Human leukocyte elastase (HLE) and cathepsin G (CG) are expressed at high levels on the surface of activated human neutrophils (PMN) in catalytically active but inhibitor-resistant forms having the potential to contribute to tissue injury. Herein we have investigated the mechanisms by which HLE and CG bind to PMN plasma membranes. $^{125}$I-Labeled HLE and CG were incubated with radiolabeled ligands. This indicates that these PMN granule proteins share binding sites on PMN and that functional active sites of HLE and CG are not required for their binding to PMN. The sulfate groups of chondroitin sulfate- and heparan sulfate-containing proteoglycans are the PMN binding sites for HLE and CG since binding of HLE and CG to PMN was inhibited by incubating PMN with 1) trypsin, chondroitin ABC, and heparitinases, but not other glycanases, and 2) purified chondroitin sulfates, heparan sulfate, and other sulfated molecules, but not with non-sulfated glycans. Thus, heparan sulfate- and chondroitin sulfate-containing proteoglycans are low affinity, high volume PMN surface binding sites for HLE and CG, which are well suited to bind high concentrations of active serine proteinases released from degranulating PMN.

HLE$^2$ (EC 3.4.21.37) and CG (EC 3.4.21.20) are serine proteinases that are contained at millimolar concentrations in the azurophil granules of PMN and are released from PMN when they are activated to degranulate (1). HLE degrades extracellular matrix components, plasma proteins, cytokines, proteinase inhibitors, and cell surface receptors in vitro (1). CG activates angiotensin I (2), processes cytokines (3), and induces platelet activation (1). Neutrophil elastase plays a critical role in host defense against Gram-negative bacteria in mice by cleaving bacterial outer membrane proteins (4, 5). Neutrophil elastase also promotes airspace enlargement and the accumulation of PMN and macrophages in the lungs of mice exposed chronically to cigarette smoke (6). CG delays wound healing in murine skin (7), and neutrophil elastase and CG both induce immune complex-mediated inflammation in murine skin and joints (8) possibly by proteolytically activating pro-inflammatory mediators.

Until recently little has been known about the mechanisms by which HLE and CG mediate their activities in vivo because 1) minimal amounts of these enzymes are freely released from viable PMN activated with biologic mediators (9, 10), 2) CG is poorly soluble in isotonic solutions (11), and 3) plasma and interstitial fluids contain abundant, high affinity inhibitors of serine proteinases. However, when PMN are activated with biologic mediators, 6-fold more active HLE and active CG are expressed on the surface of PMN than are freely released by the cells (9, 10). Membrane-bound HLE and CG have a similar spectrum of catalytic activity and efficiency as the soluble forms of the proteinases (2, 9, 12) but differ from the soluble enzymes by their resistance to inhibition by physiologic serine proteinase inhibitors (2, 12, 13). Thus, binding of HLE and CG to the surface of activated PMN not only focuses and restricts their activities to the pericellular environment but also protects them from inhibition by physiologic inhibitors by unknown mechanisms (2, 12). Thus, inhibitor-resistant membrane-bound HLE and CG are likely to be the major bioactive extracellular forms of the proteinases contributing to tissue injury during inflammatory responses.

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$^1$ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

$^2$ To whom correspondence should be addressed: Division of Pulmonary and Critical Care Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115 and Department of Internal Medicine, University of Utah Health Sciences Center and Intermountain Health Care, Salt Lake City, Utah 84132

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1 The abbreviations used are: HLE, human leukocyte elastase; CG, cathepsin G; CSPG, chondroitin sulfate (CS)-containing proteoglycan(s); GAG, glycosaminoglycan; HSA, human serum albumin; HSPG, heparan sulfate (HA)-containing proteoglycan(s); LF, lactoferrin; MPO, myeloperoxidase; PMN, polymorphonuclear neutrophil(s); PMSF, phenylmethylsulfonyl fluoride; PR3, proteinase 3; PBS, phosphate-buffered saline.
Little is known about the mechanism by which HLE and CG bind to the plasma membrane of PMN. In the current manuscript we have investigated to which PMN plasma membrane molecules HLE and CG bind. We now report that the sulfate groups of chondroitin sulfate- and heparin sulfate-containing proteoglycans (CSPG and HSPG) in PMN plasma membranes are high volume, low affinity binding sites for HLE and CG, which are well suited to bind the millimolar concentrations of serine proteinases generated at the cell surface during PMN degranulation. Cell surface binding of HLE and CG thus arms PMN with locally active HLE and CG, which contribute important activities in health and in disease processes.

EXPERIMENTAL PROCEDURES

Materials—Lactoferrin was purified from human breast milk (14). HLE and CG were purified from purulent sputum (15). Proteinase 3 (PR3) and myeloperoxidase (MPO) were purchased from Elastin Products Co., Inc. (Owensville, MO). Na125I was obtained from PerkinElmer Life Sciences. Hanks’ balanced salt solution, RPMI 1640 medium, goat anti-rabbit IgG conjugated to Alexa-546, and goat anti-murine IgG conjugated to Alexa-488 were purchased from Invitrogen. Rabbit antisera to HLE was obtained from Athens Research and Technology (Athens, GA). Polyclonal rabbit anti-human CG and non-immune rabbit IgG were purchased from Dako Corp., Inc. (Carpinteria, CA). Protease-free chondroitinase ABC was from Proteus vulgaris, heparitinases I, II, and III were from Flavobacterium heparinum, and hyaluronidase was from Streptomyces hyalurolyticus were purchased from Seikagaku Corp. (Tokyo, Japan). Heparin-conjugated agarose beads were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). All other reagents were purchased from Sigma.

