Cysteine Cathepsins Activate ELR Chemokines and Inactivate Non-ELR Chemokines

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Background: Chemokine function is regulated by proteolytic processing. Results: Cysteine cathepsins activate signaling by ELR CXC chemokines and terminate signaling by non-ELR chemokines. Conclusion: Cysteine cathepsins process CXC chemokines and promote inflammation by recruitment of CXCR2-expressing cells. Significance: This is the first comprehensive study on the processing of CXC chemokines by cysteine cathepsins.

Cysteine cathepsins are primarily lysosomal proteases involved in general protein turnover, but they also have specific proteolytic functions in antigen presentation and bone remodeling. Cathepsins are most stable at acidic pH, although growing evidence indicates that they have physiologically relevant activity also at neutral pH. Post-translational proteolytic processing of mature chemokines is a key, yet underappreciated, level of chemokine regulation. Although the role of selected serine proteases and matrix metalloproteases in chemokine processing has long been known, little has been reported about the role of cysteine cathepsins. Here we evaluated cleavage of CXC ELR (CXCL1, -2, -3, -5, and -8) and non-ELR (CXCL9–12) chemokines by cysteine cathepsins B, K, L, and S at neutral pH by high resolution Tris-Tricine SDS-PAGE and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Whereas cathepsin B cleaved chemokines especially in the C-terminal region, cathepsins K, L, and S cleaved chemokines at the N terminus with glycosaminoglycans modulating cathepsin processing of chemokines. The functional consequences of the cleavages were determined by Ca²⁺ mobilization and chemotaxis assays. We show that cysteine cathepsins inactivate and in some cases degrade non-ELR CXC chemokines CXCL9–12. In contrast, cathepsins specifically process ELR CXC chemokines CXCL1, -2, -3, -5, and -8 N-terminally to the ELR motif, thereby generating agonist forms. This study suggests that cysteine cathepsins regulate chemokine activity and thereby leukocyte recruitment during protective or pathological inflammation.

Chemokines are a group of small structurally related chemoattractant cytokines that regulate migration of leukocytes in homeostasis and inflammation but also function in other processes such as embryogenesis, angiogenesis, hematopoiesis, tumor growth, and metastasis (1). Their basic structure is conserved and includes a short unstructured N-terminal region and an extended N-loop followed by three β-strands and a C-terminal α-helix. Two disulfide bonds tether the N-loop and the N-terminal residues to the core of the protein. Based on the presence of conserved cysteine (C) residues in the sequence of the N terminus, chemokines are classified as CC, CXC, and CX₃C. CXC chemokines are further divided into ELR and non-ELR chemokines based on the presence or absence of the tripeptide glutamate-leucine-arginine motif before the first cysteine.

Upon secretion from cells, chemokines, which are highly basic proteins, bind to negatively charged glycosaminoglycans (GAGs) on the surface of cells or in the extracellular matrix, thereby creating a haptotactic gradient. However, there seems to be a considerable topological diversity in the location of the binding sites (2). Binding to GAGs not only prevents the diffusion of chemokines away from the site of release but might also protect them against proteolysis (3), although for the matrix metalloproteinasises (MMPs) there is little effect (4, 5).

The abbreviations used are: GAG, glycosaminoglycan; MMP, matrix metalloproteinase; DPPIV, dipeptidylpeptidase IV; Cat, cathepsin; C6S, chondroitin-6-sulfate; rBoc, tertiary butyloxycarbonyl; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Chemokine receptors belong to the G protein-coupled receptor superfamily and their activation has been described as a two-site model. The N-loop of the chemokine (site I) is important for binding affinity and receptor selectivity, whereas the N-terminal region (site II) mediates receptor activation (6).

Post-translational modifications such as specific and limited proteolysis, termed proteolytic processing (7) or deamination, have been established as important mechanisms for regulating chemokine activity (8). Several proteases have been demonstrated as contributing to chemokine processing, in particular MMPs, aminopeptidase/CD13, and the serine proteases thrombin, plasmin, membrane-bound peptidylpeptidase IV/CD26/DPPIV, neutrophil cathepsin G, and elastase (9).

