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Quantum Proteolytic Activation of Chemokine CCL15 by Neutrophil Granulocytes Modulates Mononuclear Cell Adhesiveness

Rudolf Richter,1,2* Roxana Bistrian,3 Sylvia Escher,* Wolf-Georg Forssmann,* Jalal Vakili,‡ Reinhard Henschler,† Nikolaj Spodsberg,§ Adjoa Frimpong-Boateng,*, and Ulf Forssmann*

Monocyte infiltration into inflammatory sites is generally preceded by neutrophils. We show here that neutrophils may support this process by activation of CCL15, a human chemokine circulating in blood plasma. Neutrophils were found to release CCL15 proteolytic activity in the course of hemofiltration of blood from renal insufficiency patients. Processing of CCL15 immunoreactivity (IR) in the pericellular space is suggested by a lack of proteolytic activity in blood and blood filtrate, but a shift of the retention time ($t_R$) of CCL15-IR, detected by chromatographic separation of CCL15-IR in blood and hemofiltrate. CCL15 molecules with N-terminal deletions of 23 ($\Delta 23$) and 26 ($\Delta 26$) aa were identified as main proteolytic products in hemofiltrate. Neutrophil cathepsin G was identified as the principal protease to produce $\Delta 23$ and $\Delta 26$ CCL15. Also, elastase displays CCL15 proteolytic activity and produces a $\Delta 21$ isofrom. Compared with full-length CCL15, $\Delta 23$ and $\Delta 26$ isofroms displayed a significantly increased potency to induce calcium fluxes and chemotactic activity on monocytes and to induce adhesiveness of mononuclear cells to fibronectin. Thus, our findings indicate that activation of monocytes by neutrophils is at least in part induced by quantum proteolytic processing of circulating or endothelium-bound CCL15 by neutrophil cathepsin G. The Journal of Immunology, 2005, 175: 1599–1608.

Chemokines are a family of small cytokines that are involved in inflammatory processes by inducing migration and activation of leukocytes. Chemokines exert their biological activities via binding to specific surface receptors, which belong to seven transmembrane G protein–coupled receptors (1). CCL15 is a human chemokine which acts predominantly on chemokine receptor CCR1 and to a lesser degree on CCR3 (2). It is a potent chemoattractant for monocytes and lymphocytes in vitro and, after i.p. application, CCL15 induces recruitment neutrophils, monocytes, and lymphocytes into mouse peritoneum (3). CCL15 is constitutively expressed in the gut and in the liver (4) and was identified to circulate in human plasma (5). The N terminus of CCL15 was found to be important to exert its full biological activity. Lee et al. (6) showed that truncation of the N terminus by at least 24 aa significantly increases the calcium flux and chemotactic activity.

One of the first essential steps in inflammation is the activation of the adhesion between endothelial cells and leukocytes. IL-1 and TNF-α are important mediators leading to increased expression of adhesion molecules E-selectin, VCAM-1, and ICAM-1 as well as expression of soluble proinflammatory cytokines, e.g., MCP-1, M-CSF, IL-6, and IL-8, by the endothelium (7–16). The subsequent recruitment of leukocytes to inflamed tissue requires their tethering and rolling as well as rapid activation of integrin-dependent arrest on the target endothelium as a checkpoint for diapedesis to the extravascular tissue (17, 18). Chemokines, present on the endothelial surface under physiologic or pathologic conditions (19–22), trigger rolling and firm adhesion by activation of G protein–coupled receptors (23–26). Tethering and rolling of leukocytes at adhesive contact zones is increased by integrin clustering raising the avidity of integrin-ligand interactions (25, 27–29). Integrin-mediated arrest of leukocytes on vascular endothelium is suggested to be induced by further interaction partners. Tetraspanins, Cytohesin-1, pentaspan, CD99/E2, and CD47 are integrin-associated molecules and are suggested either to regulate the affinity of integrins to their ligands or to interact with their own endothelial ligand (30–33).

In this study, we demonstrate a further mechanism for activation of migratory behavior of mononuclear cells by chemokines, which is associated with the degranulation of neutrophils and proteolytic activation of chemokine CCL15. Degranulation and release of proteolytic enzymes by neutrophils is induced by interaction with activated vascular endothelium, which is stimulated with IL-1 or TNF-α (34). Generally, monocyte infiltration is preceded by an initial influx of neutrophils. In cases of clinical cyclic or experimental neutropenia, complementary influx of mononuclear cells into inflammatory sites is significantly decreased and delayed. Replenishment of circulating neutrophils reestablishes the normal sequence of events in the development of inflammatory response (35, 36). In our experimental setting, activation of neutrophils is induced on semipermeable hemofilter membranes used for hemofiltration of patients with chronic renal failure (CRF)3 (37). Immediate filtration of plasma...
proteins and peptides subsequent to contact with activated leukocytes at the filter membrane allows the determination of proteolytically processed molecules in the filtrate. We were able to show that CCL15(1–92) is efficiently processed into highly active molecules by activated neutrophils. We discovered the neutrophil serine proteinases cathepsin G and elastase responsible for this proteolytic processing. A lack of proteolytic activity in blood and in the blood filtrate suggest that processing of CCL15 is a quantum proteolytic event occurring in the pericellular space before catalysis is quenched by protease inhibitors.

Materials and Methods

Reagents

All chemicals were obtained from Sigma-Aldrich unless otherwise stated. Cell culture medium was purchased from Invitrogen Life Technologies; the CCL15(1–92) variant with the N-terminal pyrogulatamic acid was obtained from Bachem Biochemica; other chemokines were obtained from R&D Systems, 123I was obtained from Amersham Pharmacia Biotech, and cell lines were obtained from the American Type Culture Collection.