Radiolabeling of HLE and CG—125I Radiolabeling of HLE and CG was achieved using the lactoperoxidase method (16). Bound 125I was separated from free 125I by gel filtration with Sephadex G25. Greater than 94% of the 125I in the labeled protein was precipitated by 20% (v/v) trichloroacetic acid. The specific activities of the 125I-labeled HLE and CG were 1020 ± 421 and 14646 ± 421 cpm/nmol, respectively.

Isoelectric Points (pl) of Unlabeled and Labeled HLE and CG—Determination of the pl values for labeled and unlabeled serine proteinases was performed by Kendrick Laboratories, Inc. (Madison, WI). Two-dimensional electrophoresis was performed using the carrier ampholine method of isoelectric focusing (17) on Coomassie Blue-stained non-equilibrium pH gradient gels. Tropomyosin (M, 33 kDa and pl 5.2) and lysozyme (M, 14 kDa and pl 11.5) were used as internal standards along with protein M, standards ranging from 14 to 220 kDa (Sigma).

Binding of Radiolabeled HLE and CG to PMN—Human PMN (>95% pure) were isolated from the peripheral blood of healthy volunteers using the Ficoll-Hypaque method (18). Approval was obtained from the Brigham and Women’s Hospital Institutional Review Board for these studies. All PMN binding assays were performed at 0 °C for 90 min in quadruplicate in 400-μl microcentrifuge tubes using 106 PMN per assay in 100 μl of binding medium (RPMI containing 10 mM HEPES and 0.1% bovine serum albumin, pH 7.4). Preliminary experiments confirmed that the binding reaction reached equilibrium within 60 min, with minimal nonspecific binding of radioiodinated serine proteinases to the tubes. To minimize receptor turnover and internalization, all assays and centrifugation steps were performed at 0 °C, and all reagents were held in melting ice in a walk-in cold room. Receptor binding studies, including the separation of bound from free ligands by centrifugation through inert oil and quantification of specific and nonspecific binding of radiolabeled ligands were performed using a gamma counter as described previously (19). All results have been corrected for nonspecific binding of radiolabeled ligands, as described previously (19).

Dissociation and Inhibition of Receptor-mediated Binding of 125I-labeled CG and HLE to PMN—To quantify dissociation of radiolabeled serine proteinases from PMN plasma membrane binding sites in the presence of excess unlabeled ligand, chilled PMN (106/assay) were suspended in 100 μl of binding medium containing 100 nM radiolabeled CG or HLE and incubated at 0 °C for 90 min. Cells were washed twice and resuspended in binding buffer, and radiolabeled HLE or CG bound to PMN was quantified on an aliquot of the cells (106 PMN at T = 0 min) by centrifugation through inert oil as described above. The remainder of the cells were incubated at 0 °C, and bound radiolabeled CG or HLE was quantified after 5 and 10 min on aliquots of the cells (106 PMN at T = −5 and T = 0 min, respectively). A 200-fold molar excess of unlabeled CG or HLE (20 μM) was then added to the remainder of the cells (at T = 0). At varying intervals thereafter, aliquots of the cells (106 PMN) were removed, bound and free radiolabeled ligands were separated by centrifugation through inert oil, and residual bound radiolabeled ligands were quantified. To quantify inhibition of binding of radiolabeled ligands by various putative inhibitors, PMN (106/assay) were incubated in quadruplicate in 100 μl of binding medium with 100 nM 125I-labeled HLE or CG in the presence or absence of a 200-fold molar excess of unlabeled HLE, CG, phenylmethylsulfonyl fluoride (PMSF)-inactivated HLE, or PMSF-inactivated CG (prepared as described previously (19)), PR3, MPO, or lactoferrin (LF). Porcine pancreatic trypsin, bovine serum albumin, and lysozyme were also tested at 20 μM as negative controls. In other experiments PMN were incubated with 100 nM 125I-labeled HLE or CG in the presence and absence of unlabeled, sulfated molecules (20 μM dextran sulfate, 100 μg/ml heparin sulfate, and 20 μM proamine sulfate) or unlabeled molecules lacking sulfate groups as negative controls (10 mM glutamine, glutamic acid, or arginine), and bound radiolabeled ligands were quantified.

