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Proteolytic Activation of Alternative CCR1 Ligands in Inflammation

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Although chemokines CCL3/MIP-1α and CCL5/RANTES are considered to be primary CCR1 ligands in inflammatory responses, alternative CCR1 ligands have also been described. Indeed, four such chemokines, CCL6/C10/MIP-related protein-1, CCL9/MIP-1y/MIP-related protein-2, CCL15/MIP-1α/hemofiltrate CC chemokine-2/leukotakin-1, and CCL23/CKβ8/myeloid progenitor inhibitory factor-1, are unique in possessing a separately encoded N-terminal domain of 16–20 residues and two additional precisely positioned cysteines that form a third disulfide bridge. In vitro, these four chemokines are weak CCR1 agonists, but potency can be increased up to 1000-fold by engineered or expression-associated N-terminal truncations. We examined the ability of proinflammatory proteases, human cell supernatants, or physiological fluids to perform N-terminal truncations of these chemokines and thereby activate their functions. Remarkably, most of the proteases and fluids removed the N-terminal domains from all four chemokines, but were relatively unable to cleave the truncated forms further. The truncated chemokines exhibited up to 1000-fold increases in CCR1-mediated signaling and chemotaxis assays in vitro. In addition, N-terminally truncated CCL15/MIP-16 and CCL23/CKβ8, but not CCL3/MIP-1α or CCL5/RANTES, were detected at relatively high levels in synovial fluids from rheumatoid arthritis patients. These data suggest that alternative CCR1 ligands are converted into potent chemoattractants by proteases released during inflammatory responses. The Journal of Immunology, 2005, 174: 7341–7351.

Among the family of β chemokines, only four members, CCL6, CCL9, CCL15, and CCL23, possess an extended N-terminal domain upstream of the chemokine body. This domain contains 16–20 aa, including multiple basic and acidic residues, and is encoded by a separate exon (1–3). The N-terminal domains of the two human chemokines CCL15 (MIP-18/hemofiltrate CC chemokine (HCC)4,2/MIP-5/leukotakin-1) and CCL23 (CKβ8/myeloid progenitor inhibitory factor (MIPF)-1/ MIP-3) are nearly identical, whereas those of the two mouse chemokines, CCL6 (C10/MIP-related protein (MRP)-1) and CCL9 (MIP-1y/ MRP-2), are very different from each other and the two human chemokines. Human forms of CCL6 and CCL9 and murine forms of CCL15 and CCL23 have not been described. All four chemokines are relatively weak ligands for CCR1 and contain precisely positioned fifth and sixth cysteine residues that appear to form a third disulfide bond (2, 4). In this report we refer to these four chemokines as the NC6 chemokine subfamily.

Although the functions of the NC6 chemokines in vivo are unknown, several lines of evidence suggest that their N-terminal domains may serve as regulators of chemokine activity. For example, the N-terminal domains seem to be removed by endogenous proteases during chemokine expression or purification. Purification of CCL23/CKβ8 from a baculovirus system in the absence of protease inhibitors yielded not only the full 99-residue chemokine, but also shorter forms with deletions of the N-terminal domain (5). rCCL15/MIP-16 produced via baculovirus was detected as two forms: 12 and 9 kDa (6). Natural (nonrecombinant) CCL9/MIP-1y was also detected as two forms, 10.5 and 9 kDa, in conditioned medium from the murine dendritic cell line XSS2 (7). The expression of CCL15/MIP-16 in yeast (2) and mammalian cells (8) resulted in one form each, but the apparent sizes (7 and 8 kDa, respectively) suggested that truncations had occurred.

Second, NC6 chemokines lacking the N-terminal domains have substantially increased potency. rCCL6/C10 with an engineered deletion of the N-terminal domain was at least 10-fold more potent than recombinant wild-type C10 in chemotaxis assays using murine peritoneal exudate cells or human PBMC (9). rCCL23/CKβ8 with a deletion of the N-terminal domain was found to exhibit >100-fold increased potency in CCR1-mediated calcium mobilization and chemotaxis in human monocytes (5), correlating with increased affinity for CCR1 (10). rCCL15/MIP-18 lacking the N-terminal domain was similarly reported to exhibit 100-fold greater potency on CCR1 transfectants (11). However, not all investigators observed increased potency after removal of the N-terminal domain (12).

