Heparin Cofactor II-Proteinase Reaction Products Exhibit Neutrophil Chemoattractant Activity

By Maureen Hoffman, Charlotte W. Pratt, Rebecca L. Brown, and Frank C. Church

The physiologic function of the plasma glycoprotein heparin cofactor II (HCII) is not well understood. An in vivo role for thrombin (IIa) inhibition by HCII in the presence of certain glycosaminoglycans (dermatan sulfate and heparin) can be proposed. Many proteins, such as complement components, can be proteolysed to generate secondary bioactive molecules. HCII is a substrate for the human neutrophil (PMN) proteinases cathespin G (CG) and elastase (LE). We found that degradation of HCII by CG or LE generated products with potent PMN chemotactic activity, which did not stimulate the PMN oxidative burst. Our results suggest that HCII may be a physiologic regulator of the acute inflammatory response.

e 1989 by Grune & Stratton, Inc.

PROTEOLYTIC activity is important in physiologic processes such as coagulation, fibrinolysis, and inflammation.1 Regulation of the proteinases that modulate these processes depends in part on the plasma proteinase inhibitors termed "serpins" (serine proteinase inhibitors).2 Heparin cofactor II (HCII) is a 65,600-dalton serpin termed "serpins." (serine proteinase inhibitors).2 Heparin cofactor II (HCII) is a 65,600-dalton serpin that is an inhibitor of the coagulation proteinase thrombin (IIa).3,4 Additionally, HCII has also been found to be an effective inhibitor of pancreatic chymotrypsin and neutrophil cathepsin G.5-9 HCII is like another serpin, antithrombin III (ATIII), because its Ila inhibitory activity is greatly enhanced in the presence of certain glycosaminoglycans, including heparin and dermatan sulfate.10,11 Unlike ATIII, \( \alpha_1 \)-proteinase inhibitor (\( \alpha_1 \)-PI), and \( \alpha_2 \)-plasmin inhibitor,3 the physiologic function of HCII is not known. HCII has been proposed to have a role in vivo as a IIa inhibitor in the presence of dermatan sulfate, especially in extravascular sites.12,13

Human neutrophils (PMN) are a major defense against invading microorganisms, and they represent a prominent component of the acute inflammatory response. An essential step to amplify the inflammatory response is through PMN recruitment. PMNs leave the vascular compartment and migrate to sites of evolving inflammation under the influence of chemotactic peptides such as elastin, collagen, and proteoglycans.17 Both proteinases may normally participate in tissue remodeling, and elastase may allow PMNs to penetrate the vascular basement membrane. However, when release of LE and CG exceeds the capacity of local proteinase inhibitors, the free proteinases can cause marked tissue damage and alter the functional levels of other biologically active protein substrates.

Serpins may have functions in addition to proteinase inhibition. For example, chemotactic activity for PMNs is generated during the proteolytic inactivation of \( \alpha_1 \)-PI by the mouse metalloproteinase macrophage elastase.18 HCII can be proteolytically inactivated by the human neutrophil proteinases CG and LE.7,9,19 We hypothesized that biologically active products would be generated from HCII during proteolysis by CG and LE. In this study, we demonstrate that the products derived from HCII by proteolysis with human CG and LE exhibit potent chemotactic activity for PMNs that is not found in the individual reaction components.

MATERIALS AND METHODS

Materials. Phorbol myristate acetate (PMA), fMLP, di-isopropylfluorophosphate (DFP), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co (St Louis). LE and CG were purchased from Elastin Products, Inc (Pacific, MO). Ila and HCII were purified from human plasma as previously described.6,13

Proteinase-inhibitor reactions. Reactions of HCII with PMN proteinases were performed by incubating 10 \( \mu \)mol/L HCII with 400 nmol/L LE or 1 \( \mu \)mol/L CG for five or 30 minutes in buffer containing 20 mmol/L HEPES, 150 mmol/L NaCl, and 0.1% (wt/vol) polyethylene glycol, pH 7.4. Proteolysis was then inhibited by the addition of DFP or PMSF to a final concentration of 1.0 to 1.25 mmol/L. The HCII-Ila complex was prepared by incubating 10 \( \mu \)mol/L HCII with 16 \( \mu \)mol/L Ila for 20 minutes at room temperature, in the above buffer, before adding DFP or PMSF. Aliquots of HCII, Ila, CG, or LE were incubated, DFP or PMSF added, and stored in parallel with the reaction mixtures.