Kf for Inhibition of Binding of Radiolabeled CG to PMN—PMN were incubated for 90 min at 0 °C with a saturating concentration of 125I-labeled CG (1 μM) in the presence or absence of varying concentrations (1 nM–20 μM) of unlabeled CG, unlabeled HLE, or unlabeled PR3. Residual bound labeled CG was quantified, and the IC50 for inhibition of binding of 125I-labeled CG by each unlabeled proteinase was calculated by nonlinear regression analysis using Sigmaplot (SSPS Inc., Chicago, IL). Kf was calculated using the formula Kf = IC50/(1 + c/KD), where c is the concentration of radiolabeled ligand, and KD is its dissociation constant.
**HLE and CG Bind to CSPG and HSPG in PMN Plasma Membranes**

*Morph* 150–200 cells per group using image analysis software (Meta-CG was quantified in arbitrary integrated fluorescence units on Nikon Instruments Inc., Sterling Heights, MI). Surface-bound HLE or (Nikon Instruments Inc. Cleveland, OH), and images of immunostained for surface-bound HLE or CG using rabbit antiserum to HLE, non-immune rabbit serum, rabbit anti-CG (3 μg/ml in PBS) or chondroitin ABC (500 μl/mil/ml, both of which had been heat-inactivated (100 °C for 15 min) or 2) trypsin (25 μg/ml in PBS) inactivated with 1 mM PMSF for 30 min at 37 °C.

Cells were washed twice in PBS, suspended in 100 μl of Hanks’ balanced salt solution, and then incubated with HLE or CG (3 μM) for 90 min at 0 °C to permit binding of serine proteinase to PMN. Cells were then washed, fixed (with PBS containing 3% paraformaldehyde and 0.5% glutaraldehyde), and immunostained for surface-bound HLE or CG using rabbit antiserum to HLE, non-immune rabbit serum, rabbit anti-CG IgG, or non-immune rabbit IgG as primary antibodies and goat-anti-rabbit IgG conjugated to Alexa-546® as the secondary antibody, exactly as described previously (12). Cells were examined using a Nikon Eclipse E8000 epifluorescence microscope (Nikon Instruments Inc. Cleveland, OH), and images of immunostained cells were captured using a Spot camera (Diagnostic Instruments Inc., Sterling Heights, MI). Surface-bound HLE or CG was quantified in arbitrary integrated fluorescence units on 150–200 cells per group using image analysis software (Meta-Morph®, Universal Imaging Corp. West Chester, PA). The data were corrected for nonspecific staining in the presence of the non-immune control primary antibody exactly as described previously (12), and the results were expressed as % binding of HLE or CG to cells incubated in the absence of glycans or trypsin. To confirm that incubating cells with glycans did not adversely affect cell viability, cell-free supernatant samples from cells incubated with or without trypsin or glycans were assayed for lactate dehydrogenase activity along with extracts of untreated PMN (prepared in PBS containing 0.04% (v/v) Triton-X100) using a commercial kit (Sigma). The results for lactate dehydrogenase activity released from the cells were expressed as percent of the cellular content of lactate dehydrogenase in extracts of PMN incubated without glycans.

**Double Immunostaining of Activated PMN for Surface Serine Proteinases and Chondroitin Sulfate (CS) and Heparin Sulfate (HS)—**PMN were activated for 15 min with 10−7 M platelet-activating factor followed by 10−7 M formylmethionylleucylphenylalanine to induce PMN degranulation and optimal binding of HLE and CG to the surface of PMN (9). PMN were then washed, fixed, and incubated for 1 h on ice in Hanks’ balanced salt solution containing 1% human serum albumin and 50 μg/ml goat IgG to block nonspecific binding of antibodies (12). Cells were then immunostained with rabbit antiserum to HLE (or non-immune rabbit serum) or rabbit anti-CG IgG (or non-immune rabbit IgG) followed by goat anti-rabbit IgG conjugated to Alexa-546®. Cells were then immunostained with murine anti-CS IgG1 or murine anti-HS IgG1 (or non-immune murine IgG1) followed by goat anti-murine IgG conjugated to Alexa-488®. Cells were analyzed with a Leica TCSNT confocal laser scanning microscope (Leica Inc., Exton, PA) fitted with air-cooled argon and krypton lasers. Fluorescent confocal micrographs were recorded under dual fluorescent imaging mode in which cells were simultaneously exposed to 488- and 568-nm light attenuated by an acousto-tunable optical filter. A band pass (530 ± 30 nm) filter was used to select light emitted from the Alexa-488®-labeled CS or HS, and a long-pass 590 nm filter was used to detect the Alexa-546®-labeled HLE or CG.

**Effect of Preincubating PMN with Purified Glycans on Binding of HLE and CG to PMN—**We incubated PMN for 15 min at 4 °C in Hanks’ balanced salt solution containing 10 mM HEPES, pH 7.4, with and without 250 μg/ml concentrations of the following purified glycans: 1) chondroitin sulfate-A, 2) chondroitin sulfate-B, 3) chondroitin sulfate-C, 4) heparan sulfate, 5) hyaluronic acid, 6) desulfated chondroitin, or 7) desulfated heparan. PMN were then incubated with 3 μM HLE or CG for 60 min at 4 °C and then washed and fixed using 3% paraformaldehyde and 0.5% glutaraldehyde for 5 min on ice (12). Bound HLE and CG were quantified by immunostaining cells with Alexa-488® for surface-bound HLE and CG and quantitative image analysis, as described above. In other experiments cells were incubated with or without purified glycans, washed twice to remove glycans, and then incubated with HLE or CG in the absence of glycans. Bound enzymes were then quantified as described above.