We examined the conditions in vivo in which NC6 chemokines might become activated by proteolytic removal of their N-terminal domains. Although little is known about NC6 chemokine biology in vivo, the use of CCR1 by these chemokines suggests that they might function in local inflammatory responses to foreign entities. Initial detection of foreign entities by resident tissue macrophages, dendritic cells, or mast cells results in, among other events, rapid secretion of the proinflammatory chemokines CCL3/MIP-1α, CCL5/RANTES, CCL11/eotaxin, and the MCPs (CCL2, -7, -8, and -13). These chemokines enter nearby blood vessels and, via chemokine receptors CCR1, -2, -3, and -5, recruit/activate effector leukocytes including neutrophils, eosinophils, and monocyte/macrophages. Mast cells and infiltrating neutrophils release high levels...
FIGURE 1. Proinflammatory proteases and fluids digest and activate the human NC6 chemokine CCL15/MIP-18. CCL15/MIP-18 was incubated for 1 or 18 h with recombinant mast cell chymase, purified neutrophil cathepsin G, purified neutrophil elastase, activated neutrophil-conditioned medium from two donors (PMN sup 1 and PMN sup 2), synovial fluids from three patients with sports-related knee injuries (synovial, synovial 2, and synovial 3) and synovial fluids from 10 patients with rheumatoid arthritis (RA1 to RA10). A, Analysis by SDS-PAGE. Portions of the digests were subjected to SDS-PAGE, then the gels (left and right panels) or a polyvinylidene difluoride filter after electroblotting (middle panel) was stained with Coomassie Blue. Digests lacking chemokine (PMN sup alone and synovial alone; same dilutions as the digests containing chemokine) and rCCL15/H9004 were shown for comparison. B, N-terminal sequence analysis. N-terminal sequences of some of the truncated CCL15/MIP-18 forms were determined by N-terminal Edman
of proteases that degrade extracellular matrix components and initiate antimicrobial events (13). Thus, if an NC6 chemokine is present at the site of inflammation, these proteases might be envisioned to remove the chemokine’s regulatory N-terminal domain.

In this study we examined whether inflammatory fluids or purified proteases released from activated mast cells and neutrophils remove the N-terminal domains from the NC6 chemokines and, in so doing, enhance their function. Each of the four NC6 chemokines was exposed in vitro to recombinant mast cell chymase, purified neutrophil cathepsin G or elastase, activated neutrophil-conditioned medium, or synovial fluids from highly inflamed knee joints. The results show clearly that such conditions result in partial or complete removal of the inhibitory N-terminal domain and activation of CCR1-mediated calcium mobilization and cell migration in vitro. In addition, synovial fluids from rheumatoid arthritis patients and subjects with knee injuries were found to contain N-terminally truncated CCL15/MIP-1α and CCL23/CK8α at concentrations more than sufficient to activate a biological response. In contrast, the traditional CCR1 ligands, CCL3/MIP-1α and CCL5/RANTES, were wholly or partially inactivated by the proteases and fluids in vitro. Moreover, CCL3/MIP-1α and CCL5/
RANTES were detected in synovial fluids from only a subset of rheumatoid arthritis patients, at concentrations insufficient to activate a biological response. Thus, we postulate that naturally occurring proteolytic processes at inflammatory sites can amplify an immune response by the ready conversion of NC6 chemokines to their activated state (designated NC6\(^\Delta\) in situ).

Materials and Methods

Chemokines, proteases, and cells

Recombinant CCL6/C10, CCL9/MIP-1\(\gamma\), CCL15/MIP-1\(\beta\), CCL15\(\Delta\)24/eukotakin (LKN)-1 (68 aa), CCL23/CK8 (22–120 aa), and CCL23\(\Delta\)24/MIPF-1 (46–120 aa) were purchased from R&D Systems. Puromycin was used for CCL15 and CCL23 is shown at the bottom. The separately encoded N-terminal domains are shown in bold, and the CC motif is boxed. The genomic exon/intron organization of CCL9 has not been determined.

Typically, 10\(\mu\)g of each NC6 chemokine was mixed with 0.1–0.4 \(\mu\)g of each enzyme, 4 \(\mu\)l of synovial fluid, or 20 \(\mu\)l PMN sup in a 40-\(\mu\)l volume reaction containing 100 mM Tris-HCl (pH 7.8) and 10 mM CaCl\(_2\). The mixtures were incubated at 37°C up to 24 h, then frozen for later analysis. For visualization of chemokine cleavage fragments, 8 \(\mu\)l of each digest was mixed with 2 \(\mu\)l of 1 M DTT (Sigma-Aldrich) and 10 \(\mu\)l Tricine sample buffer (Invitrogen Life Technologies), heated to 90°C for 3 min, and subjected to denaturing gel electrophoresis on 10–20% acrylamide Tricine gels. The gels were electroblotted onto Immobilon-P\(^{\circ}\) membranes (Sigma-Aldrich), stained with GelCode Blue and sequenced by the Stanford PAN or University of California Molecular Structure Facility (Davis, CA) facilities.

