Role of C3 Cleavage in Monocyte Activation During Extracorporeal Circulation

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Background—We previously demonstrated that inhibiting formation of terminal complement components (C5a and C5b-9) prevents platelet and neutrophil (PMN) but not monocyte activation during simulated extracorporeal circulation (SECC). This study examined whether earlier complement inhibition during SECC, blocking C3a formation, would additionally prevent monocyte activation.

Methods and Results—SECC was established by recirculating heparinized whole blood from human volunteers on a membrane oxygenator. CAB-2, a chimeric protein constructed from genes encoding the complement regulatory proteins CD46 and CD55, inactivates the C3/C5 convertases and blocks in vitro generation of C3a, C5a, and C5b-9. CAB-2 was used in 4 experiments at a final concentration of 300 μg/mL and 4 experiments at 30 μg/mL; 4 control runs used vehicle alone. Samples were assayed for C3a and C5b-9, monocyte activation (CD11b upregulation), PMN activation (CD11b upregulation and elastase release), and platelet activation (P-selectin expression and monocyte-platelet conjugate formation). CAB-2 at both doses significantly inhibited formation of C3a and C5b-9 during SECC. High-dose CAB-2 significantly blocked monocyte and PMN CD11b upregulation and PMN elastase release. CAB-2 also inhibited formation of platelet activation–dependent monocyte–platelet conjugates.

Conclusions—Blockade of complement activation early in the common pathway inhibited monocyte CD11b upregulation during SECC, suggesting that early complement components contribute most to monocyte activation during SECC. As expected, PMN and platelet activation were blocked by terminal complement inhibition. This investigation further elucidates the relation between complement and blood cell activation during simulated cardiopulmonary bypass.

Key Words: extracorporeal circulation ▪ cell adhesion molecules ▪ leukocytes ▪ platelets

Cardiopulmonary bypass (CPB) is associated with an inflammatory response consisting of humoral and cellular changes that contribute to tissue injury. Complement activation is a potential contributor to this inflammatory response,1,2 but the relation between specific complement components and the activation of inflammatory or coagulant pathways has been difficult to ascertain. Simulated extracorporeal circulation (SECC) has been used extensively in vitro to model inflammatory changes produced during in vivo CPB.3,4 SECC activates complement (C3a and C5b-9 formation), platelets (increased CD62P-positive platelets and monocyte-platelet conjugate formation), and leukocytes [neutrophil (PMN) and monocyte CD11b upregulation and PMN elastase secretion] comparable to in vivo bypass.5 We previously demonstrated that inhibiting formation of the late complement components, C5a and C5b-9, during SECC prevents PMN and platelet activation but did not clearly inhibit monocyte activation.4 In the present study, we used complement activation blocker-2 (CAB-2) to explore the role of C3a formation in monocyte activation during SECC. CAB-2, the product of a chimeric gene constructed from the genes encoding human membrane cofactor protein (MCP, CD46) and human decay accelerating factor (DAF, CD55), is a soluble, glycosylated, 110-kDa protein whose chimeric nature is confirmed by reactivity with both MCP- and DAF-specific antibodies.6 MCP cleaves complement factors C3b and C4b to their inactive forms, iC3b and iC4b, respectively. The complement regulatory activity of DAF results from its ability to dissociate C3 and C5 convertase subunits, thus downregulating formation of C3a and C5a, respectively. The chimeric CAB-2 product combines both of these activities and thus inactivates both classic and alternative C3/C5 convertases through proteolysis of C3b (through MCP) and enhancement of convertase decay (through DAF),6 thereby inhibiting formation of C3a, C5a, and C5b-9 in vitro and in vivo.
The soluble activities of CAB-2 have IC_{50} values nearly identical to soluble MCP (sMCP) and soluble DAF (sDAF). However, against cell-associated convertases, CAB-2 has greater activity than the parent proteins combined, with 150-fold more MCP activity and 10-fold more DAF activity against classical pathway-mediated sheep red blood cell hemolysis. Blockade of alternative pathway hemolysis was similarly greater with CAB-2 than that seen with a mixture of sMCP and sDAF in both a Forsmann shock model (guinea pig) and during in vitro generation of human C3a. Thus CAB-2 during SECC should block both alternate and classical complement activation, with abrogation of C3a formation together with later complement components. We have now examined the complement regulatory activity of this chimeric protein in human blood to determine its relation to leukocyte and platelet activation on SECC.