**Binding of PMN to Heparin Affinity Columns by Membrane-bound HLE and CG—**Heparin-conjugated agarose bead (6-μm diameter) slurry (3 ml) was added to LC columns (Macs Miltenyi Inc. Auburn, CA, and 2 × 3 ml of PBS containing 1% bovine serum albumin was allowed to run through the columns to reduce nonspecific binding of cells to the columns. The following cell types (fixed as described above) were then loaded onto the columns (20 × 10⁶ cells in 1 ml of PBS): 1) unstimulated PMN, 2) PMN optimally activated to induce cell surface expression of HLE and CG (see above and Refs. 9 and 10), or 3) lymphocytes isolated from the same healthy donor (by Ficoll-Hypaque density gradient centrifugation to obtain mononuclear cells followed by negative selection for lymphocytes by adherence of contaminating monocytes to tissue culture plastic overnight at 37 °C). Columns were washed with 3 × 5 ml of PBS containing 1% bovine serum albumin, and cells washed out of the columns into the eluates were counted using a hemocytometer. Data were expressed as % cells adhering to the columns.

**Statistics—**Data are expressed as the mean ± S.E. or mean ± S.D. The results for paired and unpaired data were compared using Student’s t test for parametric data and the Mann-Whitney Rank Sum test for non-parametric data; p values less than 0.05 were considered significant.

**RESULTS**

Saturable and Reversible Binding of Radiolabeled HLE and CG to PMN—To gain insights into the mechanism of binding of HLE and CG to PMN, we first quantified the binding affinity and number of binding sites for HLE and CG on the surface of
PMN. We incubated human PMN at 0 °C with varying concentrations of 125I-labeled HLE or CG and observed saturable binding of the radiolabeled ligands to PMN. The results of representative experiments are shown in Figs. 1, A and C, for HLE and CG, respectively. Expression of these data in the form of Scatchard plots (20) allowed the binding affinity and the number of binding sites for HLE and CG to be estimated (Figs. 1, B and D, respectively). The initial convex profile of the Scatchard plots suggests co-operative binding of serine proteinases at low concentrations of labeled ligand. The mean binding affinity and mean number of binding sites from four separate experiments for HLE and CG are shown in Table 1. Binding of HLE and CG to PMN did not require divalent cations to be present in the binding medium (not shown).

To test whether serine proteinase binding to PMN is reversible, we bound 125I-labeled CG to PMN and quantified the amount bound. We then incubated the cells with a 400-fold molar excess of unlabeled CG and, thereafter, quantified bound 125I-labeled CG at intervals. Radiolabeled CG rapidly dissociated from the cells after the addition of excess, unlabeled CG (supplemental Fig. 1). Dissociation of radiolabeled CG from PMN was ~90% complete within 1 min of the addition of excess unlabeled CG to the cells. Radiolabeled HLE also dissociated just as rapidly from the surface of PMN upon the addition of a 400-fold molar excess of unlabeled HLE (not shown). Together, these data indicate that HLE and CG bind in a saturable and reversible manner to PMN. In addition, the PMN binding sites for HLE and CG are numerous but have relatively low affinity for these ligands.

Table 1: HLE and CG bind to high volume, low affinity sites on PMN

| Ligand | Binding sites/cell | $K_D$ (nM) |
|--------|--------------------|------------|
| HLE    | 11.5 ± 0.9a        | $5 	imes 10^{-5}$ |
| CG     | 8.1 ± 2.7          | $5.4 	imes 10^{-5}$ |

*PMN (10^6/assay) were incubated at 0 °C with varying concentrations of 125I-labeled HLE and CG, and bound and free ligand were quantified as described under “Experimental Procedures.” The binding data were corrected for non-specific binding of ligands and then analyzed by the Scatchard method to determine the number of binding sites per cell and the binding affinity ($K_D$) of the PMN plasma membrane sites for HLE and CG on PMN.

Inhibition of Binding of HLE and CG to PMN—As expected, incubation of PMN in the presence of a 200-fold molar excess of unlabeled HLE or CG inhibited the binding of the same radiolabeled ligand to PMN (Fig. 2). A 200-fold molar excess of unlabeled HLE or CG also cross-inhibited the binding of radiolabeled CG and HLE, respectively, to PMN (Fig. 2). To determine whether a functional active site is required for the binding of HLE or CG to PMN, we tested the effects of proteinases inactivated with a low $M_i$ inhibitor specific for serine proteinases which binds irreversibly to their active sites (PMSF). Incubation of PMN with a 200-fold molar excess of unlabeled PMSF-inac-
tivated HLE or unlabeled PMSF-inactivated CG also sub-
stantially abrogated the binding of radiolabeled HLE and CG,
respectively, to PMN (Fig. 2). These data indicate that HLE and
CG share binding sites on the PMN plasma membrane and that
their binding to PMN does not require functional active sites of
the enzymes.