Calcium mobilization assay

Cells were loaded for 1 h with 2 \(\mu\)M indo-1/AM dye (Molecular Probes) in culture medium, then washed with HBSS and resuspended at 10\(^{7}\)ml in HBSS containing 0.1% BSA. Cytosolic calcium responses were determined using a Photon Technology International fluorometer, with excitation at 350 nm and dual emission at 400 and 490 nm. Alternatively, cells were loaded with 5 \(\mu\)g/ml fluo-4 AM dye (Molecular Probes), then washed and analyzed on a Fluorometric Imaging Plate Reader 384 (Molecular Devices), with 505 nm excitation and 530 nm emission.

Chemotaxis assay

L1.2-CCR1 transfectants, THP-1 cells, human monocytes, and human neutrophils were collected by centrifugation and resuspended in HBSS containing 0.1% BSA. Chemotaxis assays were performed in 96-well Chemotx microplates (NeuroProbe) as follows. Chemokines were added to the lower wells (final volume, 29 \(\mu\)l), and 20 \(\mu\)l of cell suspension (5 \(\times\)10\(^5\) cells/ml) was added to the polycarbonate filter (3-\(\mu\)m pore size for neutrophils, 5 \(\mu\)m for the other three cell types). After incubation at 37°C in the presence of 5% CO\(_2\) for 1 h (neutrophils), 90 min (monocytes), or 2 h (THP-1 and L1.2-CCR1), the filters were removed. Cells that migrated into the lower chamber were quantified using the CyQuant cell proliferation assay kit (Molecular Probes) and analyzed with a Tecxan fluorometer (excitation at 480 nm, emission at 530 nm). Data were analyzed and plotted in arbitrary units of fluorescence using PRISM (GraphPad).

Radiolabeled ligand binding assay

THP-1 cells (3 \(\times\)10\(^5\)) were mixed with dilutions of unlabeled chemokine (final concentration, 1 pm to 1 \(\mu\)M) and 0.025 \(\mu\)Ci/well [\(^{125}\)I]-labeled MIP-1\(\alpha\) (PerkinElmer) in a total volume of 200 \(\mu\)l and agitated at 4°C for 3 h. Levels of cell-bound radioactivity were determined by harvesting the cells on polyethyleneimine-treated GF/B glass filters (PerkinElmer) using a cell harvester (PerkinElmer), washing the filters twice with buffer (25 mM HEPES, 500 mM NaCl, 1 mM CaCl\(_2\), and 5 mM MgCl\(_2\) adjusted to pH 7.1), and measuring the amount of \(^{125}\)I bound to each filter (in cpm) with a TopCount scintillation counter (PerkinElmer).

ELISA

Serial 10-fold dilutions of human synovial fluids were analyzed for levels of CCL3/MIP-1\(\alpha\) and CCL5/RANTES with commercial sandwich ELISA kits (BD Biosciences) according to the manufacturer’s protocol. The fluids were also analyzed for human CCL15/MIP-1\(\beta\) and CCL23/CK8 by sandwich ELISA using Abs from R&D Systems. First, total CCL15/MIP-1 and CCL23/CK8 levels were determined using Ab pairs capable of recognizing both full-length and N-terminally truncated forms of each chemokine: capture mAb MAB363 and detector Ab BAF363 were used for CCL15/MIP-1\(\beta\), whereas capture mAb MAB371 and detector Ab BAF508 were used for CCL23/CK8. To determine whether the CCL15/MIP-1\(\beta\) and CCL23/CK8 detected were full-length, the capture mAbs were replaced with anti-CCL23 mAb clone L105 (a gift from R&D Systems), which is specific for the N-terminal domain that is nearly identical between the two chemokines. As such, this mAb recognizes the full-length forms, but not the N-terminally truncated forms of the two chemokines. Quantitation of the chemokines was determined using standard curves of full-length rCCL15/MIP-1\(\beta\) and CCL23/CK8 (for the full-length chemokine-specific assays) or N-terminally truncated CCL15/24/LNK-1 and CCL23/24/MIPF-1 (for the total chemokine assays). The CCL15/MIP-1\(\beta\) and CCL23/CK8 detected in the fluids were not factors in the functional analyses of the in vitro digests, because the synovial fluids were diluted 10-fold in the digests, and the digests were then diluted at least 100-fold in the functional assays.

Results

rCCL15/MIP-1\(\beta\) was tested for its ability to be cleaved by proteases and physiological fluids associated with inflammation. The chemokine was incubated for 1 or 18 h with recombinant mast cell
chymase, purified neutrophil cathepsin G, purified neutrophil elastase, activated neutrophil-conditioned medium from two donors, or synovial fluid from 10 patients with rheumatoid arthritis or three patients with sports-related knee injuries. SDS-PAGE analysis of the digests indicated that nearly all the proteases and fluids cleaved CCL15/MIP-1, generating truncated forms ∼2–3 kDa smaller than the parental chemokine (Fig. 1A). The truncated forms exhibited electrophoretic mobilities similar to rCCL15/MIP-1 lacking the N-terminal 24 residues (CCL15Δ24/LKN-1; Fig. 1A). N-terminal sequencing of the truncated forms indicated that the proteases and fluids partially or completely removed the CCL15 N-terminal domain, leaving three to 10 residues upstream of the conserved CC motif (Fig. 1B).