Methods

Extracorporeal Circuit Preparation

Identical to previous studies, extracorporeal circuits were assembled with the use of a pediatric membrane oxygenator (VP CML Plus, Cobe Cardiovascular) primed with 600 mL of lactated Ringers containing dextrose (4.0 g/L), mannitol (4.0 g/L), and porcine heparin (5 U/mL). The prime was circulated at 1.5 L/min with a heparin (5 U/mL). The prime was circulated at 1.5 L/min with a...
Figure 1. C3a and C5b-9 during SECC. Levels of C3a (A) and C5b-9 (B) were measured in plasma at the time points indicated. Mean±SEM from 4 control experiments (●), 4 experiments with 30 μg/mL CAB-2 (○), and 4 experiments with 300 μg/mL CAB-2 (●) are shown. CAB-2 significantly inhibited C3a and C5b-9 generation (P<0.01 for both CAB-2 doses).

μg/mL and 300 μg/mL of CAB-2, respectively (P<0.01 for both compared with control, Figure 1A). Similarly, C5b-9 formation during SECC was significantly inhibited by CAB-2, with peak levels of 269±33 ng/mL and 111±31 ng/mL after 90 minutes for 30 μg/mL and 300 μg/mL CAB-2, respectively (P<0.01 for both vs control, Figure 1B).

Leukocyte Activation
Control SECC resulted in significant monocyte activation, as measured by CD11b upregulation (Figure 2). Monocyte CD11b began to increase while the blood was maintained at 27°C (60 minutes), then increased dramatically during the 30 minutes at 37°C, peaking after 90 minutes total at 353±107% of baseline (P<0.01). CAB-2 addition at 30 μg/mL significantly blunted monocyte CD11b upregulation, which peaked at only 126±21% of baseline at 60 minutes; in particular, the late increase with rewarming to 37°C was not seen with CAB-2 addition (P<0.05, Figure 2). CAB-2 at 30 μg/mL did not significantly inhibit monocyte CD11b upregulation, with levels reaching 150±36% of baseline at 60 minutes (P=0.087, Figure 2).

As previously demonstrated, control SECC activated PMN, with CD11b levels peaking at 301±135% of baseline (P<0.01, Figure 3A) and elastase:α1-antitrypsin complex levels at 982±132% of baseline (P<0.01, Figure 3B), both at 90 minutes. CAB-2 at 30 μg/mL significantly inhibited PMN activation, with CD11b levels peaking at only 116±12% of baseline after 45 minutes (P<0.05, Figure 3A). Elastase:α1-antitrypsin complex levels at the higher CAB-2 dose were also blunted, reaching only 289±86% of baseline compared with the 9-fold increase in control experiments at 90 minutes (P<0.01, Figure 3B). CAB-2 at 30 μg/mL did not significantly inhibit PMN CD11b upregulation, with levels reaching 136±13% of baseline at 45 minutes (P=0.06). Similarly, elastase:α1-antitrypsin complex levels reached 787±158% of baseline at 90 minutes (P>0.10), not significantly different from control SECC.

Platelet Activation
The percentage of circulating P-selectin–positive platelets increased significantly but only modestly during control SECC, peaking at 90 minutes at 136±10% of baseline (P<0.01). CAB-2 at 30 μg/mL significantly inhibited the increase in monocyte CD11b (P<0.01, Figure 3A) and elastase:α1-antitrypsin complex levels at 982±132% of baseline (P<0.01, Figure 3B), both at 90 minutes. CAB-2 at 30 μg/mL did not significantly inhibit PMN CD11b upregulation, with levels reaching 136±13% of baseline at 45 minutes (P=0.06). Similarly, elastase:α1-antitrypsin complex levels reached 787±158% of baseline at 90 minutes (P>0.10), not significantly different from control SECC.

