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Cleavage of CD14 on Human Gingival Fibroblasts Cocultured with Activated Neutrophils Is Mediated by Human Leukocyte Elastase Resulting in Down-Regulation of Lipopolysaccharide-Induced IL-8 Production

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Activated polymorphonuclear leukocytes (PMNs) release various types of proteases and express them on the cell surface. The proteases play important roles in PMN-mediated events. In the present study, flow cytometric analysis revealed that CD14 expression on human gingival fibroblasts (HGF) was markedly reduced by PMA-activated PMNs in a coculture system. We found that this reduction was caused by both secreted and cell surface proteases produced by activated PMNs. A protease responsible for the reduction was found to be human leukocyte elastase (HLE) secreted from the activated PMNs by use of various protease inhibitors, although HLE was only partially involved in CD14 reduction caused by cell-bound molecule(s) on fixed PMNs. Analysis with purified HLE revealed a time- and dose-dependent reduction of CD14 on HGF, and complete reduction was observed by 20 µg/ml HLE treatment for 30–60 min, but the other molecules such as CD26, CD59, CD157, and MHC class I on HGF were only slightly reduced. This reduction of CD14 resulted from direct proteolysis by HLE on the cell surface, because HLE reduced CD14 on fixed HGF and also on purified cell membranes. As a result of CD14 proteolysis, IL-8 production by HGF was suppressed when triggered by 10 ng/ml LPS, but not by IL-1α, indicating that HLE inhibited a CD14-dependent cell activation. These findings suggested that activated PMNs have a potential negative feedback mechanism for HGF function at the inflammatory site, particularly in periodontal tissues. The Journal of Immunology, 2000, 165: 5807–5813.

In chronic inflammatory diseases, such as periodontitis and rheumatoid arthritis, hemopoietic immune cells such as T cells, B cells, monocytes, and granulocytes are recruited and accumulated at inflamed areas that are composed of resident non-hemopoietic cells such as fibroblasts and endothelial cells. Periodontitis develops with an active phase and a chronic phase repeatedly, and among immune cells, polymorphonuclear leukocytes (PMNs) are recruited from peripheral blood during the active phase of inflammation following their adhesion to endothelium and transmigration into the perivascular area and connective tissue. Although PMN activation results in increased phagocytosis, bacterial killing, release of lysosomal enzymes, and superoxide anion generation (1), PMNs are implicated as mediators of connective tissue destruction events in inflammatory diseases, i.e., periodontitis (2–4), rheumatoid arthritis, respiratory distress syndromes, and blistering skin diseases (5, 6). These diseases are believed to be caused by extensive proteolysis of extracellular matrix in connective tissue (1).

Connective tissue is a major source of fibroblasts, which are a common cell type to most tissues and organs, and are implicated in the pathological production of extracellular matrix and scar tissue in fibrotic inflammatory diseases. Fibroblasts not only function in the support of frameworks by synthesis of extracellular matrix such as collagens, fibronectin, and laminin, but also actively participate in inflammatory and immune responses by producing cytokines such as IL-1, IL-6, and chemokines, in particular IL-8 (7). Human gingival fibroblasts (HGF) produce IL-8 upon stimulation with LPS in a membrane CD14-dependent manner (8).

CD14, a 55-kDa GPI-anchored protein (9), is mainly expressed on monocytes and macrophages, and heterogeneously expressed on HGF, as reported previously (8). CD14 functions not only as a major receptor for LPS from Gram-negative bacteria (9), but also acts as a pattern recognition receptor for many bacterial components, such as lipoteichoic acids from Gram-positive bacteria, lipoparabinomannan from mycobacteria, soluble peptidoglycan from Staphylococcus aureus, and lipoproteins from Treponema pallidum and Borrelia burgdorferi (10, 11). Cell activation triggered by the bacterial components via CD14 is thought to be the first line of defense against invasive bacteria.

Activated PMNs express many molecules on the cell surface (12) and release soluble factors such as cytokines (13) and proteases (1), and interactions between fibroblasts and infiltrating PMNs have been observed at inflammatory sites (14). Although these interactions have been widely investigated with regard to the mechanism of adhesion of PMNs to fibroblasts (15–17), little is known about regulation of fibroblast function influenced by activated PMNs. To help clarify this,
we used a coculture system with PMNs and HGF, and examined how activated PMNs could influence the function of HGF with regard to cytokine production. We found that activated PMNs cleaved CD14 on HGF by direct proteolysis, and this proteolysis was mediated particularly by human leukocyte elastase (HLE, E.C. 3.4.21.11) released from activated PMNs, which resulted in down-regulation of IL-8 production by HGF triggered by LPS in a CD14-dependent manner.