To determine whether the truncated forms exhibited enhanced functional activities, the digests were subjected to several functional assays in vitro. First, the digests were analyzed for their ability to induce signaling in CCR1+ cells, as measured by changes in cytosolic calcium levels over time. At 10 nM, full-length CCL15/MIP-1 induced little calcium mobilization in murine L1.2 cells stably expressing human CCR1, whereas rCCL15Δ24/LKN-1 induced a substantially greater response (Fig. 1, C and D). At 10 nM input chemokine, all the digests similarly mobilized a greater amount of calcium than full-length CCL15/MIP-1, in some cases even more than CCL15Δ24/LKN-1 (Fig. 1, C and D). Control digests lacking CCL15/MIP-1 did not induce calcium mobilization, indicating that the signaling activity of the digests derived from the truncated chemokines and not the proteases or fluids themselves (Fig. 1, C and D).

The truncated forms were also analyzed for their chemotactic activity for CCR1-bearing cells in vitro. Human THP-1 cells were used because of their high migratory potential. Full-length CCL15/MIP-1 was a relatively weak chemotactic agent for THP-1 cells (EC50, 50 nM), whereas CCL15Δ24/LKN-1 was much more potent (EC50, 60 pM; Fig. 1E). The digests were similarly potent, exhibiting EC50 values of 50 pM (chymase), 60 pM (synovial fluid), and 250 pM (PMN sup 1; Fig. 1E). Control digests lacking CCL15/MIP-1 did not induce calcium mobilization, indicating that the signaling activity of the digests derived from the truncated chemokines and not the proteases or fluids themselves (Fig. 1, C and D).

Because the proinflammatory proteases and fluids rendered CCL15/MIP-1 even more potent than CCL3/MIP-1α and CCL5/RANTES, we tested the ability of the CCL15/MIP-1 Δg digests to function on human neutrophils, which are responsive to CCL15Δ24/LKN-1, but not to CCL3/MIP-1α or CCL5/RANTES (Fig. 2A). At 30–50 nM, the CCL15/MIP-1 Δg digests induced calcium mobilization in neutrophils, in some cases as much as that induced by CCL15Δ24/LKN-1 (Fig. 2B). Desensitization of the neutrophils with high concentrations of CCL3/MIP-1α impaired the ability of the cells to respond to the CCL15/MIP-1 Δg digests (Fig. 2C), indicating that the cleaved forms of CCL15/MIP-1 Δg signaled through CCR1 in the neutrophils. Moreover, addition of a small-molecule antagonist of CCR1 to the neutrophils 20 s before addition of the digests resulted in complete abrogation of calcium mobilization (data not shown), confirming that the N-terminally truncated forms of CCL15/MIP-1 Δg signaled through CCR1 in the neutrophils.

Because CCL15/MIP-1 Δg was consistently truncated and activated by the proinflammatory proteases and fluids, the other NC6 chemokines, CCL6/C10/MRP-1, CCL9/MIP-1γ/MRP-2, and CCL23/CKβ8/MPIF-1 (Fig. 3), were similarly tested for truncation and activation. The three other NC6 chemokines were truncated by ∼2–4 kDa by the proteases and fluids (Fig. 4A), and N-terminal sequencing of the truncated forms indicated that their N-terminal domains were mostly or completely removed (Fig. 4B). At 10 nM input chemokine, the digests were uniformly active for calcium mobilization in L1.2-CCR1 cells, whereas the undigested parental chemokines were either not active or weakly active (Fig. 4C). Control digests lacking the chemokines did not induce calcium mobilization, as seen before (Fig. 1C). Three-point titrations of selected CCL6/C10 and CCL9/MIP-1γ digests in the L1.2-CCR1 chemotaxis assay indicated that the truncated forms stimulated chemotaxis with greater potency than the full-length chemokines (Fig. 4D). A more extensive titration of the CCL23/CKβ8 digests on THP-1 cells indicated that chymase decreased the EC50 50-fold, from 2.5 nM to 50 pM, whereas PMN sup 1 decreased the EC50 to 150 pM, and synovial fluid decreased the EC50 to 250 pM (Fig. 4E). rCCL23/CKβ lacking the N-terminal 24 residues (CCL23Δ24/MPIF-1) exhibited an EC50 of 90 pM. As before, control digests lacking CCL6, CCL9, and CCL23 did not chemotact the L1.2-CCR1 or THP-1 cells, indicating that the chemotactic activity of the chemokine digests was due to the truncated chemokines and not to the proteases or fluids themselves (Fig. 4D).