Electrophoresis. Electrophoresis was performed with 10% polyacrylamide gels by the method of Laemmli.30

Cell isolation. PMN were purified on Ficoll-Hypaque density gradients (Mono-Poly Resolving Medium, Flow Laboratories Inc, McLean, VA) from the blood of healthy volunteers.31,32 The purity of the resulting preparations was determined by performing differential counts of Wright's-stained cytosin preparations. The PMN preparations were routinely 95% to 99% pure.

Chemotaxis assays. Chemotaxis was measured in modified Boyden chambers.22 PMNs were suspended at 1.5 \( \times \) 10\(^6\) cells/mL in Dulbecco's Modified Eagle Medium (GIBCO Inc, Grand Island, NY) supplemented with 1 mg/mL dextrose, and containing 10%
Fig 1. Polyacrylamide gel electrophoresis of HCII-CG and HCII-LE reaction mixtures. Reaction mixtures were prepared as described in Materials and Methods and incubated for five or 30 minutes before the addition of PMSF to stop proteolysis. From left to right the lanes represent: molecular weight standards; HCII; HCII-CG five-minute incubation; HCII-CG 30-minute incubation; HCII-LE five-minute incubation; HCII-LE 30-minute incubation.

calf serum (Sterile Systems Inc, Logan, UT). All buffers, media and sera used during the isolation of cells and performance of assays contained <1 ng/mL endotoxin. Minimizing endotoxin contamination is crucial to preventing activation and degranulation of the PMN before the chemotactic response can be evaluated.

To the top well of each Boyden chamber was added 0.25 mL of the PMN suspension. The chemoattractant solution to be tested, or medium alone, was simultaneously added to the bottom compartment. The compartments of the Boyden chambers were separated by a polycarbonate filter with 5-μm pores (Nuclepore Inc, Pleasanton, CA). The chambers were incubated for one hour at 37°C in a humidified atmosphere containing 5% CO₂. The filters were fixed in 70% ethanol, stained with Wright’s stain, mounted in immersion oil under coverslips, and the number of PMNs that had migrated across the filter was counted in ten to 20 fields per filter at 1,000× final magnification. The average number of PMNs migrating in control chambers (lower compartment contained medium only) was subtracted as background from the number migrating in test chambers, to yield the net number of PMN migrating per oil field. Positive controls assessing chemotaxis to fMLP (10⁻⁶ to 10⁻⁴ mol/L) were conducted in parallel with the experimental groups. Chemotaxis to a test substance was expressed as a percentage of the maximal chemotaxis to fMLP in the same experiment. Migration to each concentration of each potential chemoattractant was tested a minimum of three times.

Hydrogen peroxide assays. Hydrogen peroxide production was determined by the horseradish peroxidase-catalyzed oxidation of phenol red, as previously described. The assays were conducted in microtiter well plates, and the A₅₃₀ read on a plate-reading spectrophotometer (Vmax, Molecular Devices Corp, Palo Alto, CA).

RESULTS

HCII, CG, LE, and Ila alone each lacked chemotactic activity for PMN. However, significant chemotactic activity was generated by the reaction of HCII with CG, LE, or Ila. HCII is an effective inhibitor of Ila. Ila cleaves a single peptide bond in the reactive site and forms a 1:1 complex with HCII. By contrast, CG and LE can degrade HCII. Gel electrophoresis of the reaction mixtures shows that HCII is extensively degraded by these PMN proteinases (Fig 1). As shown in Fig 2, the HCII-CG reaction product(s) generated during a five-minute period of proteolysis had chemotactic activity comparable with optimal concentrations of the potent PMN chemoattractant fMLP (117% of the migration to fMLP; 43 ± 10 net PMN/oil field v 37 ± 4 for fMLP). The concentration of reaction products in the lower well of the Boyden chambers is expressed in terms of the molarity of the HCII initially added to the reaction mixture. The num-
umber of PMNs migrating to optimal concentrations of the HCII-LE reaction product(s) was lower than HCII-CG product(s) (Fig 3, 48% of the migration to fMLP, 21 ± 12 net PMN/oil field v 44 ± 13 for fMLP). The HCII-IIa complex also demonstrated significant chemotactic activity compared with medium, HCII, or IIa alone. However, migration to optimal concentrations of HCII-IIa (10⁻⁸ mol/L) was only 26% of that to fMLP (14 ± 3 net PMN/oil field v 54 ± 12 for fMLP). Migration to 10⁻⁹ mol/L and 10⁻⁹ mol/L HCII-IIa was not significantly different from control values.