Materials and Methods

Reagents

LPS of Escherichia coli O55:B5, HYBRI-MAX (serum-free and protein-free medium), αL-antitrypsin (αL-AT), PMSF, 1,10-phenanthroline, FMLP, and PMA were purchased from Sigma (St. Louis, MO). Bacillus cereus phosphatidylinositol-specific phospholipase C (PI-PLC), pepstatin (an aspartic protease inhibitor), and E-64 ([N-[(3-methoxy-carboxy-1-2-carbonyl)-1-leucyl]jagmatine], a cysteine protease inhibitor) were purchased from Boehringer Mannheim (Indianapolis, IN). FCS was purchased from Flow Laboratories (McLean, VA). HLE, human neutrophil cathepsin G, and N-methoxy succinyl-Ala-Ala-Pro-Val-chloromethyl ketone (HLE/CMK) were purchased from Calbiochem-Novabiochem (La Jolla, CA). Human leukocyte elastase (HLE) was purchased from HyTest (Turku, Finland). α-MEM and 0.25% trypsin/1 mM EDTA were from Life Technologies (Rockville, MD). Human IL-1α and human rTNF-α were obtained from Dainippon Pharmaceutical (Osaka, Japan). Anti-HLA-ABC FITC (G46-2-6, mouse IgG1), anti-CD59 FITC (p228, mouse IgG2a), and anti-CD26 mAb (M-A261, mouse IgG1) were from Pharmingen (San Diego, CA). Anti-CD14 mAb (MEM-18, mouse IgG1) was from Monosan (Uden, The Netherlands). Anti-CD14 mAb (MY4, mouse IgG2b), anti-CD157 mAb (RF3, mouse IgG1), and all isotype control mAbs were obtained from Immunotech (Marseille, France). All other reagents were obtained from Sigma, unless otherwise indicated.

Isolation of human PMNs

PMNs from heparinized (10 U/ml) peripheral venous blood were isolated by density-gradient centrifugation on Mono-Poly resolving medium (ICN Biomedical, Costa Mesa, CA) at 300 × g for 30 min at room temperature (18). The fraction containing PMNs was harvested and washed twice with PBS at 4°C. The viability of these cells was greater than 98%, as judged by trypan blue dye exclusion. The purity of PMNs was above 95% morphologically.

Human gingival fibroblast

HGF were prepared from the explants of normal gingiva from 8- to 25-year-old patients with informed consent, as reported previously (19). Explants were cut into pieces and cultured in 100-mm-diameter tissue culture dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) in α-MEM supplemented with 10% FCS with a medium change every 3 days for 10–15 days until confluent cell monolayers were formed. The cells were detached with 0.25% trypsin/1 mM EDTA, washed with PBS, and subcultured in plastic flasks (Corning Coster, Acton, MA). After three to four subcultures, the cells were collected by trypsinization, washed with PBS, and used for staining. For HLA-ABC and CD59 staining, 105 HGF were stained with FITC-conjugated mAbs or FITC-conjugated isotype-matched mouse IgG1 at 4°C for 30 min. For CD14, CD157, and CD26 staining, cells were stained with each mAb or each isotype-matched control IgG at 4°C for 30 min, followed by incubation with FITC-conjugated goat anti-mouse IgG (BioSource International, Camarillo, CA) at 4°C for an additional 30 min. Staining was analyzed on a FACScan (Becton Dickinson, Mountain View, CA). For staining fixed HGF, monolayers of HGF in 24-well multilpates were treated with 1% (w/v) paraformaldehyde for 20 min at room temperature. After washing three times with PBS, HGF were treated with HLE, as described below, and harvested by trypsinization and then stained with anti-CD14 mAb. In the coculture experiment of HGF with PMNs, the HGF population was gated on the basis of forward and right-angle scatter. Data were collected for 5000 events, which were stored in list mode and then analyzed with Lysis II software (Becton Dickinson). The arithmetic mean was used in the computation of the mean fluorescence intensity (MFI).

