBL-A/C, increased mouse IgM-sensitized human RBC hemolysis compared to serum from MBL-A/C KO mice, which contains C1q, but not MBL. The addition of mouse anti-human CD59 IgM, rhMBL and rhMASP-2 to HUVEC monolayers was sufficient to induce C4 deposition. These data suggest that the presence of both IgM and MBL can lead to activation of the MBL-dependent portion of the lectin complement pathway in vitro. Following GI/R, sIgM/MBL-A/C KO mice given WT plasma have increased ALT levels as compared to sIgM/MBL-A/C KO mice given MBL-A/C KO plasma. Similarly, C3 deposition in the intestine is greater in the sIgM/MBL-A/C KO mice given WT plasma compared to sIgM/MBL-A/C KO mice given MBL-A/C KO plasma. These data indicate for the first time that IgM and MBL-A/C can interact in an antibody-dependent manner to activate the MBL-dependent portion of the lectin complement pathway following GI/R.

Our data clearly indicate that IgM binds to MBL. IgM can be found as a pentamer or hexamer in the circulation; however, the hexameric form of IgM has been shown to be a more efficient activator of complement compared to the pentameric form (Collins et al., 2002; Hughey et al., 1998). A recent study has shown that pentameric IgM that is bound to antigen cannot bind MBL because of the presence of the J chain; however, hexameric IgM, which lacks the J chain has a planar arrangement that allows for antigen and MBL binding (Arnold et al., 2005). The presence of oligo-mannose structures at Asn-402 and Asn-563 of IgM suggest that MBL may bind to IgM at these sites (Chapman and Kornfeld, 1979a, b). However, the precise interaction sites of MBL with IgM are currently not known in our studies.

Previous studies implicating the classical pathway as the leading activator of complement following I/R have utilized non-specific means of inhibition (Buerke et al., 1998; Horstick et al., 1997; Weiser et al., 1996; Williams et al., 1999). For example, the tissue protection observed in C4 KO mice following I/R could be due to the lack of the classical or lectin pathways in these mice (Weiser et al., 1999; Williams et al., 1999). Similarly, C1 inhibitor has been shown to inhibit both the classical and lectin pathways (Cicardi et al., 2005); therefore, the protective effects of this inhibitor could be due to inhibition of either the classical or lectin pathways (Buerke et al., 1998; Horstick et al., 1997). Recent studies from our lab have clearly demonstrated that the MBL-dependent lectin pathway, not the classical pathway, activates complement following intestinal or myocardial I/R (Hart et al., 2005; Walsh et al., 2005). The present study advances our previous findings by utilizing mice lacking both sIgM and MBL-A/C to determine the role of each by reconstituting sIgM and MBL-A/C or sIgM alone. Following GI/R, complement activation and tissue injury were increased only when both sIgM and MBL-A/C were present. These data help explain the discrepancy in the current literature by showing that sIgM and MBL are both required for complement activation following I/R. These are the first data to demonstrate in vivo that MBL interacts with IgM to activate the MBL-dependent portion of the lectin complement pathway.

IgM binds to the endothelium and ischemic tissue during ischemia while C3 deposition does not occur until the tissue is reperfused (Chan et al., 2004). These data suggest that IgM may bind to hypoxia-induced neoepitopes. Previous studies have suggested that specific IgM antibodies bind to self-antigens expressed as a result of hypoxia, which have been identified as nonmuscle myosin heavy chain type II A and C (Zhang et al., 2004; Zhang et al., 2006). We demonstrate that C3 deposition in the intestine after reperfusion only occurs when both IgM and MBL are present. Therefore, we propose a model in which IgM binds to intestinal and/or endothelial neo-epitopes following I/R, making a binding site for MBL. MBL binding to this ligand complex initiates lectin complement pathway activation, which then contributes to I/R-induced tissue inflammation and injury.

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References

glycoforms that can bind to mannose-binding lectin. J. Biol. Chem. 280, 29080–29087.


