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Inter-α-Trypsin Inhibitor Attenuates Complement Activation and Complement-Induced Lung Injury

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Complement activation is a central component of inflammation and sepsis and can lead to significant tissue injury. Complement factors are serum proteins that work through a cascade of proteolytic reactions to amplify proinflammatory signals. Inter-α-trypsin inhibitor (IaI) is an abundant serum protease inhibitor that contains potential complement-binding domains, and has been shown to improve survival in animal sepsis models. We hypothesized that IaI can bind complement and inhibit complement activation, thus ameliorating complement-dependent inflammation. We evaluated this hypothesis with in vitro complement activation assays and in vivo in a murine model of complement-dependent lung injury. We found that IaI inhibited complement activation through the classical and alternative pathways, inhibited complement-dependent phagocytosis in vitro, and reduced complement-dependent lung injury in vivo. This novel function of IaI provides a mechanistic explanation for its observed salutary effects in sepsis and opens new possibilities for its use as a treatment agent in inflammatory diseases.

Inter-α-trypsin inhibitor (IaI) is a complex molecule consisting of a L chain protease inhibitor named bikunin or urinary trypsin inhibitor (UTI) and two H chains containing von-Willebrand type A (vWA) domains (6). IaI is assembled in the liver and released into the circulation, and can be found in fairly high concentrations in mammalian plasma. Little is known about its exact function in the organism, other than its ability to bind to hyaluronic acid in the extracellular matrix through its H chains. The role of IaI in sepsis was investigated in animals and human studies. IaI levels in serum of septic patients were significantly lower than levels in healthy controls (7). Among septic patients, IaI levels were also inversely correlated with physiologic scores and mortality. IaI given prophylactically or therapeutically in animal models of sepsis significantly improved survival (7–9), but the mechanism of action remains elusive.

Complement and IaI are both abundantly present in serum. We hypothesized that complement factors and IaI may be able to interact with each other for the following reasons: catalytic complement proteins are serine proteases that activate downstream complement factors through proteolytic cleavage. Additionally, complement factors contain integrin-like sites that are potential binding targets to the vWA domain of the IaI H chains. In contrast, the L chain of IaI is a serine protease inhibitor. Furthermore, IaI inhibits furin (10), a serine protease that can cleave complement factors (11). We therefore hypothesized that IaI can inhibit complement activation, as well as attenuate complement-dependent processes. We sought to test this hypothesis in vitro and in vivo, so as to provide mechanistic insights into the observed effects of IaI action in sepsis and tissue injury.

Materials and Methods

Experimental animals

The generation of bikunin-deficient mice, which are also deficient in circulating IaI, was described previously (12). Mice have been backcrossed into C57BL/6 for >10 generations. Homozygous knockout males were

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3 Abbreviations used in this paper: IaI, inter-α-trypsin inhibitor; CVF, cobra venom factor; RA, rheumatoid arthritis; UTI, urinary trypsin inhibitor; vWA, von-Willebrand type A.
FIGURE 1. Murine C5a ELISA after in vitro complement activation. Complement activation is greater after incubation with IaI-deficient plasma (C) than IaI-sufficient plasma (D). □, Control C5a levels. A, Alternative pathway (zymosan); B, classical pathway (IgG); C, comparison of IaI and Crry complement inhibition (alternative pathway) (□, p < 0.001, Tukey honestly significant difference multiple comparisons testing).