Figure 2. Monocyte CD11b during SECC. CD11b fluorescence density was measured on monocytes at the time points indicated, expressed as a percentage of the baseline fluorescence. Mean±SEM from 4 control experiments (●), 4 experiments with 30 μg/mL CAB-2 (○), and 4 experiments with 300 μg/mL CAB-2 (●) are shown. CAB-2 at 300 μg/mL but not 30 μg/mL significantly inhibited the increase in monocyte CD11b (P<0.01).

Table 1. Effect of CAB-2 on Peak Levels (Mean±SEM) of Selected Variables During SECC

<table>
<thead>
<tr>
<th>Variable</th>
<th>Vehicle Alone</th>
<th>CAB-2, 30 μg/mL</th>
<th>CAB-2, 300 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3a, ng/mL</td>
<td>4887±666</td>
<td>2188±173*</td>
<td>695±242*</td>
</tr>
<tr>
<td>C5b-9, ng/mL</td>
<td>793±192</td>
<td>269±33*</td>
<td>111±31*</td>
</tr>
<tr>
<td>Monocyte CD11b</td>
<td>353±107%</td>
<td>150±36%†</td>
<td>126±21%†</td>
</tr>
<tr>
<td>PMN CD11b</td>
<td>301±135%</td>
<td>136±13%§</td>
<td>116±12%†</td>
</tr>
<tr>
<td>Elastase:α1-antitrypsin</td>
<td>982±132%</td>
<td>787±158%</td>
<td>289±86%*</td>
</tr>
<tr>
<td>P-selectin–positive platelets</td>
<td>136±10%</td>
<td>131±24%</td>
<td>111±13%</td>
</tr>
<tr>
<td>Monocyte-platelet conjugates</td>
<td>283±54%</td>
<td>158±22%†</td>
<td>139±31%*</td>
</tr>
<tr>
<td>PMN-platelet conjugates</td>
<td>395±166%</td>
<td>213±64%</td>
<td>130±31%</td>
</tr>
<tr>
<td>Monocyte count</td>
<td>71±6%</td>
<td>81±9%</td>
<td>80±4%</td>
</tr>
<tr>
<td>PMN count</td>
<td>71±5%</td>
<td>85±2%†</td>
<td>86±2%†</td>
</tr>
<tr>
<td>Platelet count</td>
<td>76±8%</td>
<td>85±4%</td>
<td>86±3%</td>
</tr>
</tbody>
</table>

*P<0.01 vs vehicle; †P<0.05 vs vehicle; ‡P=0.087 vs vehicle; §P=0.06 vs vehicle.
CAB-2 addition did not significantly block the increase in circulating activated platelets [peak levels of $131\pm 6\%$ (30 minutes) and $111\pm 6\%$ (90 minutes) for 30 $\mu$g/mL and 300 $\mu$g/mL CAB-2, respectively, $P>0.1$ for both], Table 1]. However, the percentage of monocytes binding activated platelets is perhaps a more sensitive and biologically relevant marker of platelet activation in whole blood. Monocyte-platelet binding increased significantly during control SECC ($P<0.01$), with the percentage of monocyte-platelet conjugates peaking at $283\pm 5\%$ of baseline after 90 minutes of recirculation (Figure 4). CAB-2 at 300 $\mu$g/mL significantly inhibited this measure of platelet activation ($P<0.01$, Figure 4), with the percentage of monocyte-platelet conjugates peaking at only $139\pm 3\%$ of baseline after 90 minutes. Similarly, 30 $\mu$g/mL CAB-2 inhibited monocyte-platelet conjugate formation, peaking at only $158\pm 2\%$ of baseline at 90 minutes ($P<0.05$). PMN-platelet binding, a less robust marker of platelet activation, was not significantly inhibited by CAB-2 at either dose (Table 1).