To determine whether proteolytic cleavage increased chemokine potency in primary human cells, CCL6/C10 was chosen because, of the four NC6 chemokines, it is the least potent on human monocytes and monocyte-derived immature dendritic cells (our unpublished observations). CCL6/C10, which was unable to induce calcium mobilization in monocytes even at 100 nM, mobilized calcium at that concentration after digestion with chymase, cathepsin G, elastase, or synovial fluid (Fig. 5A). CCL6/C10 also stimulated chemotaxis of monocytes at 200 nM after digestion with chymase, cathepsin G, or elastase (Fig. 5B). Digestion of CCL6/C10 with synovial fluid resulted in a dramatic increase in potency, with the most migration occurring at 2 nM (Fig. 5B). CCL6/C10 digests with chymase, cathepsin G, elastase, synovial fluid, or PMN sup 1 were also more efficacious than full-length CCL6/C10 for calcium mobilization in immature dendritic cells derived from monocytes (Fig. 5C).

To determine whether the increased potency exhibited by the cleaved forms of CCL15/MIP-1 and CCL23/CKβ8 were due to increased CCR1 binding, the cleaved forms were compared with the full-length forms as well as CCL3/MIP-1α in a radiolabeled ligand binding assay (Fig. 6). CCL15Δ24/LKN-1 exhibited a ∼3000-fold increase in binding over full-length CCL15/MIP-1 (IC50, 0.1 vs 360 nM), whereas CCL23Δ24/MPIF-1 exhibited a 20-fold increase in binding over its parent chemokine (IC50, 0.6 vs 12 nM). For comparison, CCL3/MIP-1α exhibited an IC50 of 5 nM. The increased potency for binding CCR1 exhibited by the cleaved forms over the full-length forms of CCL15/MIP-1 and CCL23/CKβ8 correlated well with the increased potency for CCR1-mediated migration (Figs. 1 and 4), indicating that the latter is primarily due to increased levels of receptor binding and not to increased efficiency of ligand-mediated signaling.

To determine whether the chemokine-activating ability of the proinflammatory proteases and fluids was specific for the NC6 chemokines, three non-NC6 chemokines, CCL3/MIP-1α, CCL5/RANTES, and CCL25/TECK, were tested with a subset of the proteases and fluids. CCL3/MIP-1α and CCL5/RANTES were not visibly truncated by the two RA synovial fluids analyzed, whereas chymase initially truncated a portion of these two chemokines by ∼2 kDa (Fig. 7A). However, in a THP-1 chemotaxis assay, each chemokine was at least 10-fold less potent after digestion with either RA fluid or chymase (Fig. 7B). CCL25/TECK, which has
FIGURE 4. Proinflammatory proteases and fluids digest the NC6 chemokines CCL6/C10, CCL9/MIP-1γ, and CCL23/CKβ8. Each chemokine was treated for 15 min, 1 h, or 18 h with the proteases and fluids described in Fig. 1. A–C, CCL6/C10 is shown in the left panel, CCL9/MIP-1γ is shown in the middle panel, and CCL23/CKβ8 is shown in the right panel. A, Analysis by SDS-PAGE. Portions of the digests were subjected to SDS-PAGE, with subsequent Coomassie Blue staining of the gels. Digests lacking chemokine (PMN sup alone and synovial alone; same dilutions as the digests containing chemokine) are shown for comparison. B, N-terminal sequence analysis. N-terminal sequences of some of the truncated forms were determined by N-terminal Edman sequencing after SDS-PAGE and electroblotting. Residues shown in red are encoded by the additional exon (not yet defined for CCL9/MIP-1γ). C, Calcium mobilization assay. Murine L1.2 cells expressing human CCR1 were loaded with a calcium-sensitive fluorescent dye, then treated with a portion of the digests and analyzed for fluorescence over time. rCCL23/CKβ8 and digests lacking chemokine are shown for comparison. The two panels shown for each chemokine are from separate experiments. D and E, Chemotaxis assay. Dilutions of digests were tested for the ability to...
FIGURE 5. Human monocytes and dendritic cells respond to CCL6/C10 after N-terminal truncation by proinflammatory proteases and fluids. A, Calcium mobilization assay. Freshly isolated human monocytes were loaded with a calcium-sensitive fluorescent dye, then exposed to the CCL6/C10 digests at 2 nM and analyzed for fluorescence over time. rCCL15/LKN-1 is shown for comparison. B, Chemotaxis assay. Freshly isolated monocytes were exposed to the CCL6/C10 digests at 2 nM, 20 nM, or 200 nM for 90 min, after which the migrated cells were solubilized and quantified by DNA content. The p values (asterisks) are 0.004 for 2 nM CCL6 vs CCL6 plus synovial, 0.001 for 200 nM CCL6 vs CCL6 plus elastase, and <0.001 for 200 nM CCL6 vs CCL6 plus cathepsin G. C, Calcium mobilization assay. Immature monocyte-derived dendritic cells were loaded with a calcium-sensitive fluorescent dye, then exposed to the CCL6/C10 digests at 25 nM and analyzed for fluorescence over time. rCCL15Δ24/LKN-1 is shown for comparison.

the longest C-terminal tail of the β-chemokines, was rapidly cleaved and inactivated for signaling through CCR9 by all proteases and fluids tested (Fig. 7, A and C).

