A Direct Role for C1 Inhibitor in Regulation of Leukocyte Adhesion

Shenghe Cai, Vandana S. Dole, Wolfgang Bergmeier, Jennifer Scafidi, Hanping Feng, Denisa D. Wagner and Alvin E. Davis III

J Immunol 2005; 174:6462-6466; doi: 10.4049/jimmunol.174.10.6462
http://www.jimmunol.org/content/174/10/6462

References
This article cites 22 articles, 9 of which you can access for free at:
http://www.jimmunol.org/content/174/10/6462.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
A Direct Role for C1 Inhibitor in Regulation of Leukocyte Adhesion

Shenghe Cai, Vandana S. Dole, Wolfgang Bergmeier, Jennifer Scafidi, Hanping Feng, Denisa D. Wagner, and Alvin E. Davis III

Plasma C1 inhibitor (C1INH) is a natural inhibitor of complement and contact system proteases. Heterozygosity for C1INH deficiency results in hereditary angioedema, which is mediated by bradykinin. Treatment with plasma C1INH is effective not only in patients with hereditary angioedema, but also in a variety of other disease models, in which such therapy is accompanied by diminished neutrophil infiltration. The underlying mechanism has been explained primarily as a result of the inhibition of the complement and contact systems. We have shown that C1INH expresses the sialyl-Lewisx tetrasaccharide on its N-linked glycan, via which it binds to E- and P-selectins and interferes with leukocyte-endothelial adhesion in vitro. Here we show that both native C1INH and reactive center cleaved C1INH significantly inhibit selectin-mediated leukocyte adhesion in several in vitro and in vivo models, whereas N-deglycosylated C1INH loses such activities. The data support the hypothesis that C1INH plays a direct role in leukocyte-endothelial cell adhesion, that the activity is mediated by carbohydrate, and that it is independent of protease inhibitory activity. Direct involvement of C1INH in modulation of selectin-mediated cell adhesion may be an important mechanism in the physiologic suppression of inflammation, and may partially explain its utility in therapy of inflammatory diseases. The Journal of Immunology, 2005, 174: 6462–6466.

Plasma C1 inhibitor (C1INH) is one of the most heavily glycosylated plasma proteins. Its 478 amino acids contribute only 51% of its 104-kDa total apparent molecular mass. C1INH contains 13 glycosylation sites (7 O-linked and 6 N-linked). Ten of the glycosylation sites (all of the O-linked and 3 of the N-linked) are located in the amino-terminal domain (100 residues), the longest amino-terminal extension among the known serpins (1). Twenty-seven percent of the N-glycans of plasma C1INH are fucosylated (2). C1INH replacement therapy for acute attacks of angioedema has been used in patients with hereditary angioedema for 25 years. Surprisingly, treatment with C1INH is beneficial in a variety of other disease models, including sepsis, brain, and myocardial ischemia-reperfusion injury, hyperacute transplant rejection, traumatic shock, and the vascular leak syndromes associated with thermal injury, IL-2 therapy, and cardiopulmonary bypass (3). The mechanism underlying these beneficial therapeutic effects has been explained primarily as a result of the inhibition of the complement and contact systems. Such inhibition would, therefore, result in a reduction in the generation of the biologically active end products of these systems, such as the anaphylatoxins C3a and C5a, the C5b-9 membrane attack complex, and bradykinin, all of which can induce tissue injury. The beneficial effects of C1INH are accompanied by diminished neutrophil infiltration, as well as decreased expression of endothelial cell adhesion molecules (3, 4). However, the mechanisms underlying these observations were explained primarily as a result of inhibition of the complement system and the contact system because C3a, C5a, and C5b-9 each appear to enhance leukocyte adhesion (5–7). However, only C5b-9 accomplishes this via induction of increased P-selectin expression by endothelial cells (5–7). Our previous studies demonstrated that C1INH expresses the sialyl-Lewisx tetrasaccharide on its N-linked glycan, via which it binds to both E- and P-selectins and interferes with the leukocyte-endothelial cell interaction in vitro (8). Therefore, it is possible that C1INH mediates protection both via inhibition of complement and contact system activation and via this direct interaction with selectins.