We studied the effects of other proteins contained within
PMN granules (MPO, LF, and PR3) because 1) these proteins
are also expressed on the surface of activated inflammatory
cells (21–24), 2) all of these proteins are cationic (like HLE and
CG), and 3) electrostatic interactions likely play a role in the
binding of HLE and CG to PMN since these proteases can be
progressively eluted from PMN plasma membranes using solu-
tions containing increasing concentrations of NaCl (9, 10, 13).
Unlabeled MPO, LF, and PR3 when tested at a 200-fold molar
everse over radiolabeled ligands all significantly inhibited the
binding of 125I-labeled HLE or CG to PMN (Fig. 2), whereas
control proteins having low positive charge (trypsin) or nega-
tive charge (lysozyme) had minimal or no effect.

We determined the isoelectric points (pI) of unlabeled and
labeled HLE and CG and confirmed that 1) unlabeled CG (pI >
12) is more cationic than HLE (pI = 12), as previously reported
(25–27); 2) the pIs for both HLE and CG are greater than that
reported previously for PR3 (pI for PR3 = 9.1 (28)), and 3) our
labeling protocol did not change the pI of either HLE or CG.
Unlabeled CG (the most cationic of the three proteases)
inhibited the binding of labeled HLE to PMN more potently
than did unlabeled HLE or PR3 (Fig. 2A; p = 0.015 and p =
0.012, respectively). Unlabeled CG also more potently inhibited
the binding of labeled CG to PMN than did unlabeled HLE or
PR3 (Fig. 2B; p = 0.013 and p < 0.001, respectively). To further
evaluate the effects of the charge of the ligand on binding inhi-
bition, various concentrations of unlabeled HLE, CG, and PR3
were assayed for their capacity to inhibit binding of a saturating
concentration of 125I-labeled CG to PMN. The IC50 values for
each inhibitor were determined by nonlinear regression anal-
ysis (Fig. 3), and the IC50 values were used to calculate the KI
for inhibition of binding of 125I-labeled CG for each inhibitor, as
described under "Experimental Procedures." The KI values for
inhibition of radiolabeled CG with unlabeled CG, HLE, or PR3
were 0.32 × 10−6 M, 3.0 × 10−6 M, and 4.5 × 10−6 M respec-
tively. There was a direct relationship between the potency with
which each enzyme inhibited binding of labeled CG to PMN, as
determined by its KI, and the positive charge of the protease,
as indicated by its pI (see above). Thus, CG, the most positively
charged of the three serine proteinases, was the most effective
inhibitor of binding of radiolabeled CG to PMN, and PR3, the
least positively charged, was the least effective inhibitor.

**HLE and CG Bind to CSPG and HSPG in PMN Plasma Mem-
bane—**Our results further support the notion that the
high positive charge of CG and HLE is important in their bind-
ing to the plasma membrane of PMN. Thus, we next tested the
hypothesis that CG and HLE bind to proteoglycans in PMN
plasma membranes because proteoglycans are important com-
ponents of most plasma membranes, many proteoglycans are
highly negatively charged, and negatively charged plasma
membrane proteoglycans have been shown to bind various
other proteins to cells (29–33). Pretreatment of PMN with
tryptsin to remove surface proteins abrogated binding of HLE
(Fig. 4A) and CG (not shown) to PMN. Pretreatment of PMN
with chondroitinase ABC and heparitinases I-III to remove
chondroitin sulfate (CS) groups and heparan sulfate (HS)
groups, respectively, from the PMN surface all significantly
abrogated the binding of both HLE and CG to PMN (Fig. 4B).
In contrast, removal of hyaluronic acid and sialic acid residues
from the PMN surface using hyaluronidase and neuraminidase,
respectively, had no effect on the binding of HLE and CG to the
surface of PMN (Fig. 4B). Preincubation of PMN with heat-
inactivated chondroitinase ABC (not shown), heat-inactivated
trypsin (not shown), or PMSF-inactivated trypsin did not
reduce the binding of HLE (Fig. 4A) or CG (not shown) to PMN
confirming that the catalytic activity of the trypsin and gly-
canases is required to produce this effect.

The effects of chondroitinase ABC, heparitinases and trypsin
treatments of PMN on HLE and CG binding to the cells could
not be attributed to any adverse effects on PMN viability since
incubation of PMN with glycanases or trypsin caused minimal
release of intracellular lactate dehydrogenase activity from cells
when compared with PMN incubated without these enzymes
(2.9 ± 0.9% of the cellular content of lactate dehydrogenase was
released by PMN incubated in PBS alone versus 2.5 ± 0.6, 6.0 ±
1.7, and 5.6 ± 2.9% from cells incubated with trypsin, chon-
droitinase ABC, and heparitinases, respectively). Incubation of
cells with higher concentrations of chondroitinase and hep-
arinases alone than those tested in Fig. 4B did not further reduce
the amount of HLE or CG that binds to PMN (not shown). How-
ever, incubation of PMN with the optimal concen-
trations of chondroitinase ABC and heparitinases together
resulted in additive (~90%) inhibition of the binding of HLE
(Fig. 4C) and CG (not shown) to PMN. These data indicate that
HLE and CG bind to CSPG and HSPG in the plasma mem-
branes of human PMN.