The effect of a longer period of proteolysis on the generation of chemotactic activity was tested by incubating HCII with CG or LE for five and 30 minutes. Undegraded HCII was present in the reaction mixture containing CG after a five-minute incubation period, but was no longer seen after 30 minutes (Fig 1). This is consistent with the fact that HCII is a partial inhibitor of CG. Much greater amounts of the five-minute HCII-CG reaction mixture were required for maximal chemotactic activity than of the 30-minute reaction mixture (Fig 4). Under the conditions tested, LE degraded HCII rapidly, with no undegraded HCII remaining after five minutes (Fig 1). Much greater amounts of the 30-minute HCII-LE reaction mixture were required for maximal chemotactic activity than of the five-minute reaction mixture (Fig 4). This suggests that the chemotactic portion of the HCII molecule can itself be degraded by LE, resulting in a loss of chemotactic activity with longer incubations. Once the proteinases had been inhibited by DFP or PMSF, the reaction mixtures could be stored for several weeks at 4°C without significant loss of chemotactic activity. The migration of PMN to the reaction mixtures as a percentage of maximal migration was somewhat lower in the experiments shown in Fig 4 than in the experiments shown in Figs 2 and 3. This may be due to inter-donor variability in the assay, or some difference in the composition of proteolysis products of HCII between preparations. Since we have not yet determined which product(s) of HCII proteolysis are responsible for the chemotactic activity, we cannot be certain of the concentration of active product(s) in the reaction mixtures.

There was no net migration of PMN when equal concentrations of the HCII-proteinase reaction mixtures (from 10⁻⁷ to 10⁻⁹ mol/L) were placed in both the upper and lower compartments of the Boyden chambers (data not shown), thus confirming that proteolysis of HCII produces substances that are true neutrophil chemoattractants and not stimulators of random migration. None of the proteinases, proteinase inhibitors, or reaction mixtures tested had the ability to stimulate PMN hydrogen peroxide production, even though reaction mixtures that were effective chemotaxins. This is in marked contrast to fMLP, which was a good stimulus for peroxide production in addition to its activity as a chemotaxin (data not shown). Nor were any of the test substances able to inhibit or potentiate
PROTEOLYSIS PRODUCTS OF HCII ARE CHEMOTACTIC

stimulation of hydrogen peroxide production by fMLP or PMA.

DISCUSSION

The results of this study show that proteinases released from PMNs, particularly CG, generate potent chemotactic activity from HCII. At some point during the development of acute inflammation, the amounts of proteinases released would exceed the capacity of proteinase inhibitors in the local environment. The presence of HCII in the extracellular space would allow it to be cleaved by these proteinases, and act as a proinflammatory signal for the accumulation of more PMNs. The HCII-IIa complex also has chemotactic activity. This suggests that the reactive site peptide (that portion of the serpin molecule hydrolyzed by IIa) might itself have chemotactic activity. It is presently unclear whether the chemotaxins generated by CG and LE include all or part of the reactive site peptide, or arise from another portion of the HCII molecule. It appears that generation of thrombin during coagulation would generate less chemotactic activity compared with the action of PMN proteinases, especially CG.

The chemotactic activity derived from HCII has a feature that distinguishes it from the classic PMN chemotaxin fMLP. While fMLP is also a good stimulus for hydrogen peroxide generation, none of the chemotactic reaction mixtures stimulated the PMN oxidative burst. fMLP is a model compound of N-formylated bacterial proteins. It would be functional for contact with a bacterium to initiate activation of PMN bactericidal mechanisms as well as chemotaxis. By contrast, HCII-derived chemotaxins would only promote the influx of PMNs into the site of evolving inflammation. Then the phagocytes would not be stimulated to expend their bactericidal “firepower” prematurely.

The findings presented in this report suggest that HCII could play a significant role in propagation of acute inflammation by acting as a potent neutrophil chemotaxin.

REFERENCES

8. Church FC, Noyes CM, Griffith MJ: Inhibition of chymotrypsin by heparin cofactor II. Proc Natl Acad Sci USA 82:6431, 1985
11. Griffith MJ, Marbet GA: Dermatan sulfate and heparin can be fractionated by affinity for heparin cofactor II. Biochem Biophys Res Commun 112:663, 1983
Heparin cofactor II-proteinase reaction products exhibit neutrophil chemoattractant activity

M Hoffman, CW Pratt, RL Brown and FC Church