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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-Lewis\(^x\) 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.

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 inactive 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-Lewis\(^x\) 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 antiseraum (DAKO). TNF-\(\alpha\) and recombinant human E-selectin that had been isolated from Chinese hamster ovary (CHO) cells in which it was

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3 Abbreviations used in this paper: C1INH, plasma C1 inhibitor; C1INH, inactivated C1INH; dN-C1INH, N-deglycosylated C1INH; CHO, Chinese hamster ovary; CEA, carcinoembryonic Ag.
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 Phamingen. Goat anti-CEA antisera and the preimmune goat serum were purchased from Biodesigen, 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).

Mice

BALB/c mice were obtained from The Jackson Laboratory. Mice were housed in specific pathogen-free barrier facilities. The Animal Care and Use Committee of the CBR Institute approved all procedures for biomedical research. Mice 3–4 wk old (10–15 g) were used for intravital microscopy studies, and mice 6–8 wk old (20–25 g) were used for all other studies.

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 confluency. 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 antisera 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 developed for 30 min on ice. Absorbance was measured at 490 nm using a MRX microplate reader (Dynex Technologies). Specific binding of the Ab was calculated after subtracting nonspecific binding of the isotype control (preimmune goat serum). Functional blocking Ab against E-selectin was used as another control. Three independent experiments, each in triplicate, were used for statistical interpretation of the data.

Flow chamber

The interference of C1INH with leukocyte rolling under flow conditions was assessed using an in vitro flow chamber as described (11). The purified recombinant human E-selectin in PBS, pH 9.0, at a concentration of 2 μg/ml was coated onto a 35-mm circular petri dish (Costar) at 37°C for 1 h and preincubated with 2% BSA in PBS, pH 7.0, at 37°C for 1 h to block nonspecific binding. Alternatively, CHO/P cells were grown to a monolayer on the petri dishes. HL-60 cells, suspended in PBS containing 1 mM CaCl2, 1 mM MgCl2, and 0.5% (w/v) BSA, at 1 × 10⁶ cells/ml in the absence or presence of various forms of C1INH at a concentration of 300 μg/ml, were perfused through the chamber (Glycotech) for a 15-min period at a calculated shear rate of 4 dyne/cm² with a syringe pump (Harvard Apparatus). After each perfusion using different forms of C1INH, the chamber was flushed to remove any attached HL-60 cells. The same coating area was examined through all perfusions. Cell rolling was observed using an inverted microscope and was videotaped using a charge-coupled device video camera with a Super-VHS video recorder and an attached time-date generator. In addition to an EDTA control, blocking mAbs against human E-selectin (clone 68-5H11, 20 μg/ml) or P-selectin (clone AK-4, 20 μg/ml) were used as controls. To calculate velocities, the time (seconds) each rolling cell needed to travel a given distance (180.72 μm) was measured by analysis of the videotapes, and the velocity was defined as micrometers per second. Thirty randomly selected cells in each group were analyzed.

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 150–200 μm in diameter with shear stress ranging from 100 to 200 s⁻¹ was located and observed for the entire procedure with a Zeiss IM55 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 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).

Thioglycollate peritonitis

Thioglycollate was used to induce neutrophil recruitment into the mouse peritoneal cavity (13). Mice were injected i.p. with 3% thioglycollate broth (0.5 ml) (Sigma-Aldrich) immediately after C1INH infusion (5 or 15 mg/kg, i.v.). At 4 h postinjection, the mice were euthanized by CO2 inhalation, and peritoneal exudate cells were harvested using one intraperitoneal wash with HBSS (4 ml) containing 10% FCS. Peritoneal exudate cells were counted using a Coulter counter.

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-Lewisx, to CHO cells, was used as another control. Statistical analysis revealed that there was no significant difference between native plasma C1INH and reactive center cleaved C1INH. This result suggests that the reactive center region of C1INH is not essential for the inhibition of leukocyte infiltration.

To further investigate the role of C1INH in the inhibition of leukocyte infiltration, we analyzed the effect of C1INH on leukocyte rolling in vivo. 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 150–200 μm in diameter with shear stress ranging from 100 to 200 s⁻¹ was located and observed for the entire procedure with a Zeiss IM55 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 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).

Confocal 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 150–200 μm in diameter with shear stress ranging from 100 to 200 s⁻¹ was located and observed for the entire procedure with a Zeiss IM55 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 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).

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

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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 iC1INH 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\(^x\) is present on the N-linked carbohydrate of C1INH (8), we deglycosylated native C1INH with neuraminidase and N-glycosidase F. The resulting dN-C1INH 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 recombining 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 dyne/cm\(^2\)). 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 by 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 \(\sim 2\) fold \((p < 0.05)\) (Fig. 3A). Cleaved C1INH similarly increased leukocyte rolling velocity \((p < 0.05)\). However, C1INH 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-C1INH 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-\(\alpha\), 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\(^x\) moiety 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 \(\pm\) 0.19 \(\times\) 10\(^6\) \((n = 5)\) to 7.63 \(\pm\) 2.3 \(\times\) 10\(^6\) \((n = 10)\) 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 \((3.32 \pm 0.85 \times 10^6, n = 5, p < 0.05\) compared with the thioglycollate control) (Fig. 5). A similar effect was observed with cleaved C1INH \((4.01 \pm 0.6 \times 10^6, n = 4, p < 0.05\) compared with the thioglycollate control). However, treatment with N-deglycosylated C1INH (300 \(\mu\)g) had no effect \((8.39 \pm 3.22 \times 10^6, n = 10, p > 0.5\) 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 activity of C1INH in the modulations similar to those achieved during acute phase responses, in vivo following TNF-α.

Leukocyte rolling, both in vitro on purified E-selectin or P-selectin, and in vivo following TNF-α, 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 x 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.

The present results demonstrate that C1INH directly interferes with 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).

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Disclosures
The authors have no financial conflict of interest.

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