Zymosan-Stimulated Monocyte CD11b Upregulation In Vitro

Zymosan was used to stimulate complement activation in serum incubated in diluent or CAB-2. Because zymosan-induced C3a levels were 10-fold higher than during SECC (data not shown), the CAB-2 dose in these in vitro studies was increased to 3 mg/mL, 10-fold higher than the highest CAB-2 dose used during SECC. CAB-2 at 3 mg/mL significantly inhibited monocyte CD11b upregulation in whole blood exposed to zymosan-activated serum. Monocyte

| TABLE 2. Whole Blood Monocyte CD11b (Mean±SEM) After Addition of Zymosan-Activated Serum: Effect of CAB-2 |
|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| Untreated Serum | Zymosan-Treated Serum + Diluent | Zymosan-Treated Serum + CAB-2 |
| Monocyte CD11b, afu | $113\pm 36$ | $364\pm 4.9$ | $132\pm 25.5^*$ |

afu indicates arbitrary fluorescence units.

$^*P<0.01$ vs zymosan + diluent.
CD11b expression (in arbitrary fluorescence units) was 113±36 for untreated serum added to blood, 364±4.9 for diluent-incubated, zymosan-treated serum, and 132±25.5 for CAB-2-incubated, zymosan-treated serum (mean±SD for 3 experiments, \( P<0.01 \) by paired \( t \) test, Table 2).

Discussion

Complement activation is known to participate in the pathophysiology of CPB.1 however, the relation between the generation of specific complement components and cellular activation has been difficult to delineate. In this study we demonstrated that early complement blockade, preventing generation of C3 cleavage products and subsequent mediators, decreases monocyte, PMN, and platelet activation in simulated bypass. In earlier work, we used an anti-human C5 mAb to block C5a and C5b-9 formation during SECC. Simultaneous with inhibition of these terminal complement components but with persistent C3a formation, we demonstrated inhibition of platelet and PMN but not monocyte activation. From this earlier study, we concluded that terminal complement components were major contributors to the platelet and PMN activation induced during SECC. The current study confirms our earlier findings of an association between complement activation and activation of platelets and PMN during SECC. In addition, we demonstrate the added ability of earlier complement inhibition, blocking C3 cleavage, to effectively prevent in vitro monocyte activation, as manifest by CD11b upregulation.

C3a and C5a are broadly defined as anaphylatoxins,13 and C3a produces PMN activation14 and secretion of lysosomal enzymes15; the role of C3a in inflammatory conditions is less well understood. However, the presence of a receptor for C3a (C3aR) on monocytes has recently been demonstrated16; C3a binding induces monocyte calcium flux16 and causes adherent monocytes to synthesize tumor necrosis factor-\( \alpha \) and interleukin-1B.17 Ligation of either the C3aR or the iC3b receptor, CD11b/CD18, on monocytes induces nuclear translocation of nuclear factor-\( \kappa B \),18 with subsequent production of tumor necrosis factor-\( \alpha \) and interleukin-1B. However, ours is the first study to implicate C3 cleavage products in the upregulation of monocyte CD11b. The ability of CAB-2 to inhibit monocyte CD11b upregulation was confirmed with the use of zymosan-activated serum, a less complex system than SECC. In those experiments, CAB-2-incubation prevented monocyte CD11b upregulation produced by addition of zymosan-treated serum to whole blood.