FIGURE 6. N-terminally truncated forms of NC6 chemokines CCL15/MIP-1α and CCL23/CKβ8 possess enhanced binding to CCR1. Recombinant CCL15/MIP-1α, CCL15Δ24/LKN-1, CCL23/CKβ8, CCL23Δ24/MIPF-1, and CCL3/MIP-1α were analyzed for their ability to compete with radiolabeled CCL3/MIP-1α for binding to THP-1 cells as indicated in Materials and Methods.

To assess whether NC6 chemokines with N-terminal truncations exist in vivo, the panel of 13 synovial fluids was analyzed by sandwich ELISA. For CCL15/MIP-1α, an assay recognizing full-length, but not N-terminally truncated, CCL15/MIP-1α detected protein in none of the 13 synovial fluids; comparison with a standard curve of CCL15/MIP-1α defined the limit of detection as 90 pM (Table I). In contrast, an assay recognizing both full-length and N-terminally truncated CCL15/MIP-1α detected protein in all 13 fluids; comparison with a standard curve of CCL15Δ24/LKN-1 indicated that the synovial fluids contained 130–900 pM (Table I; limit of quantitation, 120 pM). Similarly, an assay recognizing full-length, but not N-terminally truncated, CCL23/CKβ8 detected protein in none of the 13 synovial fluids; comparison with a standard curve of CCL23/CKβ8 defined the limit of detection as 80 pM (Table I). An assay recognizing both full-length and N-terminally truncated CCL23/CKβ8 detected protein in 12 of the 13 fluids. Comparison with a standard curve of CCL23Δ24/MIPF-1 indicated that these 12 synovial fluids contained 400–950 pM (Table I; limit of quantitation, 220 pM). However, the possibility that the synovial fluids contained small amounts of full-length CCL15/MIP-1α (up to 90 pM) and/or CCL23/CKβ8 (up to 80 pM) makes it difficult to quantify precisely the levels of N-terminally truncated CCL15 or CCL23. In contrast, commercial ELISA kits specific for human CCL3/MIP-1α, CCL5/RANTES, and CCL7/MCP-3 detected only low levels (20–120 pM) of chemokine and in only a subset of the synovial fluids analyzed (Table I).

Discussion

In this study we have demonstrated that the N-terminal domain unique to the NC6 chemokines, murine CCL6/C10 and CCL9/MIP-1γ, human CCL15/MIP-1α, and CCL23/CKβ8, is readily removed by proteases associated with human inflammatory responses. Recombinant mast cell chymase, purified neutrophil cathepsin G, purified neutrophil elastase, conditioned medium from activated neutrophils, and synovial fluids from patients with rheumatoid arthritis or sports-related knee injuries all fully or partially removed the N-terminal domain of each of the four NC6 chemokines in vitro. The site of cleavage varied from three to 14

Figure 6 illustrates the binding of N-terminally truncated chemokines to CCR1. The graph shows the binding signal (in cpm) for different concentrations of chemokines. The x-axis represents the log of chemokine concentration (M), while the y-axis represents the binding signal (cpm). The legend includes various chemokines and their concentrations, such as CCL3/MIP-1α, CCL5/RANTES, and CCL7/MCP-3.

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residues upstream of the dicysteine motif. Each truncated chemokine was relatively resistant to further digestion by the proteases despite the presence of additional potential cleavage sites. Presumably, these additional sites are inaccessible to the proteases.

As a result of proteolytic removal of the N-terminal domain, all four NC6 chemokines became substantially more potent ligands for CCR1, as measured by receptor binding, calcium mobilization, and cell migration in vitro. We have designated the activated state of truncated NC6 chemokines as the NC6* state. Cleavage of CCL15/MIP-1α and CCL5/RANTES digests by chymase or synovial fluid increased chemokine potency in THP-1 cell migration 1000-fold, to an EC50 of 50–60 pM, similar to rCCL15/24/LKN-1, which lacks the N-terminal domain altogether. Cleavage of CCL23/CK8 by chymase increased that chemokine’s potency to 50 pM as well. These potencies are above those of the CCR1 ligands that have been historically better characterized, CCL3/MIP-1α and CCL5/RANTES, which exhibit EC50 values of ~500 pM. Protease incubation with CCL3/MIP-1α and CCL5/RANTES resulted in decreases in functional potency, presumably because neither of these two chemokines possesses an inhibitory N-terminal domain. The N-terminally truncated (NC6*) forms of CCL15/MIP-1α and CCL23/CK8 are the most potent CCR1 ligands known.