Flow cytometry

HGF were collected by trypsinization, washed with PBS (pH 7.2), and used for staining. For HLA-ABC and CD59 staining, 105 HGF were stained with FITC-conjugated mAbs or FITC-conjugated isotype-matched mouse IgG1 at 4°C for 30 min. For CD14, CD157, and CD26 staining, cells were stained with each mAb or each isotype-matched control IgG at 4°C for 30 min, followed by incubation with FITC-conjugated goat anti-mouse IgG (BioSource International, Camarillo, CA) at 4°C for an additional 30 min. Staining was analyzed on a FACSscan (Becton Dickinson, Mountain View, CA). For staining fixed HGF, monolayers of HGF in 24-well multilpates were treated with 1% (w/v) paraformaldehyde for 20 min at room temperature. After washing three times with PBS, HGF were treated with HLE, as described below, and harvested by trypsinization and then stained with anti-CD14 mAb. In the coculture experiment of HGF with PMNs, the HGF population was gated on the basis of forward and right-angle scatter. Data were collected for 5000 events, which were stored in list mode and then analyzed with Lysis II software (Becton Dickinson). The arithmetic mean was used in the computation of the mean fluorescence intensity (MFI).

Coculture of HGF monolayer with PMNs

Confluent monolayers of HGF (approximately 105 cells) in collagen I (rat tail tendon)-coated 24-well multilpates (Becton Dickinson Labware) were cocultured with the indicated number of purified PMNs in 500 μl of FCS-free α-MEM with or without 100 ng/ml PMA for 60 min at 37°C. Cells were collected by trypsinization, and expression of CD14 by HGF was analyzed on the flow cytometer. To examine the effect of supernatants from PMNs, the indicated number of purified PMNs in a microtube containing 500 μl of FCS-free α-MEM were stimulated with 100 ng/ml PMA for 60 min at 37°C or primed with 100 ng/ml TNF-α for 10 min, followed by stimulation with 1 μM FMLP for 60 min at 37°C. After incubation of PMNs, supernatants were harvested by centrifugation, and kept at −20°C until use. For fixing PMNs, PMNs were stimulated with PMA similar to cell-free supernatant and fixed for 3 min at 4°C in PBS containing 3% (v/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde (pH 7.4). After washing twice in HBSS, the indicated number of fixed PMNs were resuspended with 500 μl of FCS-free α-MEM. For protease inhibitor treatment, the indicated concentration of each inhibitor was preincubated with cell-free supernatant (500 μl) from 3 × 105 PMNs or fixed PMNs (3 × 105/μl) for 10 min at 37°C before addition onto monolayer cells. Percent inhibition for the supernatant and the fixed cells was calculated as ((MFI in the presence of supernatant and inhibitors) – (MFI in the absence of supernatant and inhibitors) × 100 (%) and [(MFI in the presence of fixed PMNs and inhibitors) – (MFI in the presence of fixed PMNs) × (MFI in the absence of fixed PMNs)] × 100 (%), respectively.

HLE treatment

Monolayers of HGF in collagen I-coated 24-well multilpates (the well contained 300 μl of serum-free media, HYBRI-MAX) were treated with the indicated concentration of HLE at 37°C for the indicated times. For HLE inhibitor (αL-AT and human serum) treatments, 20 μg/ml HLE was preincubated with 100 μg/ml αL-AT or 10% (v/v) human serum for 15 min at 37°C before addition onto monolayer cells.

Reexpression of CD14 following proteolytic cleavage

Confluent monolayers of HGF in wells of 24-well collagen I-coated plates were treated with or without 20 μg/ml HLE in HYBRI-MAX for 30 min at 37°C. After gently washing monolayers with prewarmed HYBRI-MAX, 1 ml of prewarmed HYBRI-MAX with 0.5% FCS was added to the wells and incubated at 37°C. At the indicated times, cells were analyzed for the expression of CD14 by flow cytometry.

Preparation of cell membranes and treatment with enzymes

HGF were collected by trypsinization, and cells were suspended in hypotonic buffer (10 mM Tris-HCl, pH 7.4, and 1 mM MgCl2) and incubated on ice for 30 min. Cells were then homogenized in a Dounce homogenizer by 15 strokes, and sucrose was added to a final concentration of 0.25 M. The homogenate was centrifuged at 500 × g for 5 min twice to remove nuclei. Supernatants were centrifuged at 15,000 × g for 30 min, and membrane pellets were suspended in PBS and stored at −20°C until use.