Patients

Hemofiltrate and blood plasma was collected from nine consenting patients with CRF from the Nephrologisches Zentrum Niedersachsen (Hannoversch-Münden, Germany). The patient group had a mean age of 67 ± 14 years (range, 49–84 years) and an average time on renal replacement therapy of 77 ± 54 mos. Renal disease was caused by chronic glomerulonephritis (one patient), chronic interstitial nephritis (two patients), nephrosclerosis (four patients), polycystic disease (one patient), and diabetic nephropathy (one patient).

Radioimmunoassay

CCL15(27–92) was radioiodinated using the chloramine-T method. Sep-Pak C18 cartridges (Waters) were used to separate labeled peptide from free 125I. Bound peptides were eluted using 0.01 M HCl in 80% acetonitrile. Fifty micromolar sodium phosphate buffer (pH 7.4), containing 0.025% w/v Tween 20, 0.5% w/v BSA, and 0.04% w/v NaN3 was used as RIA buffer. All reagents and samples were dissolved in RIA buffer. For incubation, affinity-purified goat anti-human CCL15 Ab (0.125 v/v/ml; Pharmacia) and 20,000 cpm/100 g/ml; R&D Systems) and 20,000 cpm/100 g/ml were obtained from the American Type Culture Collection.

Western blot analysis

For immunoblots, samples were desalted, lyophilized, and resuspended in sample buffer containing 1% w/v SDS, 7.5 M Tris-HCl (pH 8.4), 1 M EDTA, 32.4 mM DTT, 12.5% w/v glycérerol, and 0.025% w/v bromphenol blue and incubated for 7 min at 95°C (reducing conditions). The samples were separated by tricine-SDS-PAGE in 17.5% gels (38). Synthetic CCL15(1–92) and CCL15(27–92) were used as external standards. After electrophoresis, proteins were electroblotted onto hydrophobic polyvinylidene difluoride-based membranes (Pall). To minimize nonspecific binding of the Abs, blot strips were incubated with 5% w/v BSA in Tris-buffered saline (10 mM Tris and 150 mM NaCl, pH 8.0) and 0.05% v/v Triton X-100 (TBST). After washing in TBST, the membranes were incubated overnight at 4°C with affinity-purified goat anti-human CCL15 Ab (1 µg/ml). Immunoreactive proteins were visualized after incubation with alkaline phosphatase-blue tetrazolium and 5-bromo-4-chloro-3-indolylphospho-
phate as chromogens.

 Determination of chromatographic retention time (tR) for CCL15-immunoreactivity (IR)

To determine tR of CCL15-IR material, a standard chromatography method was developed. Chromatography was performed on a preparative reverse phase (RP)-HPLC, 250 mm X 10 mm inner diameter (i.d.) (Source RPC 15; Pharmacia). The separations were performed at a flow rate of 2.5 ml/min using a linear binary gradient of 100% solvent A (0.1% v/v TFA) to 60% solvent B (100% acetonitrile) and 0.1% v/v TFA) in 60 min and from 60% solvent B to 100% solvent B in 5 min. One fraction was collected each minute, starting at minute 10 of the chromatography. The fractions obtained were analyzed by CCL15 RIA. CCL15-IR in human plasma and hemofiltrate (HF) was identified by chromatography of 1.5 ml of plasma or 150 ml of HF. After collection from the hemofilter, the sterile filtrate was immediately frozen to ~80°C to prevent bacterial growth and proteolysis.

Generation of an HF peptide library

The generation of a HF peptide library was performed as described previously (39). Briefly, human HF for large-scale recovery of plasma peptides (40) was obtained from patients with CRF in quantities of 1600–2000 L/week. Ultrafiltrators used for hemofiltration had a specified molecular mass cutoff of 20 kDa. The sterile filtrate was immediately cooled to 4°C and acidified to pH 3 to prevent bacterial growth and proteolysis. For peptide extraction, batches of 1000 L of HF were conditioned to pH 2.7 and applied onto the strong cation exchanger Fractogel TSK SP 650(M) (100 × 250 mm; Merck). Batch elution was performed with 10 L of 0.5 M ammonium acetate. The eluate was stored at ~20°C until further use. For production of a peptide library, ammonium acetate extracts of 5000 L of HF were pooled and loaded on a 10-L cation exchange column (Fractogel SP 650(M); Merck). Bound peptides were eluted using seven buffers with increasing pH, resulting in seven pH pools. The seven buffers were composed as follows: I, 0.1 M citric acid monohydrate (pH 3.6); II, 0.1 M acetic acid plus 0.1 M sodium acetate (pH 4.5); III, 0.1 M malic acid (pH 5.0); IV, 0.1 M succinic acid (pH 5.6); V, 0.1 M sodium dihydrogen phosphate (pH 6.6); VI, 0.1 M disodium hydrogen phosphate (pH 7.4); and VII, 0.1 M ammonium carbonate (pH 9.0). pH pool VII was generated by washing the column with distilled water. The eight pools (pH pools) were collected and each of them was loaded onto a RP column, 125 mm × 100-mm i.d. (Source RPC, 15 µm; Pharmacia) and eluted in an 8-L gradient from 100% A (0.01 M HCl in water) to 60% B (0.01 M HCl in 80% acetonitrile). Fractions of 200 ml were collected.