For example, MMPs convert CC agonists into antagonists (10, 11), switch chemokine receptor specificity (12), inactivate CC (13) and CXC chemokines (5, 14), activate CC (15) and CXC chemokines (16), and shed CXCL1/C12/fractalkine (17).

N- and C-terminally truncated forms of both ELR and non-ELR CXC chemokines have been described in the supernatant of cultured leukocytes (8, 18–21). With an intact ELR motif, N-terminal truncations of ELR CXC chemokines result in higher activity in calcium mobilization and chemotaxis assays (16, 18, 20, 22, 23). In contrast, N-terminal processing of non-ELR CXC chemokines decreases their chemotactic activity (4, 24, 25). Even so, fragments without the first two N-terminal amino acid residues retain their angiostatic properties, but this is lost upon further truncations (19). Moderate proteolysis at the C terminus generally does not alter chemokine activity, whereas more extensive truncations can affect GAG binding ability (4, 15). Several proteases have been identified that can generate N-terminally truncated forms of CXCL5, -8, -10, and -11 (4, 9, 16). In contrast, the identity of proteases responsible for the N-terminal truncations of CXCL1–3 beyond the proline residue is still unknown except for a set of MMPs that cleave CXCL2 to generate the (5–73)-form (13, 14).

Despite the well established role of the above mentioned proteases in chemokine processing, only limited information is available for cysteine cathepsins. There are 11 human cysteine cathepsins (Cat), CatB, CatC, CatF, CatH, CatK, CatL, CatO, CatS, CatV, CatW, and CatX, which exhibit considerable redundancy and generally a broad substrate specificity (26).

Cysteine cathepsins are primarily lysosomal enzymes, and because of limited stability at neutral pH they were initially considered ineffective in the extracellular milieu. However, there are considerable differences in the stability of different cathepsins at neutral pH, ranging from a few minutes in the case of CatL to hours for CatS. Furthermore, the stability and activity of cathepsins can be significantly prolonged by binding to various ligands, including substrates and GAGs (26–28). In particular, CatB, CatK, CatL, CatS, and CatX have been linked to extracellular proteolysis in physiological as well as disease conditions (29–31). Because of a potent but relatively short-lived activity at neutral pH, their potential for protease signaling is high (32), suggesting that cysteine cathepsins are likely candidates for chemokine processing. In support of that, CatL has been identified as a CXCL8-converting enzyme in a model of stimulated fibroblasts (33), and cathepsin B has been found to cleave CCL20 (with no effect on activity) and CXCL9–14, but functional analyses were not performed on these CXCL cleavage products (34). More recently, CatS has been suggested to be involved in CXCL1/C12/fractalkine shedding in the spinal cord, which contributes to chronic pain in neuronal cells and arthritis models (35–37), and osteoblast-derived CatX in the degradation of CXCL12 (38).

To test the hypothesis that chemokines are extracellular substrates for cysteine cathepsins, we systematically analyzed the processing of human ELR CXC chemokines CXCL1/GROα, CXCL2/GROβ, CXCL3/GROγ, CXCL5/ENA-78, and CXCL8/IL-8 and non-ELR CXC chemokines CXCL9/MIG, CXCL10/IP-10, CXCL11/I-TAC, and CXCL12/SDF by human cysteine cathepsins CatB, CatK, CatL, and CatS at neutral pH and in the absence or presence of the GAG chondroitin sulfate C/chondroitin-6-sulfate (C6S). We found that cysteine cathepsins CatK, CatL, and CatS remove four or five amino acid residues from the N terminus of CXCL1–3. In addition, cysteine cathepsins generate more potent forms of CXCL5 and -8 and inactivate or degrade non-ELR CXC chemokines CXCL9–12. Collectively, our results suggest that cysteine cathepsins complement other proteases in post-translational modulation of CXC chemokine activity.