Western blotting

Membrane pellets from confluent HGF grown in 25-cm2 flasks were suspended in 100 μl of HBSS containing 20 μg/ml HLE or 5 μl/ml PI-PLC at 37°C. Membrane pellets and supernatants were separated by centrifugation at 15,000 × g for 30 min at 4°C, and supernatants were dried by vacuum centrifugation. Membrane pellets and dried supernatants were solubilized with 20 μl of Laemmli sample buffer (10% glycerol, 1% SDS, 0.0025% bromophenol blue, and 50 mM Tris-HCl, pH 6.8) at 100°C for 5 min. Samples were separated by SDS-PAGE (10%). Proteins were transferred to a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan) using a semidyndrum blot system (ATTO). The blot was blocked for 2 h with 5% BSA/PBS, followed by incubation with 2 μg/ml sheep anti-human CD14 polyclonal Ab (Genzyme/Technic, Minneapolis, MN) in 3% BSA/PBS for 2 h at room temperature. The blot was washed four times with PBS and then incubated for 2 h with HRP-conjugated affinity-purified donkey anti-sheep IgG (Jackson ImmunoResearch, West Grove, PA) at 1:2000 in 3% BSA/PBS. After washing, CD14 was visualized with diaminobenzidine. The M, of the proteins was estimated by comparison with the position of the standard (Bio-Rad Laboratories, Hercules, CA).
Detection of IL-8 by ELISA

A total of $5 \times 10^8$ HGF was cultured in α-MEM with 10% FCS in wells of 24-well collagen I-coated plates until confluent, and an additional 4-day culture was allowed. After washing with PBS three times, confluent monolayers of HGF were treated with 20 μg/ml HLE in HYBRI-MAX for 30 min at 37°C. HLE-treated monolayer cells were gently washed twice with prewarmed HYBRI-MAX, followed by addition of test stimulants in 500 μl of HYBRI-MAX without FCS for 4 h. For the blocking experiment with mAb, confluent monolayers of HGF were pretreated with 5 μg/ml dialyzed anti-CD14 mAb (MY4) or isotype control mouse IgG2b at 37°C for 30 min before addition of stimulants. After stimulation, the supernatants were collected and the level of IL-8 in the supernatants was determined with a human IL-8 ELISA kit (Endogen, Woburn, MA). IL-8 assays were performed exactly as instructed by the ELISA manufacturer. The concentration of IL-8 in the supernatants was determined using the Softmax data analysis program (Molecular Devices, Menlo Park, CA). Each sample was assayed in triplicate.

Statistical analysis

All experiments in this study were performed at least three times to test the reproducibility of the results, and representative findings are shown. In some experiments, experimental values are given as means ± SDs. The statistical significance of differences between two means was evaluated by Student’s unpaired t test.

Results

Reduction of CD14 expressed on HGF by activated PMNs

Confluent monolayers of HGF were cocultured with purified PMNs in the presence or absence of PMA, and CD14 expression was evaluated by flow cytometry using the MEM-18 mAb. When HGF were cocultured with $3 \times 10^5$ PMNs in the presence of 100 ng/ml PMA for 1 h, the MFI of CD14 was significantly ($p < 0.01$) reduced (MFI = 52% of control), and $3 \times 10^6$ of activated PMNs caused up to 95% reduction of the CD14 expression (Fig. 1A). However, unactivated PMNs lacked the activity at any number of PMNs. Next, we examined whether the reduction of CD14 expression required secreted molecules from PMNs or cell-to-cell contact between HGF and PMNs. When the HGF monolayer was incubated with the culture supernatant derived from the indicated number of PMA-activated PMNs, CD14 expression on HGF was significantly ($p < 0.01$) reduced, and the supernatant from $3 \times 10^6$ of activated PMNs reduced the expression to approximately 70% of the control value (Fig. 1B). When activated PMNs were fixed with 3% paraformaldehyde, as described previously (20), to prevent continuous secretion of molecules from PMNs during coculture with the HGF monolayer, the CD14 expression on HGF was also significantly ($p < 0.01$) reduced and $3 \times 10^6$ of fixed PMNs reduced to approximately 70% of the control (Fig. 1C). In addition to PMA stimulation, we examined whether a physiological stimulus also had a similar effect. Fig. 1D shows that when PMNs were stimulated with 1 μM FMLP for 60 min after priming with 100 ng/ml TNF-α for 10 min, the supernatant from those PMNs showed CD14 cleavage activity, to a degree approximately 80% that of the PMA supernatant, in a cell number-dependent fashion. These findings indicate that the reduction of CD14 expression on HGF was caused by molecules that were both released and expressed by activated PMNs.