Peptide purification from human HF

Fractions containing CCL15-IR were detected by CCL15 RIA and Western blot analysis. Identified CCL15-IR was further purified to homogeneity by six cycles of preparative chromatography steps A–F. Step A: preparative RP-C18 chromatography using a Vydec PrepPak RP-C18 column, 300 mm × 47-mm i.d., 15–20 µm, 300 Å (Vyded). Separations were performed at a flow rate of 40 ml/min using a linear binary gradient of 80% solvent A (10 mM HCl in 20% methanol) to 55% solvent B (10 mM HCl in 100% methanol) in 54 min and from 55% solvent B to 100% solvent B in 3 min. Fractions of 50 ml were collected. Step B: preparative cation exchange chromatography; column RP-C4, 150 mm × 7.5-mm i.d. (150-µm i.d., 50-micron i.d., 500 Å, 300 Å (Biotek Laboratories), Separation was performed at a flow rate of 5 ml/min using a linear binary gradient of 100% solvent A (50 mM sodium phosphate in water, pH 3.0) to 60% solvent B (50 mM sodium phosphate and 1.5 M NaCl in water, pH 3.0) in 60 min. Fractions of 5 ml were collected. Step C: analytical RP-C4 chromatography; column RP-C4, 250 mm × 20-mm i.d. (Biotek Laboratories), Separation was performed at a flow rate of 7 ml/min using a linear binary gradient from 90% solvent A (0.1% TFA in water) to 60% solvent B (0.1% TFA in 80% acetonitrile) within 50 min. Step D: analytical RP-C4 chromatography; column Protein & Peptide C18, 250 mm × 10-mm i.d., 5 µm, 300 Å ( Vyded). Separation was performed at a flow rate of 1.8 ml/min using a linear binary gradient from 85% solvent A (0.1% TFA in water) to 55% solvent B (0.1% TFA in 80% acetonitrile) within 50 min.

Mass spectrometry (MS)

Electrospray MS (ESMS) was conducted on an API III Triple stage quadruple mass spectrometer equipped with the articulated ion spray (PerkinElmer SCIEX). Flow injection was conducted at 5 µl/min. The masses were determined in the range from 400 to 2300 Da in the positive ion mode as described by the manufacturer. The peptide masses were calculated using the MacSpec 3.3 software (PerkinElmer SCIEX). MALDI-MS was performed using a LaserTec RBT MALDI-mass spectrometer (Perseptive/Vestec). Isolated CCL15 was applied to a stainless steel multiple sample tray as an admixture to sinapinic acid using the dried drop technique (41). Measurements were performed in linear mode. The instrument was equipped with a 1.2-m flight tube and a 337-nm nitrogen laser. Positive ions were accelerated at 30 kV and 64 laser shots were automatically accumulated per sample position. The time-of-flight data were externally calibrated for each sample plate and sample preparation.

Data acquisition and analysis were performed using GRAMS 386 version 3.04 software supplied by the manufacturer (Galactic Industries).
Sequence analysis

Sequence analyses of the isolated peptides were performed by stepwise Edman degradation using a gas-phase automated sequencer (model 473 A; Applied Biosystems). The resulting phenyl-thio-hydantoin amino acids were identified by integrated HPLC.

Synthesis

CCL15 molecules were prepared by F-moc solid-phase peptide synthesis as described elsewhere (42). The purified products were characterized by HPLC, capillary zone electrophoresis, ESMs, and sequence analysis. The net peptide content was determined by amino acid analysis.

Isolation of blood cells

PBMCs, polymorphonuclear neutrophils (PMNs) (4), and thrombocytes (43) were isolated according to established methods. For isolation of PBMCs and PMNs, 30 ml of blood was withdrawn and mixed with 10 ml of hydroxyethyl starch (Plasmasterril; Fresenius). Erythrocytes were sedimented for 45 min at room temperature. The supernatant was centrifuged at 200 × g for 20 min. The cell pellet was resuspended in 20 ml of FCS-free RPMI 1640, layered onto 15 ml of Ficol-Paque (Pharmacia Biotech), and centrifuged at 1000 × g for 15 min at room temperature without a brake. PBMCs were taken from the interface, diluted with two volumes of RPMI 1640, centrifuged at 200 × g for 20 min. The cell pellet was resuspended in RPMI 1640 at a concentration of 1 × 10^7/ml. PMNs were isolated from the cell pellet of the Ficol-Paque gradient. The pellet was resuspended in RPMI 1640 and centrifuged at 200 × g for 20 min. Erythrocytes were lysed by resuspending the pellet in 24 ml of double-distilled water for 30 s and subsequent addition of 8 ml of 3.6% NaCl solution. Cells were centrifuged at 500 × g for 8 min and resuspended in FCS-free RPMI 1640 medium at a concentration of 2 ×10^6/ml.

Platelets were isolated from 10 ml of blood using sodium citrate as anticoagulant. To gain platelet-rich plasma, blood was centrifuged at 800 × g for 5 min. The supernatant was diluted with 10% of citrate-citrate acid-dextrose solution containing 2.94 g/100 ml of trisodium citrate, 2.10 g/100 ml of citric acid, and 2.45 g/100 ml of glucose. Subsequently, the platelet-rich plasma was centrifuged at 2000 × g for 12 min, the supernatant was discarded, and the platelet pellet was gently resuspended in FCS-free RPMI 1640 medium.

Processing of CCL15

Thrombocytes, 2 × 10^9/100 µl of PBMCs, 2 × 10^9/100 µl of PMNs, or supernatant of PMNs were incubated at 37°C with 200 nM CCL15(1–92) for 1 h. Subsequently, the cells were sedimented by centrifugation at 400 × g for 5 min. The supernatant was withdrawn, acidified with 0.1 M acetic acid, desalted using cartridges packed with Source RPC15 material (Pharmacia Biotech), lyophilized, and analyzed by Western blotting. For sequence analysis, the proteins were separated by RP chromatography and fractions were investigated by RIA and MALDI-MS. N-terminal sequencing was performed by automated Edman degradation.