FIGURE 2. Complement binding and inactivation in vitro require the complete IaI molecule. A, IgG activation of complement (classical pathway) is inhibited by IaI (□), but not bikunin/UTI (□) in vitro, in both IaI-deficient plasma (□) and IaI-sufficient plasma (□) (□, p < 0.01 after Bonferroni multiple comparisons testing). B, Immunoprecipitation with anti-complement C3 or C4 Abs of IaI-deficient (left panel) and IaI-sufficient (right panel) plasma. In IaI-deficient plasma, C3 or C4 pulls down unprocessed H chains, but not UTI. In IaI-sufficient plasma, C3 or C4 pulls down IaI, and less so pre-α-inhibitor (pal). Lane 1, Input (spiked with UTI). Lane 2, C3 immunoprecipitated. Lane 3, C4 immunoprecipitated.
FIGURE 3. Inhibition of zymosan phagocytosis by IaI. A, Phagocytosis of zymosan particles by murine macrophages in vitro is inhibited by IaI. Preincubation of zymosan with IaI-depleted serum (□) leads to significantly more phagocytosis than preincubation with IaI-replete serum (▲); *p < 0.01. B, Preincubation of sera with CVF, which leads to complement depletion and abolishes the difference between IaI-depleted (□) and IaI-replete (▲) serum. This treatment also decreases overall phagocytosis, demonstrating that zymosan phagocytosis is largely complement dependent. C, Preincubation of zymosan with IaI-depleted serum (□) leads to significantly more phagocytosis than preincubation with IaI-replete serum (▲) when CD44-deficient macrophages are used (shown at 45-min incubation).

mg/ml IaI or 1 ml of 1 mg/ml UTI i.p. 1 h before instillation, and other mice received 4 U/kg CVF i.p. 12 h before instillation. Mice were sacrificed by CO2 asphyxiation 4 h after instillation, blood was collected in heparin-coated tubes, and mouse lungs were lavaged with 3 ml of normal saline. Spun plasma and lavage fluid were stored at −80°C until assayed.

FIGURE 4. Immune complex lung injury model. A and B, IaI-deficient mice (□) have significantly higher levels of C5a, total protein, and cytokines in lung lavage fluid than IaI-sufficient mice (▲). C, In histology, IaI-deficient mice (bottom panel) have significantly increased cellular inflammation than IaI-sufficient mice (top panel).
Histological analysis

Left lungs were inflated with 10% buffered formalin, paraffin embedded, and stained for H&E.

Statistics

Results are presented as means ± SEM. Statistical comparisons were performed with ANOVA and Bonferroni multiple comparisons testing using SPSS software. Differences were considered significant if \( p < 0.05 \).

Results

IaI inhibits complement activation

We initially investigated whether the presence of IaI in plasma has an effect on complement activation. We incubated plasma from IaI-deficient mice or their IaI-sufficient heterozygote littermates with either IgG (classical complement activation pathway) or zymosan (alternative pathway), and measured C5a levels as an indicator of complement activation. IaI-deficient serum led to significantly increased complement activation, evident by higher C5a levels, through both pathways (Fig. 1, A and B), indicating that IaI is able to inhibit complement activation in the classical and alternative pathway. To assess the inhibitory activity of IaI, we compared it with a known inhibitor of the alternative pathway, Crry. We found that IaI, at a physiologic concentration of 100 \( \mu \)g/ml, inhibited complement activation by \( \sim 25\% \), whereas Crry, at 10 \( \mu \)g/ml, inhibited complement activation by \( \sim 35\% \) (compared with control).

IaI H chains are required for complement binding and complement activation inhibition

The L chain bikunin is the protease-inhibitory moiety if IaI, whereas the H chains have no known protease-inhibitory capacity. We therefore investigated whether the IaI L chain bikunin is sufficient for complement inhibition in vitro. We incubated plasma from C57BL/6 mice (IaI-sufficient) or IaI-deficient mice with IgG to replicate the classical complement activation pathway, and added vehicle, IaI, or UTI to the reaction. We then measured levels of C5a as an indicator of complement activation. We found that the addition of IaI to IaI-sufficient plasma or IaI-deficient plasma leads to a further inhibition of complement activation (Fig. 2A). On the contrary, addition of the L chain bikunin (UTI) had no effect on complement activation. We immunoprecipitated IaI-deficient or IaI-sufficient plasma spiked with UTI with Abs to complement factors C3 and C4, which are precursors to C5 convertase. We demonstrate that IaI, pre-inhibitor, as well as free H chains can be detected in C3 and C4 pull-down fractions, indicating that IaI binds to complement. Importantly, free bikunin was not detected in these pull-down fractions, indicating that the H chains are essential for IaI binding to complement. Immunoprecipitation with anti-IaI Ab also yielded C3 and C4 in the pull-down fractions (data not shown).