Effect of protease inhibitors on reduction of CD14 by activated PMNs

Since activated PMNs release various proteases and induce the cell surface-bound serine proteases (20, 21), we next examined the effect of various protease inhibitors on the reduction of CD14 expression by cell-free supernatant from PMA-stimulated PMNs. A naturally occurring serine protease inhibitor, α1-AT, and a synthetic serine protease inhibitor, PMSF, showed a marked inhibitory effect (83.6% and 79.9%, respectively) on the reduction of CD14 caused by cell-free supernatant (Table I). Furthermore, HLE/CMK, a specific inhibitor of HLE, exhibited 62.1% inhibition of CD14 reduction caused by the supernatants. However, the inhibitory effect was scarcely observed in 1,10-phenanthroline (a metalloprotease inhibitor) and E-64 (a cysteine protease inhibitor), and only slight inhibition (15.9%) was observed in pepstatin (an aspartic protease inhibitor). These findings indicated that among the various proteases released from activated PMNs, serine proteases, in particular HLE, participated in CD14 cleavage caused by the cell-free supernatant.

Table I. Effect of protease inhibitors on reduction of CD14 on HGF induced by cell-free supernatant of PMNs or fixed PMNs

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Supematant</th>
<th>Fixed PMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-AT</td>
<td>200 μg/ml</td>
<td>83.6 (2.8)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>PMSF</td>
<td>1 mM</td>
<td>79.9 (14.4)</td>
<td>14.8 (3.9)</td>
</tr>
<tr>
<td>HLE/CMK</td>
<td>1 mM</td>
<td>62.1 (3.5)</td>
<td>11.1 (6.5)</td>
</tr>
<tr>
<td>E-64</td>
<td>1 μM</td>
<td>6.8 (4.5)</td>
<td>5.6 (4.3)</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>1 μM</td>
<td>15.9 (9.1)</td>
<td>4.5 (3.5)</td>
</tr>
</tbody>
</table>

* A confluent monolayer of HGF was cultured with supernatant derived from $3 \times 10^6$ PMA-activated PMNs (referred to as supernatant) or with $3 \times 10^6$ of fixed PMNs, in the presence or absence of the indicated protease inhibitors for 1 h, and MFI of CD14 expression on HGF was analyzed by flow cytometry.

* Percent inhibition was calculated as in Materials and Methods. Representative findings of three independent experiments are expressed as the mean ± SD of triplicate assays.
Since serine proteases including HLE are expressed on the PMN cell surface upon activation (20), we next examined whether cell surface HLE as well as released HLE participated in the CD14 reduction by coculturing the HGF monolayer with 3% paraformaldehyde-fixed PMNs in the presence or absence of various protease inhibitors. PMSF and HLE/CMK only partially inhibited the CD14 reduction at 14.8% and 11.1%, respectively. Moreover, α1-AT, 1,10-phenanthroline, E-64, and pepstatin showed almost no inhibitory effect. These findings indicated that although cell surface serine proteases actually participated in the reduction of CD14 expression, the degree was very low. This observation might be due to: 1) the protease inhibitors were ineffective at cell-to-cell contact proteolysis, or 2) some protease(s) that was/were not involved in the reduction. No inhibitory effect of α1-AT was supported by the study of Owen et al. (20), in which cell surface HLE was resistant to inhibition by naturally occurring protease inhibitors. The CD14-reducing activity induced by TNF-α and FMLP treatment of PMNs shown in Fig. 1D was also inhibited by HLE/CMK at approximately 90% (data not shown). Therefore, the findings shown in Fig. 1 and Table I indicate that the reduction of CD14 on HGF caused by activated PMNs was performed mainly by serine proteases, probably HLE in the supernatant and, although the degree was low, also partially performed by cell surface HLE.

Reduction of CD14 on HGF by HLE treatment

Since the major serine proteases released by activated PMNs are HLE, cathepsin G, and PR3, we next examined the effect of HLE, cathepsin G, and PR3 on CD14 expressed by HGF. Fig. 2A shows a representative FACS profile, indicating that marked reduction of CD14 expression on HGF treated with 20 μg/ml HLE in the serum-free condition for 1 h was observed compared with untreated cells. This reduction of CD14 on HGF reached 85% at 10 μg/ml (340 nM) of HLE, and was totally achieved at 20 μg/ml (680 nM) of HLE for 60-min treatment (Fig. 2B). In contrast, cathepsin G, which is also inhibited by α1-AT (22) and PMSF (23), and PR3 exhibited much less activity for CD14 reduction compared with the same concentration (nM) of HLE. These findings indicated that HLE was the main serine protease from activated PMNs responsible for CD14 reduction in the coculture experiment, as shown in Table I. The reduction of CD14 by HLE was inhibited almost completely by pretreatment with 100 μg/ml α1-AT and 10% (v/v) of human serum, which is known as a source of α1-AT (Fig. 2C). The time kinetics experiment revealed that complete reduction of CD14 was observed after 60-min treatment, and that even 30-min treatment was adequate to almost completely reduce CD14 from the cell surface (Fig. 2D).