For inhibition experiments, PMNs were preincubated with inhibitors for 30 min at 37°C. Subsequently, 200 nM CCL15(1–92) were added and incubated for an additional 1 h at 37°C. To test the capacity of granulocyte serine proteases to process CCL15, 1 µl of cathepsin G or elastase was incubated with 200 nM CCL15(1–92) for 1 h. Proteolytic products were analyzed by Western blotting and RP chromatography with subsequent MALDI-MS and amino acid sequencing.

Cathepsin G activity

Cathepsin G activity in 100 µl of plasma or HF was determined by the rate of hydrolysis of the 4-nitroanilide substrate Suc-Ala-Ala-Pro-Phe-pNA (Calbiochem). Samples were diluted with 100 µl of buffer to a final concentration of 0.1 M HEPES at pH 7.4 and 0.4 M NaCl containing 1 mM EDTA. Solutions of 0.1 M HEPES at pH 7.4 and 0.4 M NaCl containing 1 mM EDTA were used. Initial application of the cells was performed on the adhesion of mononuclear cells to fibronectin, prestimulation of PBMC with CCL15 molecules was performed for 5 min at 37°C in 5% CO2.

Statistical analysis

Data were compared by the Student t test; p values < 0.05 were considered to be significant.

Results

Processing of plasma CCL15-IR occurs during hemofiltration of blood from patients with renal insufficiency

To differentiate CCL15-IR in blood and HF, a standardized RP chromatographic method was used. The CCL15-IR elution profile from plasma of renal insufficiency patients revealed a tR of 45–50 min with a peak at tR of 50 min, which contains >50% of CCL15-IR material (Fig. 1). In HF, the CCL15-IR elution profile showed a substantial shift with a tR of 33–45 min and two main peaks at tR of 36 and 38 min. The tR of CCL15(27–92), CCL15(24–92), and CCL15(1–92) were 36, 38, and 51 min, respectively (Fig. 1), suggesting that early eluting CCL15-IR material is proteolytically processed CCL15. The CCL15-IR plasma changes in cellular fluorescence were recorded online after the addition of 50 µl of test compounds diluted in wash buffer. Calcium mobilization assays on a CCR1-transfected CHO-K1 cell line or CCR1-transfected HEK-293 cell line were performed as described previously (44).

Chemotaxis assay

CCL15 molecules were tested as potential chemoattractants for monocytes. Chemotaxis was assessed in 48-well chambers (NeuroProbe) by using polynvinylpyrrolidone-free polycarbonate membranes (Nucleopore; Neuro-Probe) with 5-µm pores. All assays were conducted in triplicate, and the migrated cells were counted in five randomly selected fields at 1000-fold magnification after migration for 1 h (monocytes).

Flow chamber experiments

Precoating of glass slides was performed for 2 h with fibronectin from human plasma (Sigma-Aldrich) diluted in PBS (2 µg/ml). Subsequently, the glass slides were assembled as the lower wall of the flow chamber (Circular Parallel Plate Flow Chamber kit; GlycoTech) and were extensively washed with RPMI 1640. The flow chamber was mounted on the stage of an inverted phase-contrast microscope (Zeiss). Cells were perfused at 10^6 cells/ml through the chamber at the desired flow rate generated with an automated syringe pump (Braun). Initial application of the cells was performed with a shear stress of 0.1 dyn/cm^2. After 10 min, the shear stress was increased to 2 dyn/cm^2 for 20 min. Subsequent to each perfusion period, adherent cells were documented by photographs of three independent fields using a gauged grid. To study the influence of CCL15 molecules on the adhesion of mononuclear cells to fibronectin, pretreatment of PBMC with CCL15 molecules was performed for 5 min at 37°C in 5% CO2.
concentration in CRF patients was 1.48 ± 0.32 nM, whereas in HF, the CCL15-IR concentration was 0.33 ± 0.08 nM, which correlates with an average sieving coefficient for CCL15-IR of ~0.2. To exclude hydrolytic degradation of CCL15 during collection of HF, the stability of CCL15(1–92) at pH 2.0 was determined. Under these conditions, CCL15(1–92) was not degraded within the investigation period of 3 days.

CCL15-IR in human HF are CCL15 molecules with N-terminal deletions of 23 (Δ23) and 26 (Δ26) aa. The peptide library was produced from peptide extracts of 10,000 L of human HF. Fractionation of the extracts by cation exchange chromatography and RP-HPLC resulted in 8 different pH pools containing 448 fractions, which were screened for CCL15-IR by a CCL15-specific RIA. CCL15-IR was predominantly detected in the fractions 16 –22 of pH pools 5 and 6. Western blot analysis of the IR fractions revealed CCL15-IR bands with apparent molecular masses of 7–9 kDa.

For characterization of the molecular structure of the identified CCL15-IR in pH pools 5 and 6, the material was purified in four chromatographic steps using a preparative RP-HPLC method, a preparative cation exchange chromatographic method and two subsequent analytical RP-HPLC methods. Purity of the material was determined by ESMS. Using this strategy, CCL15(24 –92), CCL15(24 –91), CCL15(27–92), CCL15(27–91), and CCL15(27–90) were identified by ESMS and Edman degradation (Table I).

PMNs process CCL15(1–92) to Δ23 and Δ26 CCL15 isoforms. Cathepsin G is the principal protease. Purified PBMCs, PMNs, and platelets were incubated with CCL15(1–92) for 1 h. Western blot analysis of cell supernatants suggested a processing of CCL15 by PMNs (Fig. 2A). This was also found in experiments using 125I-CCL15(1–92). Standardized RP chromatography of the PMN supernatant revealed a shift of the radioactive peak to an early retention position, whereas no shift was seen in the chromatography of PBMC and platelet supernatants (results not shown). The acellular, serum-free conditioned medium of PMNs also contained the proteolytic activity for CCL15. To identify the processing sites, PMNs were incubated with CCL15(1–92) and the supernatant was chromatographed in four steps using preparative RP-HPLC and cation exchange chromatography and two analytical RP-HPLC methods. Purity of the material was determined by ESMS. Using this strategy, CCL15(24 –92), CCL15(24 –91), CCL15(27–92), CCL15(27–91), and CCL15(27–90) were identified by ESMS and Edman degradation (Table I).