Materials and Methods

C1INH protein and other reagents

Plasma C1INH was obtained from Aventis and further purified with size filtration using Superdex 200 (Amersham Biosciences) on a fast protein liquid chromatography platform. Reactive center cleaved inactivated C1INH (iC1INH) was prepared by incubating native C1INH (1 mg) with trypsin-agarose (50 μl) (Sigma-Aldrich) at room temperature for 30 min. N-Deglycosylated C1INH (dN-C1INH) was prepared by treatment with neuraminidase and N-glycosidase F (New England Biolabs) according to the manufacturer’s protocols. The iC1INH and dN-C1INH were purified using Superdex 200, quantitated using ELISA, and further characterized using a complex formation assay (8). C1INH, like many other serpins, reacts with target proteases (such as C1s) to form high m.w. SDS-resistant complexes. This complex formation assay is an index of the protease inhibitory activity of serpins. Native C1INH reacts with C1s to form a 200-kDa complex. Deglycosylated C1INH retains such activity although the size of the complex is smaller. However, reactive center cleaved C1INH loses the ability to complex with target proteases and is therefore regarded as inactive. The absence or presence of sialyl-Lewisx was tested using mAb HECA-452 on Western blot, as described (8). The deglycosylated C1INH with N-linked glycan removed does not react with HECA-452, although native or cleaved C1INH preparations have such reactivity. All C1INH used in these studies was purified to the extent that each showed a single sharp peak in Superdex 200, a single band on SDS-PAGE stained with Coomassie Blue, and a single band on immunoblot probed with anti-C1INH antisera (DAKO). TNF-α and recombinant human E-selectin that had been isolated from Chinese hamster ovary (CHO) cells in which it was
expressed were obtained from EMD Biosciences. Purified carcinoembryonic Ag (CEA) was purchased from Chemicon. Functional blocking mAb against human E-selectin (clone 68-5H11) and P-selectin (clone AK-4) were obtained from BD Pharmingen. Goat anti-CEA antiserum and the preimmune goat serum were purchased from Biosied, and HRP-conjugated anti-goat IgG and mouse IgG1 were obtained from Pierce.

Cell culture

HL-60 cells, a promyelocytic cell line, were obtained from American Type Culture Collection and cultured in RPMI 1640 (Invitrogen Life Technologies) plus 10% FBS. CHO cells that express human P-selectin (CHO/P) or E-selectin (CHO/E) (9, 10) were cultured in αMEM (Invitrogen Life Technologies) containing methotrexate (Sigma-Aldrich).

In vitro binding assay

To assess the effect of C1INH on the interaction of E-selectin and its ligand CEA, CHO/E cells were plated onto a 96-well plate and grown to confluence. The expression of E-selectin on the cell surface of CHO/E was confirmed by FACS. CEA, at 1 μg/ml, in the absence or presence of various forms of C1INH at 50–400 μg/ml, was incubated with CHO/E cells for 2 h on ice, which is known to reduce nonspecific binding. After a gentle wash with PBS containing 1 mM CaCl2 and 1 mM MgCl2, cells were incubated with 4% paraformaldehyde at room temperature for 30 min. The cells were then washed with ice-cold PBS containing 1% BSA and incubated with goat anti-human CEA antiserum on ice for 2 h. After washing with PBS, cells were incubated with HRP-labeled secondary Ab on ice for 1 h. After another PBS wash, o-phenylenediamine dihydrochloride (Sigma-Aldrich) substrate was added, and the color reactions were observed for the entire procedure with a Zeiss IM35 inverted microscope (Hamamatsu Photonic Systems). The same wash was observed throughout the experiment. Rolling leukocytes were quantitated by counting the number of cells passing a given plane perpendicular to the vessel axis in 1 min. Baseline rolling was determined during the first 10 min after surgery by taking a minimum of four 1-min counts. The various forms of C1INH (300 μg per mouse) then were administered by i.v. injection, and changes in leukocyte rolling were quantitated over the subsequent 30 min. Relative leukocyte rolling was calculated as the number of leukocytes rolling at various time points normalized to the number of leukocytes rolling at 0 min (before injection of C1INH or PBS).