We next compared the sensitivity of CD26, CD59, CD157, and MHC class I as well as CD14 expressed on HGF by HLE. Since CD26 is induced by IL-1α stimulation on HGF (19), the HGF monolayer was stimulated with IL-1α for 5 days before analysis of CD26 sensitivity. HLE treatment (20 μg/ml) of HGF for 1 h reduced the expression of CD14 completely, but only slight reduction of CD26, CD59, CD157, and MHC class I (at most 12%, 28%, 7%, and 24% reduction, respectively) was observed (Fig. 3), indicating that HLE preferentially reduced CD14. Next, we examined whether CD14 could be reexpressed on the cell surface after reduction of CD14 by HLE. CD14 was gradually reexpressed in the normal culture condition, and approximately 75% of CD14 was recovered within 48 h (Fig. 4).

Reduction of CD14 on paraformaldehyde-fixed HGF by HLE treatment

These findings suggest that proteolytic cleavage of CD14 on the cell surface by HLE was the most plausible mechanism, although there remain two other possibilities that CD14 was internalized or shed by endogenous enzymes following HGF activation by HLE. To clarify this, HGF were fixed with 1% paraformaldehyde before HLE treatment. As shown in Fig. 5, after fixation of HGF, HLE was still capable of effectively cleaving CD14 (p < 0.01). Considering that the shedding and the internalization of CD14 should require activating endogenous enzyme(s) or cell activation, this
finding suggests that HLE cleaved CD14 on the cell surface proteolytically.

**Immunoblot analysis of CD14 expressed on HGF**

Another approach was to examine the proteolytic cleavage of CD14 by HLE. Purified cell membranes of HGF were treated with HLE, and both the cell membrane and extracellular supernatant were analyzed by Western blotting using anti-CD14 polyclonal Ab. As a positive control, PI-PLC, an enzyme that specifically removes GPI-anchored proteins from the cell surface (9), was used in this experiment. In untreated cell membranes, a strong 55-kDa CD14 band was detected, whereas this band disappeared after HLE treatment and partial reduction of the band was observed after PI-PLC treatment (Fig. 6). This partial reduction of the CD14 band in the PI-PLC-treated cell membrane was consistent with flow cytometry in which CD14 expression was partially reduced (MFI, 75.7 (control) vs 28.9 (PI-PLC treatment)). In the extracellular supernatant, a strong 55-kDa band was detected in the PI-PLC-treated extracellular supernatant, whereas no bands were detected in HLE-treated extracellular supernatant or in controls. This finding and Fig. 5 indicate that reduction of CD14 by HLE resulted from direct proteolysis, but not from shedding or internalization following cell activation, and could be degraded into multiple CD14 fragments that were not detected by polyclonal Ab for CD14.

**HLE treatment of HGF resulted in the reduction of IL-8 production by HGF in response to LPS**

The observation that HLE cleaved CD14 on HGF raises the question whether HLE inhibits LPS-induced IL-8 production by HGF, since it was reported that HGF produce IL-8 in response to LPS stimulation in a membrane CD14-dependent pathway (8). Preincubation of HGF with 20 µg/ml HLE for 30 min markedly suppressed IL-8 production from HGF upon stimulation with 10 ng/ml LPS (Fig. 7A). Anti-CD14 mAb (MY4) inhibited LPS (10 ng/ml)-induced IL-8 production by HGF to background levels, but isotype-matched control Ab did not exhibit any inhibitory effect. We performed similar experiments with IL-1α as a CD14-independent stimulant. As shown in Fig. 7B, HLE pretreatment with HGF did not significantly change IL-8 production triggered by IL-1α. MY4 and isotype-matched Ab were both unable to inhibit IL-8 production by HGF triggered with IL-1α. Thus, these findings indicate that HLE treatment of HGF specifically inhibited the CD14-dependent cell activation triggered by LPS.

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**FIGURE 4.** Reexpression of CD14 on HGF after reduction by HLE treatment. HGF monolayer cells were treated with 20 µg/ml HLE for 30 min at 37°C. After washing three times, monolayer cells were recultured in serum-free medium (HYBRI-MAX) supplemented with 0.5% FCS for the indicated times. After harvesting by trypsinization, cells were stained with MEM-18 and analyzed by flow cytometry. The findings are expressed as the percentage of MFI of control cells incubated for the same time without pretreatment of HLE. Representative findings of three independent experiments are expressed as the mean ± SD of the MFI (% of control) of triplicate assays.