**Experimental Procedures**

**Chemokines and Proteases**—All chemokines were synthesized using Boc (tertiary butyloxycarbonyl) solid phase chemistry as described previously (39). Recombinant human cysteine cathepsins were expressed in *Escherichia coli* (CatB (40)) or *Pichia pastoris* (CatK (41), CatL, and CatS (42)). CatB and CatS were activated in 100 mM phosphate buffer, pH 6.0, and CatK and CatL in 100 mM acetate buffer, pH 5.5, all in the presence of 5 mM DTT at room temperature for 5 min.

**Chemokine Processing Assays**—Chemokine processing assays were performed in 50 mM HEPES, pH 7.4, at an enzyme to substrate ratio of 1:20 (w/w), in the case of CatB, CatK, and CatS, and 1:40 (w/w), in the case of CatL, at 37 °C. The final concentration of chemokine in the reaction mixture was 0.1 mg/ml (43). Additional processing reactions were performed at an enzyme to substrate ratio of 1:500 or 1:600 (w/w) or at acidic pH in 100 mM citrate buffer, pH 4.5. To investigate the effect of GAGs on the processing, C6S was added to the reaction mixture at a final concentration of 0.01, 0.05, 0.25, or 1.0 mg/ml. Samples of the reaction mixture were taken after 15, 30, or 60 min. The reaction was terminated by adding E64d, a broad spectrum cysteine cathepsin inhibitor, at a final concentration of 50 μM. Samples were then analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a Voyager-DE STR (Applied Biosystems) in sinapinic acid matrix. To degrade C6S, which interfered with MALDI-TOF MS analysis, hyaluronidase from bovine testis type NS (Sigma) was added to the reaction mixture at a final concentration of 0.1 mg/ml for 1 min after the reaction was terminated with E64d and before the sinapinic acid matrix was added. In addition, spotted samples were washed with 1 μl of dH2O. PeptID software was used to identify the truncated fragments in the MS spectra (43). Reaction mixtures were also analyzed with 15% Tris-Tricine SDS-PAGE, and gels were silver-stained (43).
CXC Chemokine Processing by Cysteine Cathepsins

**Transfected and Isolated Cells**—Human CXCR3- and CXCR2-transfected B300-19 cells (44), kindly provided by B. Moser (Bern, Switzerland), were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μM β-mercaptoethanol, and 1.0 mg/ml G418. Human neutrophil granulocytes were isolated from peripheral blood of healthy volunteers as approved by the Clinical Ethics Review Board of the Republic of Slovenia. Blood was drawn into sodium citrate-treated Vacutainer tubes and layered on Lympholyte®-poly solution (Cedarlane) according to the manufacturer’s instructions.

**Calcium Mobilization**—CXCR2- and CXCR3-transfected B300-19 cells were stained with 2 μM Fluo-4 AM (Molecular Probes) for 30 min at 37 °C. The assay was performed as described previously (4).

**Chemotaxis**—Chemotaxis assays were performed in 96-well chemotaxis chambers of MBA96 series (Neuro Probe) across polycarbonate filters with 5-μm pores for CXCR3-transfected B300-19 cells and 3-μm pores for neutrophil granulocytes. Cells and chemokines were diluted in RPMI supplemented with 1% (w/v) BSA, 20 mM HEPES, and 2 mM L-glutamine. Serial dilutions of a chemokine ranging from 100 to 0.1 nM were prepared in lower chambers, and 0.2 × 10⁶ cells were added to upper chambers. After incubation at 37 °C for 60 min with neutrophil granulocytes and 90–120 min with B300-19 cells, the upper chambers were aspirated and washed twice with dH₂O. Migrated cells in lower wells were transferred to a black 96-well plate and lysed by freeze-thawing, and the total cell numbers were quantified against a standard curve using a CyQuant assay according to the manufacturer’s instructions (Molecular Probes). Chemotactic index was calculated as the ratio of cells migrating in response to chemokines compared with buffer control.