PMNs process CCL15(1–92) to Δ23 and Δ26 CCL15 isoforms. Cathepsin G is the principal protease.

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Analysis of the molecules was performed by N-terminal Edman sequencing and ESMS. The C-terminal amino acid sequence was deduced from the published amino acid sequence and the MS results. Cysteine residues are not sequenced by Edman degradation (X).

FIGURE 2. Proteolytic processing of synthetic CCL15 by neutrophil cathepsin G. A. 200 nM CCL15(1–92) was incubated with 2 × 10⁹/100 µl of PMNs, 2 × 10⁹/100 µl of PBMCs, or 100 µl of thrombocyte suspension for 1 h at 37°C. Western blot analysis of CCL15-IR revealed proteolytic processing by PMNs. B. Inhibition of CCL15(1–92) processing by PMNs in the presence of different protease inhibitors: lane 2, AEBSF (4 mM); lane 3, EDTA (1 mM); lane 5, leupeptin (10 µM); lane 6, pepstatin (10 µM); lane 7, 1,10-phenanthroline (1 mM); lane 8, E-64 (2.5 µM). Supernatant of PMNs without CCL15 or protease inhibitors was applied to lane 1. Supernatant of PMNs incubated with CCL15(1–92) was applied to lane 10. C, Identification of specific neutrophil serine proteases: CCL15(1–92) was incubated with PMNs with or without specific serine protease inhibitor SLPI (2.5 µM), AEBSF (4 mM), and elastatinal (50 µM). D, Time course of proteolytic processing of CCL15 on coincubation with PMN. Two × 10⁹/100 µl of PMNs were coincubated with 2 µM CCL15(1–92) for the time period indicated. Supernatants were subsequently acidified with 1 M acetic acid and desalted using a RP column (Source RPC15; Pharmacia) and lyophilized. Aliquots of the extracts were separated by SDS-PAGE and CCL15-IR was detected by Western blot analysis. One hundred nanograms of CCL15(1–92) and CCL15(27–92) was run in parallel for comparison. Data from one of three representative experiments are shown.
AEBSF (Pefabloc) and the serine protease inhibitors. The serine protease inhibitors on serine, cysteine, aspartate, and metalloproteases were used. The activity of PMNs was tested. First, class-specific inhibitors active CCL15, a range of protease inhibitors on the CCL15-processing and 11.8%, respectively. CCL15(27–92), CCL15(29–92), and CCL15(25–92) or tra. The percentages of CCL15(22–92), CCL15(24–92), and CCL15(27–92) were identified by RIA, MALDI-MS, and amino acid sequencing. These molecules generated from CCL15(1–92) by proteolytic processing with purified PMNsa and their respective MALDI-mass spectra. The percentages of CCL15(22–92), CCL15(24–92), CCL15(27–92), CCL15(29–92), and CCL15(25–92) or CCL15(24–91) in the PMN supernatant were 41.7, 27.9, 12.4, 6.3, and 11.8%, respectively.

To identify the protease(s) responsible for the conversion of CCL15, a range of protease inhibitors on the CCL15-processing activity of PMNs was tested. First, class-specific inhibitors active on serine, cysteine, aspartate, and metalloproteases were used. The active inhibitors, identified by Western blot analysis, were among the serine protease inhibitors. The serine protease inhibitors AEBSF (Pefabloc) and α1-antitrypsin, as well as the serine- and cysteine protease inhibitor chymostatin and the endoprotease inhibitor α2-macroglobulin, blocked processing of CCL15(1–92). Another serine protease inhibitor, leupeptin, did not inhibit the processing of CCL15. Inhibitors of metalloproteases (EDTA, 1,10-phenanthroline), cysteine proteases (pepstatin), and aspartic proteases (E-64) had no activity in this assay (Fig. 2B). The concentration of the potent serine protease inhibitor leupeptin (0.02 mM) used in this assay is expected to inhibit most serine-like proteases, with the exception of a few enzymes, e.g., cathepsin G and elastase (45, 46), that are known to be relatively resistant to this inhibitor.

This led us to hypothesize that one or more granulocyte serine proteases, elastase, cathepsin G, and proteinase 3 are involved in the processing of CCL15. To test this hypothesis, the specific elastase inhibitor elastatin and secretory leukocyte proteinase inhibitor (SLPI), which is known to inhibit cathepsin G and elastase, but not proteinase 3, were tested (47). Whereas elastatin only partially inhibited the processing of CCL15, SLPI inhibited the processing of CCL15 almost totally (Fig. 2C). These results suggest that granulocytes process full-length CCL15 by cathepsin G and elastase. Therefore, we investigated whether cathepsin G and elastase were able to process full-length CCL15. Western blot analysis of the processed CCL15 products suggests that these enzymes are able to process CCL15. RP-chromatography of the processed molecules with subsequent MALDI-MS of the collected fractions, and amino acid sequencing of the identified molecules revealed that cathepsin G processed synthetic CCL15(1–92) predominantly to CCL15(24–92), CCL15(27–92), and CCL15(29–92), whereas elastase produces CCL15(22–92) (Table III). To analyze the time course on the processing of CCL15(1–92), 2 × 10^4/100 µl of PMNs were cocultivated with 2 µM CCL15(1–92). Separation by SDS-PAGE revealed that CCL15(1–92) was completely converted to a molecule similar in size to CCL15(27–92) within 15 to 30 min (Fig. 2D).