To further evaluate the roles of plasma membrane CSPG and
HSPG in the binding of HLE and CG to the surface of activated
PMN, we double immunostained cells for HS or CS groups and
HLE or CG endogenously expressed on the PMN plasma mem-

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**FIGURE 3.** KI for inhibition of binding of radiolabeled CG by cold CG, HLE, and PR3. PMN were incubated for 90 min at 0 °C with 1 M 125I-labeled CG in the presence of varying concentrations of unlabeled CG (solid circles), HLE (open triangles), or PR3 (solid squares). Bound 125I-labeled CG was quantified, and the IC50 values were calculated for each inhibitor, as described under "Experimental Procedures."
brane and examined the cells by confocal microscopy. The results show that on the surface of activated PMN, HLE strongly co-localizes with both HS- and CS-containing proteoglycans (supplemental Figs. 2, A and B, respectively). CG also strongly co-localizes with CS and HS groups on the surface of PMN (not shown). In addition, treatment of PMN with optimal concentrations of chondroitinase ABC and heparitinases together reduced the co-localization of HLE and HS when PMN were activated to induce surface expression of endogenous serine proteinases, whereas hyaluronidase treatment of PMN did not abrogate surface expression of HLE on activated PMN (Fig. 5).

Next, we tested whether membrane-bound HLE and CG on activated PMN bind PMN to columns containing heparin-conjugated agarose beads. Activated PMN, which express very high levels of membrane-bound HLE and CG (10, 12), bound avidly to these columns (Fig. 6). In contrast, unstimulated PMN, which express minimal amounts of membrane-associated HLE or CG (9, 10), and lymphocytes, which do not express either enzyme, bound poorly to heparin-conjugated agarose beads. Thus, serine proteinase endogenously expressed on the surface of activated PMN bind the cells to negatively charged glycosaminoglycan (GAG)-conjugated affinity supports.

To provide further assurance that CSPG and HSPG bind HLE and CG to the PMN surface, we tested the effects of purified GAGs on binding of HLE and CG to PMN. Incubation of PMN with purified CS and HS significantly inhibited the binding of exogenous HLE and CG to PMN, whereas purified hyaluronic acid was ineffective (Figs. 7, A and B). CS and HS inhibited the binding of the proteinases to PMN probably by binding to them in solution since incubating cells with these glycans but washing them with buffer to remove soluble glycans before adding exogenous HLE failed to inhibit subsequent binding of HLE to PMN (not shown).

The Sulfate Groups of Plasma Membrane CSPG and HSPG Bind HLE and CG to PMN—Because HS and CS are highly sulfated GAG and sulfate groups are responsible for their high

**FIGURE 4. Effects of trypsin and glycanases on the binding of HLE and CG to PMN.** In A PMN were incubated for 60 min with or without 1) porcine pancreatic trypsin (1 μm) or 2) PMSF-inactivated porcine pancreatic trypsin (1 μm). NS, not significant. In B PMN were incubated for 60 min with or without 1) chondroitinase ABC (Chond; 500 units/ml), 2) heparitinases I, II, and III (Hep; all at 500 units/ml), 3) hyaluronidase (Hyal; 500 units/ml), and 4) neuraminidase (Neur; 500 milliunits/ml). In C PMN were incubated at 37 °C for 60 min with 1) heparitinases I, II, and III alone (Hep; 500 units/ml), 2) chondroitinase ABC alone (Chond; 500 units/ml), or 3) the same concentrations of heparitinases I-III and chondroitinase ABC together (Hep + Chond). Data are the mean values ± S.E. (n = 100–150 cells/group). * indicates p < 0.01; **, p < 0.001 compared with cells incubated without enzymes.

**FIGURE 5. Effects of chondroitinase ABC and heparitinases versus hyaluronidase on the binding of endogenous HLE to PMN.** In A PMN were incubated at 37 °C for 60 min without glycanases, in B with hyaluronidase (Hyal; 500 units/ml), or in C with both heparitinases I-III and chondroitinase ABC (all at 500 units/ml). PMN were washed and then activated at 37 °C for 15 min with 10⁻⁷ M platelet-activating factor and then for 30 min with 10⁻⁷ M formylmethionylleucylphenylalanine to induce PMN degranulation and optimal surface expression of endogenous HLE, as described under “Experimental Procedures.” Cells were then double-immunostained with murine IgG1 antibodies and goat anti-murine IgG conjugated to Alexa-488 for heparin sulfate (2nd panels) or with rabbit antiserum to HLE and goat anti-rabbit IgG conjugated to Alexa-546 for heparin sulfate (3rd panels). Cells were examined using bright field (first panels) or epifluorescence microscopy (second and third panels). Cells incubated with isotype control primary antibodies showed minimal staining (not shown).
negative charge, we next tested the possibility that the sulfate groups of HSPG and CSPG bind the positively charged HLE and CG to the surface of PMN. We preincubated PMN with and without purified CS or HS versus desulfated chondroitin or desulfated heparan and then quantified the binding of exogenous HLE and CG to PMN. In marked contrast to CS and HS, the desulfated moieties (like hyaluronic acid, which is also not sulfated) were completely ineffective at inhibiting the binding of HLE and CG to PMN (Figs. 7, A and B). In addition, other naturally occurring and synthetic sulfated glycans (including heparin sulfate, dextran sulfate, and protamine sulfate) also strongly inhibited the binding of exogenous HLE to PMN (Fig. 7C), whereas negatively charged but non-sulfated molecules (such as glutamic acid in Fig. 7C, lysozyme in Fig. 2, and albumin; not shown) and molecules having neutral or positive charge at neutral pH (glutamine and arginine, respectively, in Fig. 7C) had no significant effect. These data confirm that HLE and CG bind to the sulfate groups of CSPG and HSPG in PMN plasma membranes.