**Results**

**Processing of CXC Chemokines by CatB, CatK, CatL, and CatS at Neutral pH**—Human CXCL1–3, -5, and -8–12 were incubated with activated human CatB, CatK, and CatS at an enzyme to substrate ratio of 1:20 (w/w) and with CatL at an enzyme to substrate ratio of 1:40 (w/w) for 15 min at neutral pH. Analysis by Tris-Tricine SDS-PAGE (Fig. 1), with confirmation and identification of cleavage sites by MALDI-TOF MS (Table 1, Fig. 2, and supplemental Fig. 1), showed that in the absence of C6S, the four cathepsins specifically cleaved these chemokines by processing or degradation. The only exception was CXCL1, which was not processed by CatB. Moreover, the presence of bands of slightly reduced molecular weight indicated that CXCL1–3, -5, and -8 were specifically processed by cathepsins, whereas CXCL9–11 were mostly degraded, as judged on the bands of considerably lower molecular weight. CXCL12 was completely degraded, as no smaller processing products were observed with any of the cathepsins.

In the presence of C6S, the proteolytic profile changed. Chemokine processing by CatB was largely diminished, whereas processing by CatL and CatS was also reduced but to a smaller extent. In contrast, processing of chemokines by CatK was increased in the presence of C6S, in agreement with the known effect of GAGs on the proteolytic activity of CatK on protein substrates. However, the effect of C6S was also chemokine-specific. In the presence of C6S truncated forms of CXCL1–3, and to a smaller extent CXCL10 and -11, were more readily detected by Tris-Tricine SDS-PAGE and confirmed by MALDI-TOF MS than in the absence of C6S. In contrast, there was little effect of C6S on the cleavage of CXCL5, -8, -9, or -12. Similar effects on chemokine processing were also observed in the presence of C4S (results not shown).

With the exception of CXCL5, CatB cleaved all chemokines at the C terminus (Table 1 and Fig. 2). CatK, CatL, and CatS processed chemokines at sites N-terminal to the conserved CXC sequence. The resultant truncated forms of CXCL1–3, and to a smaller extent CXCL10 and -11, were more readily detected by Tris-Tricine SDS-PAGE and confirmed by MALDI-TOF MS than in the absence of C6S. In contrast, there was little effect of C6S on the cleavage of CXCL5, -8, -9, or -12. Similar effects on chemokine processing were also observed in the presence of C4S (results not shown).

In the presence of C6S, the proteolytic profile changed. Chemokine processing by CatB was largely diminished, whereas processing by CatL and CatS was also reduced but to a smaller extent. In contrast, processing of chemokines by CatK was increased in the presence of C6S, in agreement with the known effect of GAGs on the proteolytic activity of CatK on
To evaluate the efficiency of cleavage, we investigated chemokine processing with reduced amounts of proteases. Figs. 3 and 4, which present MALDI-TOF MS spectra for selected cathepsin/chemokine combinations, illustrate that although the processing efficiency was diminished, characteristic chemokine fragments were generated after as little as 15 min of incubation with cathepsins.

Cathepsins are normally functional within the acidic environment of lysosomes, and thus we also investigated the processing of selected chemokines at pH 4.5. MALDI-TOF MS spectra for the processing of CXCL5 by CatK (Fig. 3A) and CXCL10 by CatS (Fig. 3B) are presented. At acidic pH, CatK was found to be a potent protease both in the presence and absence of GAGs, generating even smaller CXCL5 fragments than at neutral pH. In contrast, CatS was a less potent protease at acidic pH, although it generated similar cleavage products as at neutral pH.

The Effect of C6S on Chemokine Processing—To better understand the effect of GAGs on chemokine processing, processing of CXCL1, -2, and -11 was performed at four different C6S concentrations (0.01, 0.05, 0.25, and 1.0 mg/ml, Fig. 5). As visualized by Tris-Tricine SDS-PAGE, the presence of CatL- or CatS-derived proteolytic products of CXCL1 and -2 was attenuated at acidic pH, whereas high C6S concentrations reduced with increasing concentration of C6S. In contrast, the processing of these two chemokines by CatK was increased at low C6S concentrations, whereas high C6S concentrations diminished it. In the case of CXCL11, the effect of C6S was only observed at low C6S concentrations, whereas high C6S concentrations attenuated it. In the case of CXCL11, the effect of C6S was only minor even at the highest concentration used.