IaI blocks phagocytosis in vitro via complement inhibition

We then investigated whether inhibition of complement activation by IaI has a physiologic effect in vitro, and examined zymosan phagocytosis by murine macrophages in the presence or absence of IaI. We chose zymosan, because its phagocytosis is known to be complement dependent (13). We showed that absence of IaI leads to increased zymosan phagocytosis by J774 murine macrophages (Fig. 3A). Depletion of complement by preincubation with CVF led to overall lower phagocytic index, thus confirming the complement-dependent nature of zymosan phagocytosis (Fig. 2B). More importantly, preincubation of sera with CVF abolished...
the effect of IaI on zymosan phagocytosis (Fig. 3B). Because IaI can bind to CD44, a cell surface receptor that participates in phagocytosis (14), we examined whether the effect of IaI on phagocytosis is CD44 dependent. We found that IaI-dependent difference in zymosan phagocytosis is present in CD44-deficient peritoneal macrophages as well (Fig. 3C). Inhibition of zymosan phagocytosis by IaI is therefore complement dependent, but CD44 independent.

IaI ameliorates complement-dependent lung injury in vivo

We next asked whether the IaI affects complement-dependent processes in vivo. We examined IaI-deficient mice and IaI-sufficient littermates and used the well-described model of immune complex lung injury, in which immune complex-mediated complement activation leads to localized expression of cytokines, such as TNF-α, cytokine-induced neutrophil chemoattractant/KC, MCP-1, and MIP-2, and influx of inflammatory cells (for a review of the mechanism, mediators, and regulation of immune complex lung injury, see Ref. 15). We found that IaI-deficient mice have significantly increased levels of C5a in the lavage fluid compared with IaI-sufficient mice, indicating that complement activation is also inhibited by the presence of IaI in vivo (Fig. 4A). Total protein levels in the lung lavage fluid were also significantly increased in IaI-deficient mice compared with IaI-sufficient mice, indicating increased vascular permeability (Fig. 4B). IaI-deficient mice showed an increase both in relevant cytokines (Fig. 4B) and cellular inflammation (Fig. 4C), indicating that the presence of IaI attenuates complement-dependent lung injury. Complement depletion via CVF pretreatment attenuated lung injury in IaI-deficient mice (Fig. 5, A and B). Intrapleural injection of IaI 1 h before lung injury attenuated inflammation in IaI-deficient mice (Fig. 5C). Interestingly, i.p. injection of UTI 1 h before lung injury also decreased inflammation (Fig. 5D). We measured lung lavage TNF-α as a representative proinflammatory cytokine. IaI-deficient mice treated with IaI had similar TNF-α levels as IaI-sufficient mice, and both groups of mice had significantly lower levels than IaI-deficient mice (p < 0.01 after Bonferroni multiple comparisons correction). IaI-deficient mice pretreated with UTI had a trend toward lower TNF-α levels, which was not statistically significant after Bonferroni correction (Fig. 5E). C5a levels in IaI-treated mice were similar to IaI-sufficient mice. UTI-treated mice again had a trend toward lower C5a levels, which was not statistically significant after Bonferroni correction (Fig. 5F).

Discussion

The present study describes a novel important role of IaI in inflammation and tissue injury, via complement inhibition. Inflammation is increasingly recognized as an important factor for morbidity and mortality in sepsis and tissue injury, and anti-inflammatory treatment is now at the forefront of therapeutic interventions in septic patients. Activation of complement, such as C5 is a central event in inflammatory tissue injury (16), and occurs in the complement activation cascade, but also spontaneously through hydrolysis, mediated by thrombin (1), or neutrophil elastase (2). Inhibition of complement activation is therefore necessary to prevent overshooting inflammatory responses and tissue injury in health as well as in disease. Complement inhibitors such as C1 inhibitor, soluble CR1, or compstatin occur naturally in the blood, but are mostly specific to certain complement factors (17). IaI fills an important niche by being a nonspecific inhibitor of diverse activation pathways.