**Cathepsin G proteolytic activity is not detectable in blood plasma and HF**

For detection of cathepsin G activity in plasma and HF, the substrate Suc-Ala-Ala-Pro-Phe-pNA was used. This substrate is not hydrolyzed by leukocyte elastase (48). The absorbance of hydrolytic substrate products at 405 nm was suppressed in plasma, compared with the control medium, by ~15%. In plasma of healthy donors and in plasma and HF of CRF patients, no intrinsic cathepsin G activity was detectable. Plasma compounds significantly inhibited the activity of 1 mU of cathepsin G added to 100 µl of plasma, whereas 100 µl of HF did not contain the capacity to inhibit this proteolytic activity (Fig. 3A). In experiments with 100 µl of HF, 0.062 mU of cathepsin G displayed residual proteolytic activity, whereas 100 µl of plasma totally inhibited this activity. In incubation experiments, HF revealed no proteolytic activity for CCL15(1–92) (Fig. 3B).

**Compared to CCL15(1–92), Δ23 and Δ26 CCL15 isoforms reveal increased calcium flux-inducing activity, chemotactic activity, and adhesiveness to fibronectin**

The calcium flux-inducing activity of CCL15(1–92), CCL15(24–92), and CCL15(27–92) were tested on the CHO-K1 cell line expressing CCR1, monocytes, and THP-1 cells. On CHO-K1 cells, the analysis revealed that N-terminal processing of CCL15(1–

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Peptide</th>
<th>Determined N-Terminal Sequence</th>
<th>Detected M_r MALDI-MS</th>
<th>Theoretical Average M_r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastase</td>
<td>CCL15(22–92)</td>
<td>VLNShFAADAXX</td>
<td>7764.5</td>
<td>7767.1</td>
</tr>
<tr>
<td></td>
<td>CCL15(24–92)</td>
<td>NSHFHAADAXX</td>
<td>7556.6</td>
<td>7554.8</td>
</tr>
<tr>
<td></td>
<td>CCL15(27–92)</td>
<td>HFAADXTSYI</td>
<td>7207.1</td>
<td>7206.6</td>
</tr>
<tr>
<td>CCL15(29–92)</td>
<td>AADX</td>
<td>6921.1</td>
<td>6922.1</td>
<td></td>
</tr>
</tbody>
</table>

The N-terminus, determined by Edman sequencing, and the M_r, determined by MALDI-MS, are presented.
Proteolytic activity of cathepsin G in plasma of healthy donors and in plasma and HF of CRF patients with substrate Suc-Ala-Ala-Pro-Phe-pNA. Regulation of cathepsin G activity by protease inhibitors was estimated by addition of 1 nM of purified enzyme to plasma and HF. Proteolytic activity was detected after 3 h of incubation in three different plasma or HF samples.

FIGURE 3. Proteolytic activity of cathepsin G in plasma and HF. A. Detection of proteolytic activity of cathepsin G in plasma of healthy donors and in plasma and HF of CRF patients with substrate Suc-Ala-Ala-Pro-Phe-pNA. Regulation of cathepsin G activity by protease inhibitors was estimated by addition of 1 nM of purified enzyme to plasma and HF. Proteolytic activity was detected after 3 h of incubation in three different plasma or HF samples. B: Proteolytic activity for CCL15 processing in HF. One microgram of CCL15(1–92) was applied to 1 ml of HF. After 0 min, 30 min, 60 min, and 120 min × 100 μl of HF was withdrawn, acidified to pH 2.0, and chromatographed with the standardized RP method.

Discussion

The present study demonstrates an efficient processing of the chemokine CCL15 by neutrophils and consequences for the activation of adhesive properties of PBMCs. Processing of CCL15 was induced in two different models: 1) by incubation of CCL15 with isolated PMNs and 2) by hemofiltration of blood from renal insufficiency patients, a treatment in which substantial activation of PMNs and monocytes by interaction with the filter membrane has been described previously (49, 50). Proteolytic processing of plasma CCL15 during hemofiltration is suggested by different compositions of CCL15-IR material in plasma and HF of CRF patients. The compositions were analyzed by the use of a highly sensitive and reproducible two-dimensional method, which links chromatographic separation of CCL15-IR molecules and their IR. This method was essential for the study since it allowed us to differentiate CCL15 isoforms in 250 μl of plasma containing ~300 fmol of CCL15-IR. Such a level of sensitivity for analysis of a molecule and its isoforms in the complex mixture of plasma molecules has not been achieved with other methods until now. Using this method, a significant shift of CCL15-IR retention position on the chromatogram was observed (49, 50). ELISA measurements showed that CCL15-IR peaks in HF. The shift of CCL15-IR was not attributable to accumulation of CCL15-IR with early tR and retention of CCL15 with late tR in blood, since under these supposed conditions the high concentrations of CCL15-IR peaks in HF. The shift of CCL15-IR tR was not attributable to accumulation of CCL15-IR with early tR and retention of CCL15 with late tR in blood plasma. In contrast, isolated CCL15-IR materials from HF were always identified as CCL15 molecules by amino acid sequencing.
and mass spectrometric analysis, indicating that CCL15-IR in HF and blood represents CCL15 molecules. A sieving coefficient of 0.2 for CCL15-IR does not completely exclude an entire retention of the CCL15-IR peak at $t_R$ of 50 min, but a sieving coefficient of 0.0 for proteins and peptides was rarely found (51–53), indicating that this peak also represents CCL15.