Because pH and GAGs considerably modulated chemokine processing by cysteine cathepsins, we evaluated their effects on CXC Chemokine Processing by Cysteine Cathepsins

Fig. 2. Cysteine cathepsin cleavage sites in human CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, CXCL9, CXCL10, and CXCL11.

CXCL1 1-72
1 ASVATLRCQCLOTL...LNSDQS K
K/L/S
CXCL2 1-73
1 APLATLRCQCLOTL...LNKGKS K/S K/L/B
CXCL3 1-73
1 AVSVELRCQCLOTL...LNKGST K/L/S
CXCL5 1-78
1 AGPAAVLRELRCVCLQT...ILDGGNK K/B K/L/S
CXCL8 1-77
1 AVLPRASKELRCQCKT...FLKRAEN K/L/S K/S
CXCL9 1-103
1 TPVRKGRSCSIS...KVLKVRKORS K/S S/K
CXCL10 1-77
1 VPLSRVTCTCISINO...KERSK RP K/L/S S/L B
CXCL11 1-73
1 FPMKFRGRCCLCIPG...I1KVKERN K/S K/L/S
TABLE 1

| Chemokine | m/z [M+H]+ | Measured | Predicted | Predicted activity | Cathepsin |
|-----------|------------|----------|-----------|-------------------|-----------|
| CXCL1-(1–72) | 7751 | 7751 | ↑ Ref. 18, Figs. 6A | *K*, *L*, *S and 7A |
| CXCL1-(5–72) | 7422 | 7422 | ↑ Ref. 18, Figs. 6A | *K*, *L*, *S and 7A |
| CXCL2-(1–73) | 7893 | 7893 | ↑ Ref. 20, Fig. 7B and C | *K*, *L*, *S |
| CXCL3-(6–73) | 7651 | 7649 | | ND |
| CXCL5-(1–78) | 8357 | 8357 | | | |
| CXCL9-(1–90) | 10139 | 10140 | | | |
| CXCL9-(100) | 11525 | 11523 | | | |
| CXCL9-(110) | 12915 | 12913 | | | |
| CXCL9-(120) | 14305 | 14303 | | | |
| CXCL10-(1–75) | 8461 | 8462 | | | |
| CXCL10-(1–77) | 8461 | 8462 | | | |
| CXCL10-(1–79) | 8461 | 8462 | | | |
| CXCL10-(1–81) | 8461 | 8462 | | | |
| CXCL10-(1–83) | 8461 | 8462 | | | |
| CXCL10-(1–85) | 8461 | 8462 | | | |
| CXCL10-(1–87) | 8461 | 8462 | | | |
| CXCL10-(1–89) | 8461 | 8462 | | | |
| CXCL10-(1–91) | 8461 | 8462 | | | |
| CXCL10-(1–93) | 8461 | 8462 | | | |
| CXCL10-(1–95) | 8461 | 8462 | | | |
| CXCL10-(1–97) | 8461 | 8462 | | | |
| CXCL10-(1–99) | 8461 | 8462 | | | |
| CXCL10-(1–101) | 8461 | 8462 | | | |
| CXCL10-(1–103) | 8461 | 8462 | | | |
| CXCL10-(1–105) | 8461 | 8462 | | | |
| CXCL10-(1–107) | 8461 | 8462 | | | |
| CXCL10-(1–109) | 8461 | 8462 | | | |
| CXCL10-(1–111) | 8461 | 8462 | | | |
| CXCL10-(1–113) | 8461 | 8462 | | | |
| CXCL10-(1–115) | 8461 | 8462 | | | |
| CXCL10-(1–117) | 8461 | 8462 | | | |
| CXCL10-(1–119) | 8461 | 8462 | | | |
| CXCL10-(1–121) | 8461 | 8462 | | | |
| CXCL10-(1–123) | 8461 | 8462 | | | |
| CXCL10-(1–125) | 8461 | 8462 | | | |
| CXCL10-(1–127) | 8461 | 8462 | | | |
| CXCL10-(1–129) | 8461 | 8462 | | | |
CXC Chemokine Processing by Cysteine Cathepsins