CD11b/CD18 is a \( \beta \)-2-integrin on monocytes and PMN, whose activation-dependent upregulation permits its binding to endothelial cell intracellular adhesion molecule-1 as a prelude to vascular egress.19,20 As noted earlier, monocyte CD11b also serves as a receptor for iC3b, facilitating binding and phagocytosis of complement-opsonized particles.21 Thus it is not surprising that a product released early in complement activation should stimulate upregulation of this monocyte integrin. Complement components C3a, iC3b, and C3c stimulate prostaglandin release by monocytes22; blockade of one or all of these early components may be important in preventing CD11b upregulation on CPB. Although monocyte CD11b upregulation was demonstrated in both the present study, our previous SECC investigation,4 and during in vivo CPB, earlier work using a different SECC system23 did not find monocyte CD11b upregulation to be caused by SECC itself. Monocyte CD11b upregulation in that study23 was found to be more dependent on temperature change, with higher CD11b levels at 37°C; this finding is consistent with the CD11b increase noted during rewarming in the present study. On the basis of these studies, it is reasonable to postulate that monocyte CD11b upregulation on SECC is predominantly a consequence of early complement activation and is potentiated at 37°C compared with 27°C. The role of activated monocytes in complications of CPB is not well defined but likely includes both proinflammatory effects through monocyte cytokine synthesis18 and prothrombotic potential through tissue factor expression.24

Activated PMN can induce tissue injury by local release of toxic oxygen species and granule contents including elastase. In addition to facilitating transendothelial migration, PMN CD11b also amplifies the inflammatory response, with oxidative burst activity linked to receptor occupancy.25,26 Studies probing for a C3a receptor on PMN have produced conflicting results,16 and functional effects of C3a on PMN are unclear. Indeed, C3a-induced stimulation of PMN may be secondary to eosinophil activation in blood.28 By contrast, C5a is a potent PMN activator, producing significant CD11b upregulation.29 On the basis of our previous work,4 C3a formation does not cause significant PMN CD11b upregulation during SECC. PMN elastase, not measured in our earlier work, was significantly decreased in this study by CAB-2 addition. Both C3a3 and contact activation29 stimulate elastase release; the decrease in elastase measured here may also result in part from inhibition of C3a or one of the contact activation pathway components. Potent inhibition of contact activation during SECC has been shown to inhibit neutrophil elastase release30,31 despite ongoing complement activation. It is possible that products of both contact and complement activation play a synergistic role in PMN activation during CPB, and inhibition of either pathway reduces PMN activation.

Complement component C5b-9 induces platelet P-selectin expression.32 Both CAB-2 doses significantly inhibited monocyte-platelet conjugate formation, a consequence of platelet P-selectin expression in whole blood.8 P-selectin mediates activated platelet binding to monocytes/PMN through P-selectin glycoprotein ligand (PSGL-1),33 and the platelet-monocyte conjugate has the potential for both procoagulant34 and proinflammatory35 effects. In vivo studies have demonstrated increases in leukocyte-platelet conjugates in both stable34 and unstable35 coronary artery disease and during CPB.5 In both in vitro4 and in vivo36,37 studies of platelet activation, the monocyte-platelet conjugate formation consistently exceeds PMN-platelet conjugates; this may partly result from higher surface PSGL-1 density on the monocyte (Rinder, unpublished observations). Furthermore, potent activation of PMN but not monocytes decreases P-selectin–dependent PMN-activated platelet binding12 through PSGL-1 clustering, thereby reducing its binding availability.38 SECC-induced formation of platelet-PMN conjugates was blunted by CAB-2, but this decrease did not reach
statistical significance. Although the increase in unbound P-selectin–positive platelets was also not inhibited by CAB-2, this may be partly due to the modest level of platelet activation produced by control SECC in this study, with P-selectin–positive platelets increasing to only 130% of baseline. As an alternative explanation, the degree of C5b-9 inhibition by high-dose CAB-2 (77%) was less than demonstrated in our previous study with an anti-C5 mAb (>90%). It is possible that the small amounts of C5b-9 still formed in the present study may, in part, be responsible for the lack of inhibition of platelet P-selectin expression on SECC.

In conclusion, this study extends in vitro (SECC) investigations of the role of C3a in monocyte activation; early complement blockade has a significant role in preventing monocyte CD11b upregulation induced during the rewarming phase of extracorporeal circulation. Blockade of early (C3a) and late (C5b-9) complement components effectively blocks monocyte, neutrophil, and platelet activation under conditions that simulate human in vivo CPB.

Acknowledgments

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