Due to its potency, the NC6* form of CCL15/MIP-1α was functional on human neutrophils, which are poorly responsive to CCL3/MIP-1α (14, 15) or CCL5/RANTES (14) in vitro. The specificity of CCR1 use on the neutrophils was confirmed by pretreating the cells with a CCR1-specific, small-molecule antagonist or with high concentrations of CCL3/MIP-1α. Although CCL3/MIP-1α did not induce calcium mobilization on its own, it partially or completely desensitized the cells to subsequent exposure to the NC6* forms of CCL15/MIP-1α. CCL3/MIP-1α also signals through CCR5, but neutrophils do not express CCR5. Interestingly, neutrophils from half

FIGURE 7. Proinflammatory proteases and fluids do not activate the non-NC6 chemokines CCL3/MIP-1α, CCL5/RANTES, and CCL25/TECK. Each chemokine was treated for varying times with selected proteases and fluids as described in Fig. 1. A, Analysis by SDS-PAGE. Portions of the digests were subjected to SDS-PAGE, with subsequent Coomassie Blue staining of the gels. B, Chemotaxis assay. THP-1 cells were exposed to titrations of the CCL3/MIP-1α and CCL5/RANTES digests for 2 h, after which the migrated cells were solubilized and quantified by DNA content. C, Calcium mobilization assay. Molt4 cells were loaded with a calcium-sensitive fluorescent dye, then exposed to the CCL25/TECK digests at 1 nM and analyzed for fluorescence over time.
(four of eight) of the donors tested exhibited markedly reduced responses to CCL15Δ24/LKN-1, although responses to CXCL8/IL-8 were nearly uniform for all donors. The explanation for this variation in CCL15Δ24/LKN-1 responsiveness is currently being studied.

Because digest mixtures, not purified cleavage fragments, were applied to the cells in our studies, it is possible that the proteases altered the cells, rendering them more responsive to the chemokines. However, because calcium mobilization was observed within 5 s after addition of the digests, any such modification of the cells would have had to occur very rapidly. Moreover, because recombinant chemokines CCL15Δ24 and CCL23Δ24, lacking N-terminal domains, exhibited similar potencies in the absence of proteases, it is unlikely that protease-mediated cellular changes contributed substantially to the results.

Proteases have been reported to both activate and inactivate chemokine function. Chemokine activation after proteolytic removal of N-terminal residues has been described for two other CCR1 ligands, CCL14/HCC-1 (16, 17) and the MIP-1α isofrom LD78β (18), as well as for CXC chemokines CXCL1–3, -5, and -8 (19–24). However, none of these chemokines possesses separately encoded, extended N-terminal domains, and all truncations were relatively small (<10 aa residues removed). Interestingly, the CXCR2 ligand CXC7/neutrophil-activating protein-2 is generated by proteolytic removal of the N-terminal 11 aa of β-thromboglobulin (25), which itself is inactive on CXCR2. A synthetic form of neutrophil-activating protein-2 containing five additional N-terminal residues was shown to be inactive in vitro due to folding of the extended N terminus over the glutamic acid-leucine-arginine region near the dicysteine motif (26).

Not surprisingly, proteolytic removal of N-terminal residues has also been shown to inactivate CC and CXC chemokines (27–38). In many cases, these truncated chemokines were still able to bind their receptors and, as a result, functioned as antagonists in chemotaxis assays in vitro (27, 30–34, 36) and exhibited anti-inflammatory properties in vivo (27, 32).

It is interesting that in the absence of exogenously added chemokines, certain proinflammatory proteases have been shown to induce leukocyte chemotaxis in vitro and in vivo. Injection of human chymase or trypsin into guinea pig skin or mouse peritoneal cavity resulted in substantial neutrophil and eosinophil recruitment within 3 h (39, 40). Injection of human chymase into mouse ear also resulted in polymorphonuclear cell recruitment (41, 42). Moreover, a chymase inhibitor significantly impaired cell recruitment in several experimentally induced and natural dermitis models, indicating that chymase plays a role in dermal inflammatory reactions (41–44). These in vivo studies indicate that either chymase activates a resident, dormant chemokine, or that chymase itself is chemotactic. Although chymase was not found to be chemotactic in our study, two groups reported that this enzyme was chemotactic for human leukocytes in vitro (41, 42, 45). However, enzymatic activity was required for chemoattraction in each of these studies, raising the possibility that the actual chemoattractant might have been a chymase-derived cleavage fragment of an inactive chemokine secreted by the cells. Chymase can cleave endothelins, producing 21- and 31-residue vasoactive peptides that are chemotactic for human monocytes, neutrophils (46), and macrophages (47). With regard to other proteases, neutrophil protease cathepsin G has also been shown to be chemotactic for human neutrophils, monocytes, and macrophages in a manner likewise dependent on enzyme activity (48, 49). Injection of cathepsin G into mouse skin resulted in neutrophil and macrophage recruitment (49). In addition, CD13/endopeptidase N, an ectoenzyme and T cell chemoattractant (50), was observed at elevated levels on alveolar macrophages in two separate rat models of airway T cell inflammation (50, 51). CD13 was also observed at elevated levels on synovial fibroblasts and in synovial fluid from patients with rheumatoid arthritis (52).