Proteolytic degradation of CCL15-IR in HF by bacterial contamination or low pH was excluded by immediate freezing of the samples at $-80^\circ$C and by the evidence of the hydrolytic stability of CCL15(1–92) at pH 2.0. Therefore, we investigated the processing of CCL15(1–92) by isolated PBMCs, PMNs, and platelets. Our results indicate that at least one activity for proteolytic processing of CCL15(1–92) is released by activated PMNs (54). Experiments with proteasome inhibitors indicate conclusively that this activity is constituted by the neutrophil granule serine proteases cathepsin G and elastase. These two proteinases are known to be liberated from PMNs during blood filtration into the plasma (49, 55) and play a central role during inflammation by processing inflammatory modulators (56–61). Cathepsin G was shown to process CCL15(1–92) to CCL15(24–92), CCL15(27–92) and CCL15(29–92) and elastase processes CCL15(1–92) predominantly to CCL15(22–92). Amino acids Leu-23, Phe-26, and Phe-28 at the P1 site indicate a chymotrypsin-like processing, which is common for cathepsin G (62). The amino acid Val-21 at P1 is a common processing site for elastase (63). The predominant N-terminal processing sites Leu-23/Asn-24 and Phe-26/His-27 for the isolated CCL15 molecules from blood filtrate support the hypothesis of a cathepsin G-like processing in the course of hemofiltration.

Processing of CCL15 by PMN resulted in a molecule with a $M_r$ 7442.4, which fits to CCL15(25–92) or CCL15(24–91). Recently Youn et al. (64) described CCL15(25–92), which resulted from proteolytic processing between Asn-24 and Ser-25 during expression in a cabbage looper insect cell line. For this unusual processing site, legumain is a putative protease which has a highly restricted specificity requiring an asparagine at the P1 site (65). Legumain is a lysosomal protease, appears to be expressed in response to stress (66), and may be secreted from cells under some conditions (67), and like cathepsin L it may be active in the pericellular environment (68). Legumain is expressed in various organs (69) and was found in macrophages (70), dendritic cells (71), and in vivo in tumors with high invasive and metastatic potential (66). Under these conditions, legumain may be responsible for processing of CCL15 to the molecule CCL15(25–92) and may than support the immune response or the malignant character of a tumor.

Quantum proteolytic processing (72) of CCL15 in the close proximity of activated neutrophils by millimolar concentrations of cathepsin G and elastase, when released from single azurophil granules of neutrophils before catalysis by pericellular inhibitors, is suggested by two different facts: 1) In HF; neither proteolytic activity for processing of additionally added CCL15 nor intrinsic cathepsin G activity was detected. 2) In blood plasma, no proteolytic activity for processing of additionally added CCL15, no proteolytic products CCL15(24–92) and CCL15(27–92), and no intrinsic cathepsin G activity were detected. Also, described in Materials and Methods. Each panel is representative of at least three independent experiments. The chemotaxis experiments represent mean ± SEM for triplicate wells. E, Adhesion rate of PBMC to plasma fibronectin induced by CCL15(1–92), CCL15(24–92), and CCL15(27–92) at shear stresses of 0.1 and 2.0 dyn/cm$^2$ (*, $p < 0.05$; **, $p < 0.0001$). Values are the mean for at least nine independent determinations.
blood plasma from healthy volunteers and CRF patients shows a high capacity to inhibit the activity of additionally added cathepsin G, suggesting that granulocyte cathepsin G liberated into blood plasma during blood filtration is immediately inactivated by high concentrations of protease inhibitors in plasma (49, 55, 73–75).

A fast rate of activation of CCL15 is also suggested by entire processing of 2 nmol/ml CCL15(1–92) by 2 × 10^7/ml neutrophils within 15–30 min, indicating significantly faster kinetics than for the processing of other chemokines by neutrophils (76). In addition, an increased ratio of neutrophils (2–5 × 10^5/ml) to CCL15-IR concentrations (1.5 pmol/ml) in blood by a factor of 100–300 may result in a much faster processing rate for filtered CCL15, which then occurs within seconds before millimolar concentrations of cathepsin G and elastase are diluted and inhibited by protease inhibitors.

To evaluate the functional relevance of N-terminal processing, CCL15(1–92) and the identified N-terminally truncated CCL15 molecules were synthesized and investigated for their chemotactic, calcium-mobilizing, and adhesive properties. Calcium mobilization assays with monocytes, CCR1-transfected CHO-K1 cells, and THP-1 cells displayed a remarkable increase in agonistic potency of the N-terminal truncated CCL15(24–92) or CCL15(27–92) compared to full-length CCL15. For monocytes, EC50 values for full-length CCL15 are above 1 nM, and decrease below 1 nM for CCL15(24–92) and CCL15(27–92). Chemotaxis assays with the different CCL15 molecules on monocytes do not show such a remarkable difference of their chemotactic potency. CCL15(1–92) induced a maximum chemotaxis activity at 1 nM, whereas CCL15(24–92) and CCL15(27–92) had a potency of 1 nM. The efficacy to induce chemotaxis was ~30% higher for CCL15(24–92) and CCL15(27–92) compared with CCL15(1–92). To assess the relevance of human neutrophil elastase in activation of CCL15, CCL15(1–92) was digested with the protease and subjected to calcium mobilization assays. Our results show that also elastase digestion significantly increases the potency of CCL15 to induce an intracellular calcium flux via CCR1. In contrast, preliminary results indicate that CCL15(22–92), the predominant molecule produced by elastase digestion, in comparison to CCL15(24–92) and CCL15(27–92), has an reduced calcium flux-inducing activity.

The results are consistent with the findings of Lee et al. (6), who compared calcium flux-inducing and chemotactic activity of CCL15(1–92) and eight different N-terminally truncated CCL15 forms on CCR1-transfected human osteosarcoma cells. They found that N-terminally truncated forms CCL15(25–92), CCL15(28–92), and CCL15(29–92) are strong inducers of calcium flux and chemotaxis. In their investigations, CCL15(1–92) and CCL15(30–92) carry at least a 100-fold lower potency to induce calcium flux and CCL15(1–92) a 10-fold lower potency to induce chemotactic migration than CCL15(25–92), CCL15(28–92), and CCL15(29–92). Additionally, for CCL15(30–92), a significantly reduced chemotactic activity was found (6, 77). Thus, the results demonstrate that truncation of CCL15 N-terminally to aa 22–29 results in a significant increase of the biological potency of the molecule.

