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Tissue proteases convert CCL23 into potent monocyte chemoattractants in chronic rhinosinusitis

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To the Editor

Although chronic rhinosinusitis with nasal polyps (CRSwNP) is known to be characterized by eosinophilia, other inflammatory cells including macrophages and dendritic cells (DCs) are also widely accepted to have a pathogenic role in CRSwNP.[1-4] However the mechanisms leading to accumulation of macrophages and DCs have yet to be identified. We recently found that chemokine CCL23 is produced from eosinophils, upregulated in eosinophilic CRSwNP and that mRNA expression of CCL23 positively correlates with its receptor CCR1 in NPs.[5] However, mature CCL23 (residues 22-120; referred to as CCL23) shows low affinity for CCR1, although it has chemotactic activity for monocytes and DCs at high concentration.[6, 7] Therefore an elevation of CCL23 protein alone cannot fully explain the positive correlation between CCL23 and CCR1 in NPs. Recent publications suggest that CCL23 can be post-translationally modified by several proteases and truncated forms of CCL23 (residues 46-120 and 47-120; referred to as CCL23(46-120) and CCL23(47-120)) show 10-100 times higher CCR1 binding activity than CCL23.[8, 9] However, whether post-

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translational modification of CCL23 can occur in NPs and whether this modification generates active forms of CCL23 has not been investigated.

To determine whether CCL23 protein can be post-translationally modified in NPs, we incubated recombinant CCL23 with NP extracts and detected CCL23 protein by Western blotting. Detailed methods are given in the Online Repository. We found that CCL23 was cleaved by NP extracts (Fig. 1A). Importantly, a cleaved CCL23 was detected even after only 1 hour exposure to NP extracts and the molecular weight was similar to the known active form, CCL23(46-120) (Fig. 1AB). We also found that truncation of CCL23 by NP extracts was inhibited by a protease inhibitor cocktail (PIC), Nafamostat, Leupeptin and TLCK but not by various other inhibitors (Fig. 1C and E1A), suggesting that truncation of CCL23 in NPs occurs in a trypsin-like serine protease dependent manner.

We next examined the potential role of proteases released from inflammatory cells on the truncation of CCL23 in NPs. We found that CCL23 was truncated by supernatants from mast cells and neutrophils but not by supernatants from eosinophils or macrophages (Fig. 1D). Since trypsin-like serine proteases have a major role in the truncation of CCL23 in NPs and inhibitors of neutrophil elastase (SSR69071) and MMPs (Marimastat), that are known to cleave CCL23,[8,9] did not block the truncation (Fig. 1C), we focused on a mast cell trypsin-like serine protease, tryptase. We found that CCL23 was truncated by tryptase and chymase (a positive control)[8], and tryptase-dependent truncation was inhibited by TLCK (Fig. 1E and E1B). This suggests that mast cell tryptase may be involved in the truncation of CCL23 in NPs.

To examine whether NP-treated CCL23 manifests chemotactic activity, we initially used peripheral blood mononuclear cells to determine the time-course of migration (Fig. E2). We then utilized THP-1 cells, a human monocytic cell line that expresses CCR1, to desire a more sensitive assay. We found that NP-treated CCL23 displayed typical bell-shaped migration responses, and that 1 nM NP-treated CCL23 had the highest migration activity on THP-1 cells although the same concentration of NP extract without CCL23 and BSA-treated CCL23 did not show a response (Fig. 1F). We also found that 1 hour NP treated CCL23 had significantly higher chemotactic activity for THP-1 than mature CCL23 (Fig. E3A), indicating that migration activity was controlled by truncation of CCL23. Importantly, a CCR1antagonist, CCX8960, completely blocked NP-treated CCL23-dependent but not CCL2 (a CCR2 ligand)-dependent migration of THP-1 cells indicating that truncation of CCL23 by NP extracts results in increased CCR1-dependent migration of THP-1 cells (Fig. E3B).

Since previously known active truncated forms of CCL23 are formed by N-terminal truncations, we initially assessed N-terminal protein sequences. The N-terminal sequence of the truncated form was Phe-His-Ala-Thr-Ser, indicating that the N-terminal cleavage site of CCL23 by NP extracts was between residue 46 and 47 (Fig. 2AD). We also utilized MALDI-TOF mass spectrometry to assess the truncation products. We found that two N-terminus truncation products with spectral peaks having *m/z* value of 2,933 and 2,449 were time-dependently increased by NP extracts (Fig. 2BC) suggesting that a 4 amino acid truncation also occurred at the N-terminus. Since we could not detect migration activity of

THP-1 cells induced by CCL23(22-46) (data not shown), we focused on C-terminal end truncation products. We detected three peaks (*m/z* 8,414, 8,170 and 8,014), representing CCL23(47-120), CCL23(47-118) and CCL23(47-117), appearing after 1 hour incubation with NP extracts, and CCL23(47-117) was a major product at 6 hour incubation (Fig. 2BC). We also detected CCL23(22-117) and CCL23(26-117). Synthesis of all truncation products by NP extracts was blocked by the serine protease inhibitor Nafamostat (Fig. 2BC and E4). These results suggest that truncation of CCL23 in NPs can occur from both N- and C-terminus by serine proteases and that the novel truncated form, CCL23(47-117), may be the major active product in NPs (Fig. 2D).