Our demonstration that NC6 chemokines are activated to NC6* states by proinflammatory proteases in vitro raises the distinct possibility that this type of regulation also occurs in vivo. In mice, CCL9/MIP-1γ mRNA is expressed constitutively in a wide variety of tissues in the absence of exogenous stimuli and is also expressed in murine myeloid cell lines (53–55), in contrast to CCL3/MIP-1α mRNA, which is expressed only in select tissues and only after the addition of exogenous stimuli such as LPS (53). CCL9/MIP-1γ protein has also been detected in multiple tissues (53) and in a Langerhans cell line (7), and can reach 1 μg/ml (90 nM) in normal murine serum (53). CCL6/C10 mRNA is expressed in normal peritoneal cells (56), and full-length CCL6/C10 protein is expressed in normal lung macrophages, smooth muscle cells, and fibroblasts (57). The constitutive expression of CCL9/MIP-1γ and CCL6/C10 suggests that NC6 chemokines might be present during the very first steps of inflammation, when mast cells or neutrophils degranulate. In fact, the full-length NC6 chemokines could serve as inhibitors of CCR1+C cell migration in the absence of inflammation, although we observed that full-length NC6 chemokines possess weak CCR1 binding affinity in vitro. When an inflammatory event is triggered, released proteases might activate CCL6/C10 or CCL9/MIP-1γ, setting up an immediate gradient of an extremely potent CCR1 ligand. However, no studies have been performed to
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Determine whether this phenomenon occurs in vivo. In the only published study of CCL9/MIP-1y function in vivo, the chemokine was implicated in CD11b+ dendritic cell recruitment to murine Peyers patches (58). In vivo studies of the function of CCL6/C10 focus on mRNA and protein induction in murine inflammation models; in some models, the induction occurs early and is required for leukocyte recruitment (57, 59, 60), whereas in other models, CCL6/C10 is induced late and is required for resolution of inflammation (56, 61). In addition, in these models only the full-length chemokine was detected, because the polyclonal anti-C10 antiserum used did not recognize N-terminally truncated forms (9), presumably due to the high density of charged amino acids in the N-terminal domain.

In humans, no studies have been published that demonstrate CCL15/MIP-16 or CCL23/CX3CR1 protein expression in vivo. We now show that both CCL15/MIP-16 and CCL23/CX3CR1 are present in nearly all synovial fluid samples studied. (The CCL15/MIP-16 and CCL23/CX3CR1 present in the fluids were not factors in the functional analyses of the in vitro digests, because the synovial fluids were diluted 10-fold in the digests, and then the digests were diluted at least 100-fold in the functional assays.) Using Ab pairs that can discriminate between full-length and N-terminally truncated chemokines, we observed that most (if not all) of each chemokine is N-terminally truncated and present at concentrations (150–950 pM) well above those required to elicit chemotaxis of CCR1-bearing cells in vitro. In contrast, concentrations of CCL3/MIP-1a and CCL5/RANTES in the fluids were always <120 pM and often below 20 pM, far below the concentration required for chemotaxis in vitro. The presence of substantially more CCL15/MIP-16 and CCL23/CX3CR1 than CCL3/MIP-1a and CCL5/RANTES in the synovial fluids is consistent with our observation that the former two chemokines are activated by the synovial fluids in vitro, whereas the latter two chemokines are inactivated. These results raise the possibility that in inflamed synovial tissue, where CCR1 is thought to play a critical role in leukocyte infiltration (62), N-terminally truncated CCL15/MIP-16 and CCL23/CX3CR1, rather than CCL3/MIP-1a or CCL5/RANTES, actively recruit CCR1-bearing monocytes and neutrophils. In pilot experiments in vitro, we have observed that the synovial fluids procoagulant THP-1 cells in a CCR1-dependent manner: small-molecule antagonists specific for CCR1, but not those specific for CCR2 or CXCR4, completely blocked chemotaxis. However, Abs specific for CCL3/MIP-1a, CCL5/RANTES, CCL15/MIP-16, and CCL23/CX3CR1 were not able to block chemotaxis of THP-1 cells to the synovial fluids, even when all four Abs were pooled. This result suggests that the Abs are not neutralizing for the natural (i.e., nonrecombinant) forms of the NC6 chemokines.

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Disclosures

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