**FIGURE 5.** Effect of fixation on the reduction of CD14 on HGF by HLE treatment. HGF monolayer cells were fixed with 1% paraformaldehyde for 10 min at room temperature. After washing, untreated or fixed cells were incubated with 20 µg/ml HLE for 1 h at 37°C. Expression of CD14 on HGF was assessed by flow cytometry. Representative findings of three independent experiments are expressed as the mean ± SD of the MFI (% of control) of triplicate assays. Statistical significance is shown (+, p < 0.01 vs respective control).

**FIGURE 6.** Immunoblot analysis of CD14 expressed on and released by HGF after HLE treatment. Purified cell membrane of HGF was treated with 20 µg/ml HLE or 5 U/ml PI-PLC for 1 h at 37°C. After the cell membrane and the supernatant were separated by ultracentrifugation, both specimens were then solubilized with Laemli sample buffer, subjected to 10% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. The blot was probed with an anti-CD14 polyclonal Ab. Molecular mass markers (kDa) are shown on the left. Findings are representative of two independent experiments.

**FIGURE 7.** HLE treatment of HGF resulted in the reduction of IL-8 production by HGF in response to LPS. The amount of IL-8 in the supernatants was analyzed by ELISA. Findings are representative of two independent experiments.
Discussion
In the present study, activated but not unactivated PMNs reduced CD14 expression on HGF by coculture of HGF and PMNs in a manner mediated by both the cell-free supernatant from activated PMNs and the fixed PMNs. The CD14 reduction by the supernatant was caused mainly by a serine protease, HLE, with use of various protease inhibitors, which were serine, cysteine, aspartic acid, and metalloprotease inhibitors. Previous studies demonstrated that cathepsin G (E.C. 3.4.21.20) and PR3 (both are other major serine proteases) are released from PMNs (1, 24). However, the present study showed that the purified HLE, but not cathepsin G and PR3, efficiently reduce CD14 on HGF (Fig. 2), which suggested that cathepsin G and PR3 are only partially involved in the reduction of CD14 expression.

We next focused on the mechanism of the reduction caused by HLE released from PMNs upon activation. HLE exhibited a dose- and time-dependent proteolysis of CD14 on the cell surface. As a consequence of CD14 cleavage, CD14-dependent IL-8 production triggered by LPS was inhibited. In many cases, the shedding of cell surface molecules followed cell activation (25–27). Since HLE induces cell activation, such as activation of MAP kinase in epithelial cells (28) and chemokine production by macrophages (29), the possibility that CD14 reduction is mediated by cell activation was examined using fixed HGF and purified HGF membrane. It was clearly demonstrated that reduction of CD14 by HLE was due to the direct proteolysis of CD14 on the cell surface (Figs. 5 and 6). It is unlikely that reduction of CD14 expression on HGF resulted from the direct action of PMA to HGF because no change in CD14 expression on HGF was observed when the HGF monolayer was stimulated with 100 ng/ml PMA for 1 h in the absence of PMNs (data not shown). Furthermore, multiple fragmentation of CD14 might occur, since polyclonal CD14 Ab did not detect the products by Western blotting analysis. These findings suggested that CD14 molecules proteolyzed by HLE had no ability to work as a soluble CD14 (sCD14), CD59 and CD157, other GPI-anchored glycoproteins expressed on HGF, were not reduced as efficiently as CD14 by HLE (Fig. 3), which excluded the possibility that HLE preferentially cleaves GPI-anchored molecules on the cell surface. Note that the trypsinization itself to harvest HGF did not affect the CD14 expression. Moreover, when HLE-treated HGF immediately before confluence was harvested by EDTA without trypsin, CD14 expression was reduced by as much as that by trypsinization (data not shown). These observations ruled out the possibility that HLE alters the accessibility of CD14, which is then released at trypsinization.

Since HLE is induced on the cell surface upon activation (20, 30), this suggested that cell-bound HLE was also involved in the reduction. To clarify this possibility, fixation of the PMNs was a suitable procedure, because 1) the fixation does not affect the enzymatic activity (20, 30), 2) the fixation is necessary to diminish the tight adhesion of PMNs to HGF since the tight adhesion can create microenvironments from which inhibitors are excluded (31, 32), 3) the fixation prevents leakage of endogenous proteases. However, CD14 reduction by the fixed PMNs was only slightly inhibited by PMSF and HLE/CMK, and the other protease inhibitors did not significantly inhibit the reduction (Table I). The lack of an effect of α1-AT on fixed cell-induced CD14 reduction (Table I) was consistent with a previous study that reported that cell-bound serine proteases are remarkably resistant to inhibition by naturally occurring high m.w. protease inhibitors such as α1-AT (20). However, low m.w. synthetic inhibitors such as PMSF and HLE/CMK substantially inhibit cell surface protease activities (20), and 85% inhibition of cell-bound HLE (fixed PMNs) activity was shown with 60 μM of HLE/CMK at 5 × 10^5 fixed PMNs. In the present study, the more effective condition of 1 mM of HLE/CMK for 3 × 10^6 of fixed PMNs was used. Therefore, it was suggested that only slight inhibition of CD14 reduction by PMSF and HLE/CMK was not due to the resistance of serine proteases against these inhibitors, and that some protease(s) that was/were not inhibited by the protease inhibitors used in this study might be involved in the reduction.