A  CatK : CXCL5
1:20  pH 7.4

15 min

30 min

60 min

1:500  pH 7.4

no GAG  GAG

1:500  pH 4.5

no GAG  GAG

B  CatS : CXCL10
1:20  pH 7.4

no GAG  GAG

1:20  pH 4.5

no GAG  GAG
the catalytic efficiency of cysteine cathepsins using Z-FR-AMC, a small fluorogenic substrate. C6S prolonged the activity of all tested cysteine cathepsins on small fluorogenic substrates at acidic and neutral pH (data not shown), likely through the structural stabilization of the proteases, in agreement with previous studies using different GAGs (46, 47).

Calcium Mobilization of CXCR2- or CXCR3-transfected B300-19 Cells—The effect of cathepsin processing of chemokines on chemokine receptor activation was evaluated by a calcium mobilization assay, performed on human CXCR2- or CXCR3-transfected B300-19 cells with C6S, full-length or C6S/CatS-processed CXCL2, -5, -9, or -10 (Fig. 6). The two receptors were selected based on their binding of ELR (CXCR2) (48) or non-ELR (CXCR3) (44) chemokines. CatS was selected as a model cathepsin because it generated the same major fragments as CatK and CatL. ELR CXC chemokines CXCL2 and -5 induced detectable calcium influx in CXCR2-transfected cells only after cleavage with CatS. Full-length CXCL2-(1–73) exerted no significant activity at chemokine concentrations of up to 100 nM, whereas C6S/CatS-generated CXCL2-(5–73) showed calcium mobilization activity at concentrations as low as 1 nM (Fig. 6A). Similarly, full-length CXCL5-(1–78) had no activity, whereas C6S/CatS-truncated CXCL5-(9–78) induced calcium influx at concentrations above 10 nM (Fig. 6B). In contrast, Ca$^{2+}$ influx in CXCR3-transfected cells in response to non-ELR chemokine was decreased after cysteine cathepsin processing. Truncated CXCL9-(6–89, 6–90) generated by C6S/CatS had considerably reduced activity (Fig. 6C). Similarly, full-length CXCL10-(1–77) induced a strong calcium influx above 5 nM, whereas truncated CXCL10-(5–77) had no detectable

**FIGURE 3.** Representative MALDI-TOF spectra of chemokine CXCL5 processed by CatK (A) and CXCL10 processed by CatS (B). Cleavage assays were performed at an enzyme to substrate ratio of 1:20 or 1:500 (w/w) (CatK) and 1:40 or 1:600 (w/w) (CatL) in 50 mM HEPES buffer, pH 7.4, in the absence or presence of 0.05 mg/ml GAG C6S for 15 or 30 min. Samples were analyzed by MALDI-TOF MS on a Voyager-DE STR (Applied Biosystems) in sinapinic acid matrix. Processing was efficient, and similar chemokine fragments were generated at high or low cathepsin to chemokine ratios.

**FIGURE 4.** Representative MALDI-TOF spectra of chemokines CXCL1 processed by CatL (A) and CXCL2 processed by CatK (B). Cleavage assays were performed at an enzyme to substrate ratio of 1:20 or 1:500 (w/w) (CatK) and 1:40 or 1:600 (w/w) (CatL) in 50 mM HEPES buffer, pH 7.4, in the absence or presence of 0.05 mg/ml GAG C6S for 15 or 30 min. Samples were analyzed by MALDI-TOF MS on a Voyager-DE STR (Applied Biosystems) in sinapinic acid matrix. Processing was efficient, and similar chemokine fragments were generated at high or low cathepsin to chemokine ratios.
CXC Chemokine Processing by Cysteine Cathepsins