The findings indicate a dissociation of the dose-response curves for chemotaxis and calcium mobilization for the investigated CCL15 molecules. Whereas CCL15(1–92) induces chemotaxis, reaching a maximum at a concentration of 10 nM, it does not induce an efficient calcium mobilization on monocytes, the monocyte cell line THP-1, or on CCR1-transfected cells. In contrast, CCL15(24–92) and CCL15(27–92) induce maximum chemotaxis at concentrations of 1 nM as well as a robust calcium flux. It is not clarified in detail how chemokine-mediated cell migration is related to the transient rise in intracellular free Ca2+2, which is necessary for the cytoskeletal remodeling events that control cell motility and contractility. Different studies on chemokines suggest that there is no strict correlation of calcium influx and migratory activity (78–81).

Since calcium mobilization is associated with detachment, arrest, and locomotion of leukocytes on vascular endothelium (78), we investigated the influence of the different CCL15 molecules on the adhesion of PBMCs to fibronectin in flow chamber experiments. Fibronectin was chosen as a substrate for the adhesion assays since fibronectin ligands on leukocytes are indispensable for the coordinated interaction of leukocytes with endothelial cells and can be functionally assessed this way in a reliable fashion (17, 82). Compared with controls, CCL15(1–92) only modestly increased the adhesiveness of PBMCs, whereas the N-terminally truncated CCL15(24–92) and CCL15(27–92) significantly increased their adhesiveness to fibronectin. This finding suggests that N-terminally truncated CCL15 molecules activate at least one of the integrins αβ1, αβ2, or αβ3, which are expressed on PBMCs and are ligands for plasma fibronectin (82–88). The chemokines MIP-1α and RANTES, which act also via the chemokine receptors CCR1 and CCR3, are known to activate the integrin αβ2 (VLA-5), suggesting that CCL15 also activates αβ2 (VLA-4), another integrin activated by MIP-1α and RANTES, is a weak ligand for plasma fibronectin due to limited exposure of the VLA-4-specific binding sequence CS-1 (89) excluding this integrin as a candidate for increased adhesiveness to fibronectin.

Our findings lead us to the assumption that cathepsin G and elastase, released from activated PMNs, e.g., during their adhesion to endothelium (90, 91) at sites of tissue trauma, infection, or inflammation, proteolytically activate circulating or endothelium-bound CCL15 and support monocyte infiltration into inflammatory sites. This activation might also support the recruitment of neutrophils to sites of tissue injury, since neutrophils, exposed to IFN-γ and GM-CSF, were found to up-regulate CCR1 and CCR3.

### Table IV. Functional parameters of PBMCs, monocytes, CCR1-transfected CHO-K1 cells, and THP-1 cells for CCL15(1–92), N-terminally truncated CCL15 molecules, and the reference chemokine RANTES

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Monocytes (EC50 ± SEM)</th>
<th>CCR1 (EC50 ± SEM)</th>
<th>THP-1 (EC50 ± SEM)</th>
<th>Maximal Migration in Chemotaxis Assay: Monocytes (μM)</th>
<th>Efficacy of Migration: Monocytes (cell-high power field ± SEM)</th>
<th>Adhesion to Fibronectin: PBMCs (0.1 dyn/cm², 2.0 dyn/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL15(1–92)</td>
<td>≥1000 nM</td>
<td>&gt;1000 nM</td>
<td>&gt;333 nM</td>
<td>10 nM</td>
<td>126 ± 11</td>
<td>106.6 ± 22.6, 126.0 ± 18.2</td>
</tr>
<tr>
<td>CCL15(24–92)</td>
<td>0.84 ± 0.15 nM</td>
<td>4.7 ± 3.0 nM</td>
<td>35.2 ± 13.9 nM</td>
<td>1 nM</td>
<td>166 ± 9</td>
<td>247.9 ± 29.6, 273.1 ± 36.0</td>
</tr>
<tr>
<td>CCL15(27–92)</td>
<td>0.62 ± 0.12 nM</td>
<td>3.4 ± 2.1 nM</td>
<td>17.5 ± 2.4 nM</td>
<td>1 nM</td>
<td>177 ± 8</td>
<td>331.9 ± 64.3, 371.4 ± 87.3</td>
</tr>
<tr>
<td>RANTES</td>
<td>ND</td>
<td>34.9 ± 25.0 nM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*For detection of maximal chemotactic activity, four different concentrations of the CCL15 molecules were tested. Efficacy of migration was detected at concentrations of 10 nM for CCL15(1–92) and 1 nM for CCL15(24–92) and CCL15(27–92). Adhesion of PBMCs to plasma fibronectin is given as a percentage of the control for shear stresses of 0.1 and 2.0 dyn/cm². Values are the mean for at least three independent determinations.*
and increase their receptiveness to CCR1 and CCR3 ligands (2, 92, 93). Processing of CCL15 by neutrophils is locally limited to the close proximity of the cells by immediate inactivation of cathespins G and elastase by blood-derived protease inhibitors. Given that N-terminally truncated CCL15 molecules are not detectable in blood plasma, it might be possible that these molecules immediately bind to and activate monocytes. This might support the development of atherosclerosis, which is a significant cause of morbidity and mortality for dialysis patients (94, 95). Therefore, inhibition of its receptors CCR1 and CCR3 could be an alternative focus for treatment of atherosclerosis in dialysis patients.

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Disclosures
The authors have no financial conflict of interest.

References