HGF not only functions to support frameworks by synthesis of extracellular matrix, but also participates in inflammatory and immune responses to bacterial components such as LPS from periodontitis-associated Gram-negative bacteria. Activation of HGF via the LPS-CD14 pathway induces chemokines such as IL-8 (8), which attract PMNs from the peripheral blood to the inflammatory site to form a first line host defense, indicating that this mechanism is of great importance for innate immunity. A previous study reported (33) that HLE was able to digest IL-8 and abolish the chemotactic activity, which suggested the possible mechanism of the down-regulatory cascade of inflammation by HLE. The present study showed that production of IL-8 was inhibited via CD14 cleavage by HLE, which might be another mechanism for the down-regulatory cascade of inflammation by HLE. Since HLE is released from PMNs via the β₂ integrin-ICAM-1 pathway (34, 35), and ICAM-1 is cleaved by HLE (36), HLE is likely to auto-down-regulate HLE production from PMNs possibly by impairment of the β₂ integrin-ICAM-1 pathway, which is a negative feedback effect of HLE.

HLE is an essential factor for host defense against bacteria (37), and also has the capacity to degrade a wide variety of extracellular matrix including elastin, proteoglycan, denatured collagen, fibronectin, and laminin (38, 39), which are characteristics of several pathological conditions, including periodontitis (2–4), rheumatoid arthritis, respiratory distress syndrome, and blistering skin diseases (5, 6). Recently, HLE was reported to affect on the various functions of resident nonhemopoietic cells and hemopoietic immune cells by proteolytic activity. For example, HLE treatment results in the release of basic fibroblast growth factor and heparan sulfate proteoglycans from pulmonary fibroblasts (40, 41) and the release of TGFβ1 from epithelial and endothelial cells (42), all of which could influence the tissue repair mechanism. In immune cells, specific leukocyte Ags, such as CD2, CD4, CD8 (43), CD43 (44), and CD54 (36), are cleaved by HLE. Recently, treatment of monocytes with HLE resulted in CD14 reduction, followed by inhibition of TNF-α production triggered by LPS (45), and it was demonstrated that sCD14 can be proteolyzed into multiple fragments by HLE. Considering that no CD14-expressing HGF are activated with LPS in a serum-derived sCD14-dependent manner (46), HGF activation with LPS could be inhibited by HLE in a sCD14-dependent manner as well as a cell surface CD14-dependent manner.

It is unclear whether reduction of CD14 on HGF by HLE could also occur in vivo. Previous studies demonstrated that activated PMNs may release up to 380 nM HLE into the extracellular medium (47), and the number of PMNs may increase by even 100-fold at the inflammatory site (48, 49). In addition, although serum contains abundant naturally occurring protease inhibitors, pericellular concentrations of HLE exceed that of naturally occurring inhibitors by approximately 2 orders of magnitude (31, 32), resulting in an escape from the action of protease inhibitors. Reduction of CD14 on HGF by HLE released from activated PMNs under the coculture condition shown in the present study (Fig. 1) was close to the in vivo condition. Considering the present findings and those of previous studies, reduction of CD14 on HGF caused by activated PMNs is likely to occur in vivo.
In conclusion, the present study demonstrated that direct interaction between HGF and PMNs regulated the inflammatory responses induced by LPS. This mechanism could exist in physiologic conditions as a new negative feedback mechanism of inflammation. Early-onset periodontitis is characterized by rapidly progressive, early-onset alveolar bone loss and familial aggregation (50). PMNs from early-onset periodontitis patients have been reported to produce much higher HLE than controls (51). This may lead to an altered physiological balance regulated by the negative feedback mechanism shown in the present study. The present findings suggest that interaction of HGF and PMNs leading to the regulation of inflammatory responses may provide an additional viewpoint to understand the mechanism of onset and development of inflammatory diseases such as periodontitis.

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References