DISCUSSION

PMN express high capacity, low affinity binding sites for HLE and CG that are predominantly plasma membrane CSPG and HSPG. The cationic HLE and CG bind by electrostatic interactions to the negatively charged sulfate groups in the GAG side chains of these proteoglycans. These binding sites are well suited to sequester the millimolar concentrations of HLE and CG generated close to the plasma membrane when PMN degranulate and likely render the proteinases resistant to inhibition by physiologic inhibitors in the extracellular space (see below).
HLE, CG, and other cationic proteins stored within PMN granules (PR3, myeloperoxidase, and lactoferrin). Likely HLE and CG compete with each other and with these other cationic proteins for the binding sites when PMN degranulate. However, the effect of this competition is likely mitigated by the very large number of binding sites present on the PMN surface. Our data show that the more basic the ligand, the greater the avidity with which it binds to sulfate groups in GAGs on the surface of PMN. However, it is likely that the local arrangement of surface charge on each ligand (rather than its global isoelectric point) determines the affinity with which each ligand binds to surface GAGs since acidic and basic fibroblast growth factor both bind avidly to heparin and heparan sulfate (34–36).

The binding sites for CG on PMN have not been studied previously, but Dwenger and Tost (37) reported that PMN express one class of high affinity, low capacity binding sites ($K_d = 1.4 \times 10^{-9}$ m and $8.3 \times 10^4$ sites/cell) and a second class of low affinity, high capacity sites ($K_d = 2.4 \times 10^{-7}$ m, $14.3 \times 10^6$ sites/cell). The latter binding sites agree with our data, but we did not identify high affinity, low capacity HLE binding sites. However, Dwenger and Tost (37) performed the binding experiments at room temperature, and Cai et al. (38) reported low capacity binding of HLE to PMN CD11b/CD18 integrins at 37 °C. Thus, at above room temperature, a small proportion of HLE molecules may bind to CD11b/CD18 integrins on PMN (see below). Alternatively, in Dwenger’s study (37), performing the binding assays at room temperature may have permitted internalization of labeled HLE by PMN, which was misinterpreted as a second class of high affinity binding sites for HLE. The characteristics of the PMN binding sites we found for HLE and CG are consistent with those reported previously for various cationic proteins binding to several inflammatory cell types (23), but the surface molecules binding cationic proteins to the cells were not identified in these earlier studies.

**HLE and CG Bind to Sulfate Groups in HSPG and CSPG—**

Our results show that HLE and CG bind to CSPG and HSPG in the plasma membranes of PMN because 1) treatment of PMN with trypsin, chondroitinase ABC, and heparitinase abrogates binding of HLE and CG to PMN, 2) soluble CS and HS inhibit binding of HLE and CG to PMN, 3) HLE and CG strongly colocalize with CS and HS groups on the surface of activated PMN, and 4) activated PMN, which express high levels of membrane-bound HLE and CG, bind avidly to heparin-conjugated agarose beads, whereas unstimulated PMN, which have minimal surface-bound HLE or CG, bind poorly. The sulfate groups which impart the high negative charge on CSPG and HSPG bind the cationic HLE and CG to PMN since sulfated carbohydrates inhibit binding of HLE and CG to PMN, but GAGs or other negatively charged molecules lacking sulfate groups are ineffective. We could not test the effects of agents (e.g. sodium chloride) that inhibit sulfation of newly synthesized proteins on serine protease binding to PMN because mature PMN have limited capacity to synthesize new proteins.

The positive charge on HLE is imparted by 19 Arg, 18 of which are located on the external surface of HLE in a “cluster shoe”-like arrangement around the active site cleft, with several forming clusters of 2–4 Arg residues (39), which are balanced by only 9 acidic residues, 3 of which are buried in the molecule. Similarly, the positive charge of CG (40) is due to its high number of basic amino acids (33 Arg and 3 Lys). Soluble HLE binds via ionic interactions to soluble-sulfated GAGs including heparin, HS, and CS (41–44), and the greater the degree to which the GAG is sulfated, the greater the affinity with which HLE binds. When HLE binds to heparin, the Arg residues around the active site of HLE bind to the sulfate groups of heparin (45, 46). It is likely that not all of the basic residues in HLE and CG (or in the other cationic proteins that compete with HLE and CG for the GAG binding sites on the surface of PMN) are required to bind these proteins to the PMN surface GAGs. This would render the other basic residues in these surface-bound cationic proteins available to anchor activated PMN to the sulfate groups in heparin-arosane affinity supports (Fig. 6).