Intravital microscopy

The effect of C1INH on leukocyte rolling in vivo was examined using intravital microscopy (12). TNF-α (0.5 μg, i.p.) was administered 3.5 h before leukocyte rolling was evaluated. The mesentery was exteriorized through a midline abdominal incision in anesthetized mice. A venule of 150- to 200-μm diameter with shear stress ranging from 100 to 200 s⁻¹ was isolated and observed for the entire procedure with a Zeiss IM35 inverted microscope connected to a Super-VHS video recorder (Panasonic AG-6720A; Matsushita Electric) using a charge-coupled device video camera (Hamamatsu Photonic Systems). The same venule was observed throughout the experiment. Rolling leukocytes were quantitated by counting the number of cells passing a given plane perpendicular to the vessel axis in 1 min. Baseline rolling was determined after the first 10 min after surgery by taking a minimum of four 1-min counts. The various forms of C1INH (300 μg per mouse) then were administered by i.v. injection, and changes in leukocyte rolling were quantitated over the subsequent 30 min. Relative leukocyte rolling was calculated as the number of leukocytes rolling at various time points normalized to the number of leukocytes rolling at 0 min (before injection of C1INH or PBS).

Statistical analysis

Data were expressed as mean ± SEM. Two-way ANOVA (Tukey multiple comparisons) test was used for analysis of the results. Statistical significance was defined as p < 0.05.

Results

C1INH interferes with the interaction of E-selectin and its ligand CEA

To further investigate a possible role for C1INH in inhibition of leukocyte infiltration, we prepared different forms of C1INH to test in several cell adhesion models. Native plasma C1INH was effective in each model. We then tested both reactive center cleaved C1INH, which loses its ability to inactivate target proteases, and N-deglycosylated C1INH in these models. We first investigated the effect of C1INH on the interaction of E-selectin with its ligand, CEA. The binding of CEA, which expresses sialyl-Lewisα, to CHO cells that express E-selectin on their surface (CHO/E) was assessed using a cellular ELISA. C1INH interfered with the interaction of CEA with the CHO/E cells in a dose-dependent manner (Fig. 1). Significant inhibition (~50%) was achieved with a concentration of 200 μg/ml or higher.
Like most other serpins, the protease inhibitory activity of C1INH depends on its three-dimensional structure. Treatment with limiting concentrations of trypsin cleaves C1INH at its reactive center, which results in a dramatic conformational rearrangement and loss of the ability to react with target proteases, such as C1s, to form high m.w. complexes (Fig. 2). This reactive center cleaved iC1NH inhibited the binding of CEA to the CHO/E cells to as great an extent as did the same concentration of native C1INH (Fig. 1). Because we previously showed that sialyl-Lewis\textsuperscript{x} is present on the N-linked carbohydrate of C1INH (8), we deglycosylated native C1INH with neuraminidase and N-glycosidase F. The resulting dN-C1NH showed the expected decrease in m.w. but retained protease-inhibitory activity as shown by its ability to complex with C1s (Fig. 2). However, it completely lost the ability to inhibit the binding of CEA to the CHO/E cells (Fig. 1). These data demonstrate that the ability of C1INH to prevent the binding of CEA to CHO/E cells is independent of its protease-inhibitory function but requires the presence of N-linked carbohydrate.

**C1INH inhibits leukocyte-endothelial cell adhesion under flow condition**

We characterized the effect of C1INH on leukocyte rolling in vitro under flow conditions using a flow chamber. Purified human recombinant E-selectin (2 \mu g/ml) was immobilized on a petri dish. Under flow conditions, a portion of the leukocytes roll on the immobilized E-selectin. We used a shear rate similar to that of a postcapillary venule (4 dyn/cm\textsuperscript{2}). This system mimics the first step in leukocyte adhesion during acute inflammation. The specificity of the system was confirmed by reversal of rolling with EDTA and in inhibition with the mAb 68-5H11, which is specifically directed against human E-selectin (data not shown). Treatment with C1INH at a concentration similar to those observed during acute inflammation (300 \mu g/ml) increased the leukocyte rolling velocity by 2-fold (p < 0.05) (Fig. 3A). Cleaved C1NH similarly increased leukocyte rolling velocity (p < 0.05). However, C1NH preparations with N-linked carbohydrate removed had no effect on velocity. Similarly, we investigated the effect of C1INH on leukocyte rolling on P-selectin-transfected cells. Native or cleaved C1INH, at a concentration of 300 \mu g/ml each increased the leukocyte rolling velocity by approximately 2.5-fold (p < 0.05) whereas dN-C1NH did not significantly alter the leukocyte rolling velocity (Fig. 3B).