Although IaI has been known for >50 years, its exact function has been elusive. The IaI L chain bikunin is a nonspecific and relatively weak serine protease inhibitor, and no specific role for its protease-inhibitory function has been discovered in vivo. The only known function for IaI to date had been its binding to hyaluronic acid and stabilizing extracellular matrix, particularly in the cumulus oophoros (12). However, IaI is found in relatively high concentrations in plasma and extravasates into injured tissue along with other plasma proteins. The vWA domain of the H chains confers binding capacity to many integrin-containing proteins. It may therefore be necessary to see IaI under a different light, namely as a versatile protease inhibitor that can act as an anti-inflammatory agent, specifically as a complement activation inhibitor. IaI has been shown to ameliorate LPS-induced sepsis in animal models, and the infusion of IaI reduced mortality in animal models of septic shock (9, 18). IaI levels were directly correlated with survival in patients with sepsis (7). We hereby provide a mechanism that may explain these observations at least partly. Other potential IaI-complement interaction sites are possible. For example, complement is known to participate in the pathogenesis of rheumatoid arthritis (RA) (19). IaI has been found in the synovial fluid of RA patients in high concentrations (20, 21), and it has been speculated that it may act as an anti-inflammatory mediator in these sites. Complement inhibition may be a possible function of IaI in RA and other autoimmune diseases in which complement plays a role (22).

Our results imply that the mechanism of direct complement inhibition by IaI is dependent on both the H and L chain components of IaI. In vitro, the full IaI molecule was required for complement inhibition. Additionally, our immunoprecipitation assay demonstrated that IaI and H chains, but not bikunin, are pulled down with C3 or C4. Of note, complement binding to IaI probably varies depending on conditions, e.g., divalent ion concentration. Our immunoprecipitation should therefore be seen as proof-of-principle for the interaction of complement and IaI, rather than quantification of the strength of the interaction. We hypothesize that H chains are needed for binding to complement, and that they do so through their vWA domain. Indeed, previous studies have shown that the vWA domain on factor B can bind complement C3 (23) and the vWA domain on complement C2 can bind complement C3 and C4 (24, 25). The bikunin L chain acts as the protease-inhibitory moiety. However, we also showed in vivo that UTI (the equivalent of bikunin) was sufficient to attenuate immune complex lung injury in a murine model. UTI/Bikunin also led to a trend toward decreased complement activation in our in vivo model, although this did not reach statistically significant levels in multiple comparisons testing. Several explanations for this can be offered. It is possible that although bikunin does not inhibit complement in vitro, it is able to do so in vivo either through stochastic proximity or mediated by an intermediary protein. More importantly, it must be remembered that although the effects of immune complex lung injury in the murine model are complement initiated, they are ultimately mediated by other factors such as neutrophil elastase, plasmin, kallikrein, or cathepsin G, which bikunin does inhibit (26). In turn, many of these factors can cause complement activation. We therefore hypothesize that bikunin/UTI may in vivo indirectly lead to a decrease in complement activation. Furthermore, bikunin inhibits phosphorylation of p38, ERK1/2, and JNK, and reduces TNF-α and IL-1β production by murine macrophages after endotoxin challenge (27), which would help explain the observed decrease in TNF-α levels in UTI-treated mice. In aggregate, we believe that the observed effects of bikunin and IaI in vivo can be explained as follows: direct complement inhibition by IaI; indirect decrease of complement activation by bikunin and IaI through inhibition of complement activators such as elastase, kallikrein, and cathepsin G; and decrease in TNF-α production by IaI and bikunin through downstream effects.
In summary, in this study, we demonstrate that IaI inhibits complement activation through both classical and alternative pathways, and that this inhibition requires the complete IaI molecule. In vitro, complement inhibition by IaI blocks complement-dependent phagocytosis. In vivo, IaI attenuates tissue inflammation in a complement-dependent mouse model of lung injury through direct and indirect complement inhibition, and also probably through complement-independent anti-inflammatory effects. In aggregate, these results indicate a novel and important role for IaI in inflammation and inflammatory tissue injury, and support its use as a treatment agent in human sepsis.

Disclosures
The authors have no financial conflict of interest.

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