**Effect of Binding of HLE and CG to the Plasma Membrane GAGs on Serine Protease Function—**

Previous studies have shown that binding of soluble HLE to the sulfate groups in sulfated GAGs inhibits HLE activity in vitro (41, 43, 46). However, HLE and CG also bind to soluble high $M_p$ HSPG (including syndecans-1 and -4) shed into bronchial secretions from patients with bronchiectasis and into acute human dermal wound fluids (47, 48), and HLE and CG bound to these syndecans retain their catalytic activity against extracellular matrix substrates (47). The number of active molecules of HLE and CG we previously calculated to be endogenously expressed on the surface of activated PMN (9, 10) is very similar to the number of radiolabeled, exogenous, active HLE and CG molecules that bound to the surface of PMN in the current study. Moreover, membrane-bound HLE and CG have a similar spectrum of catalytic activity as the soluble forms of the proteases, with membrane-bound HLE degrading fibronectin (12) and elastin<sup>3</sup>, membrane-bound HLE and CG stimulating goblet cell degranulation in airway epithelium (49), and membrane-bound CG activating angiotensin I as efficiently as the soluble form of the protease (2). Thus, as for HLE and CG bound to syndecans in biologic samples, binding of these serine proteases to HSPG and CSPG in PMN plasma membranes does not significantly compromise their catalytic activities.

The major difference between the soluble and membrane-bound forms of these proteases is their susceptibility to inhibition by serine proteinase inhibitors. Soluble HLE and CG are very efficiently inhibited by physiologic serine proteinase inhibitors. In contrast, membrane-bound HLE and CG on activated PMN are remarkably resistant to inhibition by physiologic inhibitors, and there is an indirect relationship between inhibitor size and its effectiveness against membrane-bound HLE and CG (2, 12). Membrane-bound HLE and CG are almost completely resistant to inhibition by high $M_p$ inhibitors such as $\alpha_1$-proteinase inhibitor and $\alpha_1$-antichymotrypsin but are only partially resistant to inhibition by intermediate $M_i$ inhibitors such as secretory leukocyte proteinase inhibitor (SLPI). We initially hypothesized that this was due to steric hindrance of large inhibitors, resulting in impaired complex formation between large extracellular inhibitors and enzymes stericly confined on the PMN surface. However, the binding of soluble HLE and

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<sup>3</sup> C. A. Owen, unpublished observation.
soluble CG to soluble heparin in vitro renders the proteinases resistant to inhibition by α1-proteinase inhibitor and α1-antichymotrypsin (50, 51) but promotes inhibition of CG by SLPI (52) via a heparin-induced conformational change in SLPI (53). Moreover, serine proteinases complexed to syndecans in biologic fluids are resistant to inhibition by α1-proteinase inhibitor and α1-antichymotrypsin (by reducing the affinities of the proteinases for their inhibitors as indicated by reductions in second order rate constants for associations for inhibitors and proteinases bound to syndecans (47, 48, 54)). This suggests that the resistance of membrane-bound HLE and CG on activated PMN to inhibition by physiologic inhibitors could be directly due to HLE and CG binding to sulfated GAGs on the PMN surface.

Cai et al. (38) showed that HLE is a ligand for CD11b/CD18 integrin on PMN, and binding of HLE to this integrin detaches activated PMN from fibrinogen-coated surfaces. Cai et al. (38) did not suggest that CD11b/CD18 is the major binding site for HLE on the PMN surface, since the number of CD11b/CD18 molecules expressed per cell is low relative to the number of HLE (and CG) molecules bound per cell. It is also noteworthy that Cai et al. (38) demonstrated that the active site of HLE is required for its binding to CD11/CD18, whereas our data show that HLE and CG binding to the PMN surface does not require a functional active site. A unifying hypothesis for the findings in our study and that of Cai et al. (38) is that CSPG and HSPG serve as a reservoir to initially sequester via low affinity ionic interactions the large quantities of HLE and CG released from degranulating PMN. This binding leaves the active sites of HLE (and possibly CG) free to bind CD11b/CD18 integrins and to regulate PMN adhesion likely by inducing a conformational change in the integrins.

The binding of other molecules to cell surface proteoglycans induces outside-in signaling and regulates the activities of the ligands. The binding of platelet factor 4 to CSPG induces PMN degranulation (29). HSPG on endothelial cells and fibroblasts binds to platelet factor 4 to CSPG induces PMN degranulating PMN. This binding leaves the active sites of HLE (and possibly CG) free to bind CD11b/CD18 integrins and to regulate PMN adhesion likely by inducing a conformational change in the integrins.

Conclusions—Our data provide new insights into the mechanism by which HLE, CG, and other cationic proteins contained within the granules of PMN bind to the PMN surface and retain their activity. Serine proteinase binding to HSPG and CSPG focuses the activities of these potent enzymes to the pericellular environment and also preserves their activities by protecting them from inhibition by high Mₙ, naturally occurring inhibitors. Thus, the CSPG and HSPG binding sites for HLE and CG on PMN may be new targets for developing treatment strategies for diseases in which HLE and CG play important pathogenic roles.

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