Although CCL23(46-120) and CCL23(47-120) have potent chemoattractant activity,[8, 9] there are no reports of the existence and activity of CCL23(47-117). Therefore we synthesized recombinant CCL23(47-117) (Fig. E5) and examined its activity using THP-1 cells. We found that 1 nM CCL23(47-117) had the highest migration activity upon THP-1 cells and the activity of CCL23(47-117) was significantly higher than the known active metabolite, CCL23(46-120) at 0.1 nM and more than 100 fold higher than that of mature CCL23 (Fig. 2E). CCL23(47-117)-dependent migration was completely blocked by CCX8960 (Fig. 2F) suggesting a CCR1 dependent mechanism.

The presence of CCR1 on macrophage and DC subsets had not yet been established in NPs. We found that CCR1 was expressed on both M1 and M2 macrophages (Fig E6). We also found that CCR1 was expressed on myeloid DC type 1 (mDC1) and type 2 (mDC2), that are elevated in NPs[3], but not on plasmacytoid DCs or B cells (negative control cells) in NPs (Fig. E7). This indicates that active CCL23 metabolites may be involved in the recruitment of macrophages and mDCs in NPs.

In conclusion, we report here that CCL23 is post-translationally modified by trypsin-like serine proteases and CCL23(47-117) may be a major active form of CCL23 in NPs. Our findings indicate that the overproduction and cleavage of CCL23 may play important roles in the inflammation in CRSwNP via the recruitment of macrophages and mDCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CRS	Chronic rhinosinusitis
CRSwNP	CRS with nasal polyps
DCs	dendritic cells
mDCs	Myeloid DCs
MMP	matrix metalloprotease
NP	Nasal polyp

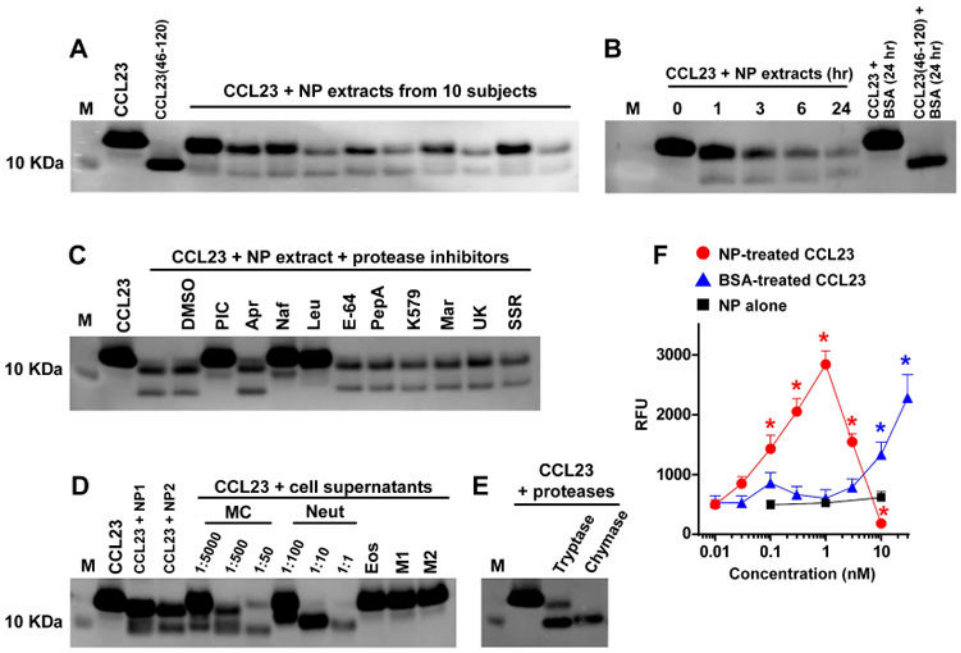
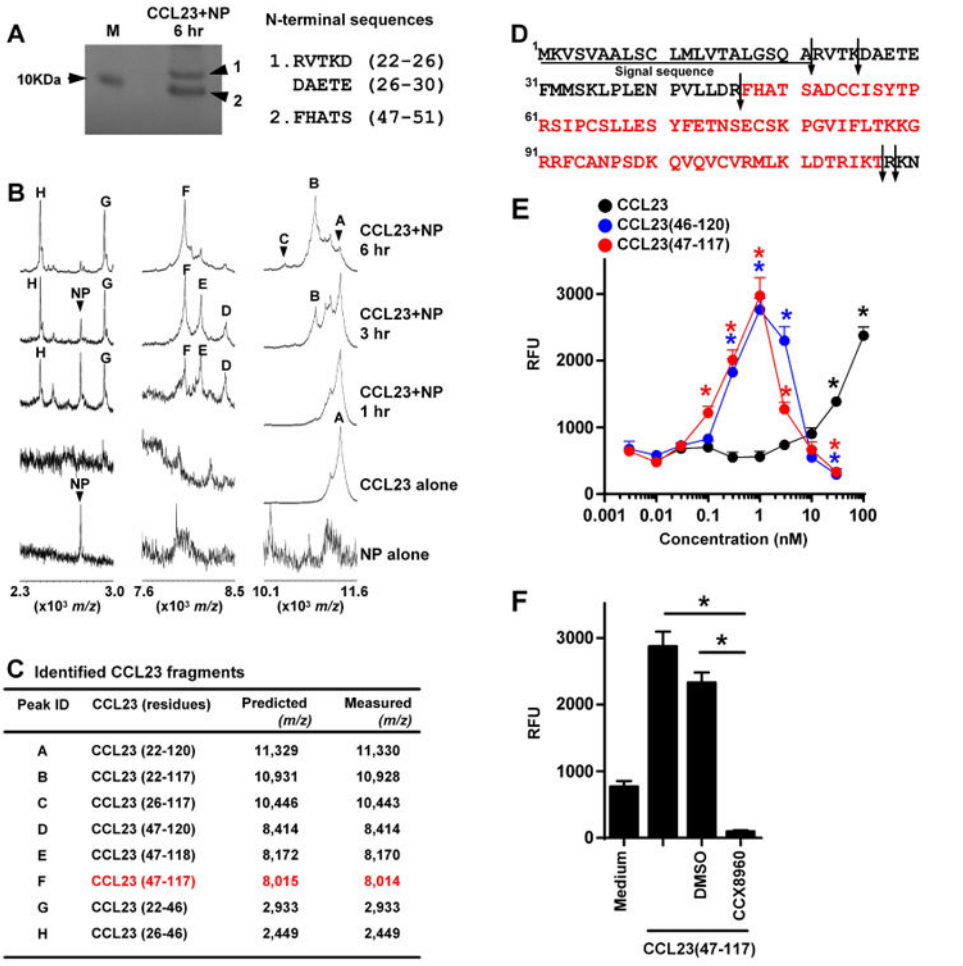