**C1INH interferes with TNF-\alpha induced leukocyte rolling in vivo**

Intravital microscopy was used to visualize the effects of the various forms of C1INH on leukocyte rolling in vivo. Before treatment with TNF-\alpha, a few leukocytes were observed rolling on the endothelium. TNF-\alpha administration (0.5 \mu g, i.p.) induces systemic inflammation, and at 3.5 h posttreatment the rolling leukocyte numbers dramatically increased. Administration of either native or reactive center cleaved inactive C1INH reduced the number of rolling leukocytes whereas administration of N-deglycosylated C1INH had virtually no effect on leukocyte rolling (Fig. 4). These data demonstrate that the activity of C1INH in blocking TNF-\alpha-induced leukocyte rolling is independent of its protease-inhibitory function and suggest that it is dependent on the sialyl-Lewis\textsuperscript{x} moieties on its N-glycans.

**C1INH blocks leukocyte infiltration in the thioglycollate peritonitis model**

We used the thioglycollate peritonitis model to determine the effect of C1INH on leukocyte infiltration in vivo. Intraperitoneal injection of thioglycollate-induced (0.5 ml of 3% thioglycollate in PBS) leukocyte infiltration with the total leukocyte number increased from 3.66 ± 0.19 × 10\textsuperscript{6} (n = 5) to 7.63 ± 2.3 × 10\textsuperscript{6} (n = 10) at 4 h. The majority of leukocytes are neutrophils as revealed by microscopic inspection. C1INH (300 \mu g per mouse) blocked thioglycollate-induced leukocyte infiltration (3.32 ± 0.85 × 10\textsuperscript{6}, n = 5, p < 0.05 compared with the thioglycollate control) (Fig. 5). A similar effect was observed with cleaved C1INH (4.01 ± 0.6 × 10\textsuperscript{6}, n = 4, p < 0.05 compared with the thioglycollate control). However, treatment with N-deglycosylated C1INH (300 \mu g) had no effect (8.39 ± 3.22 × 10\textsuperscript{6}, n = 10, p > 0.5 compared with the control).

**Discussion**

Previous studies have shown that C1INH modulates leukocyte-endothelial cell adhesion in vivo during early reperfusion (14) or during experimental endotoxemia (15, 16). However, the effects of C1INH in all these reports have been assumed to result from the ability of C1INH to inhibit complement activation. Although there is no doubt that complement is activated in these circumstances,
Mice were treated with TNF-α and the selectin molecules. Therefore, in addition to its anti-inflammatory form. The observation that the reactive center cleaved inactive form was as potent as the native form. Because in every model tested, the concentration of leukocyte rolling in these experiments was not related to blocked leukocyte infiltration. The activity of C1INH in the modulation of leukocyte adhesion in these experiments was not related to its protease-inhibitory function because in every model tested the reactive center cleaved inactive form was as potent as the native form. The observation that TNF-α-induced leukocyte rolling in vivo. Mice were treated with TNF-α (0.5 µg, i.p.) for 3.5 h before administration (i.v.) of C1INH (300 µg per mouse) or an equal volume of PBS. Leukocyte rolling was observed using intravital microscopy. Numbers of rolling leukocytes in venules were measured before (0 min) and over a 30-min period after i.v. injection of native C1INH (A), cleaved C1INH (B), dN-C1INH (C), or PBS (D). Relative leukocyte rolling was calculated as the number of leukocyte rolling at various time points normalized to the number of leukocyte rolling at 0 min (before injection of C1INH or PBS) \( n = 3–7 \). *, \( p < 0.05 \) and **, \( p < 0.001 \) vs 0 min).