Fig. 1. Post-translational modification by NP trypsin-like serine proteases controls CCL23 activity

Recombinant CCL23 was incubated with 1 mg/ml NP extracts, 1 mg/ml BSA (control), supernatants from activated mast cells (MC), neutrophils (Neut), eosinophils (Eos), M1 macrophages (M1) and M2 macrophages (M2), tryptase βII or skin chymase for 6 (A, C-E) or 0-24 (B) hours. CCL23 was incubated with NP extract in the presence of DMSO, PIC, Aprotinin (Apr), Nafamostat mesylate (Naf), Leupeptin (Leu), E-64, Pepstatin A (PepA,), K579, Marimastat (Mar), UK370106 (UK), SSR69071 (SSR), TLCK or TPCK for 6 hours (C). CCL23, cleaved CCL23 and CCL23(46-120) were detected by western blot using an anti-CCL23 antibody. The results are representative of three separate experiments with separate donors (B-D). THP-1 cells were incubated with NP-treated CCL23 (closed circle), the same concentration of NP extracts without CCL23 (closed square) or BSA-treated CCL23 (closed triangle) for 2 hours using a micro chemotaxis plate (n=4) (F). * $p<0.05$, one-way ANOVA, compared to medium control (A and B). RFU, relative fluorescence units.



Peak ID	CCL23 (residues)	Predicted (m/z)	Measured (m/z)
A	CCL23 (22-120)	11,329	11,330
B	CCL23 (22-117)	10,931	10,928
C	CCL23 (26-117)	10,446	10,443
D	CCL23 (47-120)	8,414	8,414
E	CCL23 (47-118)	8,172	8,170
F	CCL23 (47-117)	8,015	8,014
G	CCL23 (22-46)	2,933	2,933
H	CCL23 (26-46)	2,449	2,449

Fig. 2. CCL23(47-117) is an active CCL23 metabolite in NP extracts

NP extract-treated CCL23 proteins were separated by SDS-PAGE. N-terminal protein sequences of each product were detected using an Edman-based sequencer (A). CCL23 was incubated with NP extracts for 1-6 hours. Truncated products were detected by MALDI-TOF MS (B). Summary table of identified truncated products assigned by comparison of measured with predicted *m/z* by MALDI-TOF MS and N-terminal sequencing (C). Protein sequence of CCL23. Arrows indicate identified cleavage sites. Red color indicates the sequence of a potential major active metabolite, CCL23(47-117), in NPs (D). The results are representative of three separate experiments with separate donors (A-C). THP-1 cells were incubated with CCL23, CCL23(46-120) or CCL23(47-117) for 2 hours using a micro chemotaxis plate (n=4) (E). THP-1 cells were incubated with 1 nM CCL23(47-117) for 2 hours using a micro chemotaxis plate in the presence of 0.02% DMSO (vehicle control) or 100 nM CCX8960 (n=3) (F). * $p<0.05$, one-way ANOVA, compared to medium control (E). RFU, relative fluorescence units.