the observation that plasma C1INH may contain sialyl-Lewis^a moieties (8) suggested a potential additional mechanism through which C1INH might modulate the interaction of leukocytes with endothelial cells. In this study, we have shown that plasma C1INH directly suppresses leukocyte rolling both in vitro and in vivo. The present results demonstrate that C1INH directly interferes with the interaction of both E- and P-selectins with the natural ligand, CEA. Leukocyte rolling, both in vitro on purified E-selectin or P-selectin, and in vivo following TNF-α treatment, was inhibited by C1INH. In the thioglycollate peritonitis model, C1INH, at concentrations similar to those achieved during acute phase responses, blocked leukocyte infiltration. The activity of C1INH in the modulation of leukocyte adhesion in these experiments was not related to its protease-inhibitory function because in every model tested the reactive center cleaved inactive form was as potent as the native form. The observation that N-deglycosylated C1INH was inactive in every model is consistent with the interpretation that inhibition is a result of the interaction of sialyl-Lewis^a on C1INH with the selectin molecules. Therefore, in addition to its anti-inflammatory activities mediated via inhibition of complement and contact system proteases, these data suggest that plasma C1INH also plays a direct role in modulating leukocyte adhesion during inflammation.

By guest on July 25, 2017 http://www.jimmunol.org/ Downloaded from

C1INH inhibits TNF-α-induced leukocyte rolling in vivo. Mice were injected (i.v.) with various forms of C1INH at a single dose of 100 or 300 µg immediately followed by an injection (i.p.) with 0.5 ml of 3% thioglycollate. Mice were sacrificed 4 h later, and the number of leukocytes in the peritoneal cavity was counted. TG, thioglycollate. *, \( p < 0.05 \).

FIGURE 4. C1INH inhibits TNF-α-induced leukocyte rolling in vivo. Mice were treated with TNF-α (0.5 µg, i.p.) for 3.5 h before administration (i.v.) of C1INH (300 µg per mouse) or an equal volume of PBS. Leukocyte rolling was observed using intravital microscopy. Numbers of rolling leukocytes in venules were measured before (0 min) and over a 30-min period after i.v. injection of native C1INH (A), cleaved C1INH (B), dN-C1INH (C), or PBS (D). Relative leukocyte rolling was calculated as the number of leukocyte rolling at various time points normalized to the number of leukocyte rolling at 0 min (before injection of C1INH or PBS) \( n = 3–7 \). *, \( p < 0.05 \) and **, \( p < 0.001 \) vs 0 min).

The selectins play a significant role in the regulation of cell adhesion as well as in cell signaling, particularly in leukocyte rolling, the initial step of leukocyte infiltration (18). All selectins (L, P, and E) recognize the sialyl-Lewis^a moiety (19–21). Lower affinity appears to be a common feature of the interaction of selectins with their physiological ligands (19). The affinity of the interaction of plasma C1INH with the selectins is unknown. However, our previous binding data suggest that very likely it is also quite low (8). This may, at least in part, explain the observation that large doses of plasma C1INH, usually 3000–5000 units, are required for therapeutic application in various inflammation settings (3). However, the concentration of C1INH in plasma is relatively high, with a normal plasma level of 80–195 µg/ml (22). At these concentrations, little or no effect on adhesion is observed. The concentration may increase up to 2.5-fold at 24–48 h after the onset of inflammation to ~200–500 µg/ml (23). Therefore, it is possible that the interaction of plasma C1INH with selectin adhesion molecules plays a role in the normal physiologic suppression of acute inflammation. These studies also provide a novel mechanistic basis for the therapeutic application of C1INH in inflammatory diseases.

It appears likely that C1INH plays multiple roles in modulation of inflammation. It regulates activation of all three complement activation pathways which results in diminished generation of all the complement activation products such as C3a, C5a, and the C5b-9 membrane attack complex. It regulates activation of the contact system and thereby suppresses the generation of bradykinin. Last, the data presented here suggest that it may interact with selectins, which may serve to concentrate C1INH at sites of inflammation and to inhibit the transmigration of leukocytes across the endothelial surface. It will be important to delineate the relative contributions of these different mechanisms in more complex models of inflammation, such as hyperacute transplantation rejection or ischemia-reperfusion injury.

Disclosures
The authors have no financial conflict of interest.

References

FIGURE 5. C1INH blocks leukocyte infiltration in thioglycollate-induced peritonitis. Mice were injected (i.v.) with various forms of C1INH at a single dose of 100 or 300 µg immediately followed by an injection (i.p.) with 0.5 ml of 3% thioglycollate. Mice were sacrificed 4 h later, and the number of leukocytes in the peritoneal cavity was counted. TG, thioglycollate. *, \( p < 0.05 \).
6466 C1 Inhibitor Directly Suppresses Leukocyte Adhesion