Although the involvement of extracellular cysteine cathepsins in inflammation-associated diseases is well established (49), a comprehensive analysis of their potential as chemokine-processing enzymes has not been published previously. Herein, we show that cysteine cathepsins CatB, CatL, CatS, and CatK rapidly processed CXC ELR (CXCL1–3, -5, and -8) and non-ELR (CXCL9–12) chemokines, with several cathepsins generating the same truncated forms, and we also show that the processing was functionally relevant. The differential processing of ELR and non-ELR chemokines by cysteine cathepsins, generating more active forms of the former and inactivating the latter, suggests that cathepsins are potential regulators of chemokine activities in vivo. Several other proteases have already been found to process CXC chemokines, including plasmin, thrombin, several MMPs, and DPPIV. We found that CatS, CatL, and CatK process several of the chemokines in a unique way, with the most interesting being N-terminal processing of CXCL1, which generates the (5–72)-form, and of CXCL2 and CXCL3, which generates the (5–73)-forms. Notably, MMP1, -9, -12, and -25 also generate the CXCL2-(5–73) form (13, 14). However, other proteases that generate fragments of CXCL1–3 with increased chemotactic activity in the supernatant of cultured monocytes (8, 18, 20) have not been identified.

Although cysteine cathepsins have broad substrate specificity, consistent with their function in lysosomal degradation (28), they specifically generated CXCL1–12. The differential processing of ELR and non-ELR chemokines by cysteine cathepsins, generating more active forms of the former and inactivating the latter, suggests that cathepsins are potential regulators of chemokine activities in vivo. Several other proteases have already been found to process CXC chemokines, including plasmin, thrombin, several MMPs, and DPPIV. We found that CatS, CatL, and CatK process several of the chemokines in a unique way, with the most interesting being N-terminal processing of CXCL1, which generates the (5–72)-form, and of CXCL2 and CXCL3, which generates the (5–73)-forms. Notably, MMP1, -9, -12, and -25 also generate the CXCL2-(5–73) form (13, 14). However, other proteases that generate fragments of CXCL1–3 with increased chemotactic activity in the supernatant of cultured monocytes (8, 18, 20) have not been identified.

Discussion

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Calcium mobilization is increased in response to cysteine cathepsin-processed ELR CXC chemokines and decreased in response to cysteine cathepsin-processed non-ELR chemokines. Calcium mobilization of CXCR2-transfected B300-19 cells is increased in response to CatS-truncated CXCL2-(5–73) (A) and CatS-truncated CXCL5 (B). In contrast, calcium mobilization of CXCR3-transfected B300-19 cells is decreased in response to CXCL9 processed by CatS (C), which generates (6–89, 6–90)-fragments, or in response to CatS-truncated CXCL10-(5–77) (D). Cysteine cathepsins were incubated with chemokines at the enzyme to substrate ratio of 1:20 (w/w) for CatS in 50 mM HEPES buffer, pH 7.4, in the presence of 0.05 mg/ml GAG C6S for 15 min. Calcium concentrations were calculated from relative fluorescence based on calibration with ionomycin and EGTA.

Chemotactic migration is increased in response to cysteine cathepsin-processed ELR CXC chemokines and abolished in response to cysteine cathepsin-processed non-ELR chemokines. Chemotaxis of neutrophil granulocytes is increased in response to CXCL1 processed by CatK, which generates the (5–72)-fragment (A), and in response to CXCL2 processed by CatL (B) or CatS (C), which generates the (5–73)-fragment. In contrast, chemotaxis of CXCR3-transfected B300-19 cells is abolished in response to CXCL10 processed by CatS (D) or CatL (E), which generates the (5–77)-fragment, or in response to CXCL11 processed by CatL (F), which generates the (6–73)-fragment and also degrades CXCL11. Cysteine cathepsins were incubated with chemokines at an enzyme to substrate ratio of 1:20 (w/w) for CatS and 1:40 (w/w) for CatL in 50 mM HEPES buffer, pH 7.4, in the presence of 0.05 mg/ml GAG C6S for 15 min. Chemotaxis was measured across 3-μm pore filters for 60 min with neutrophil granulocytes and across 5-μm pore filters for 90–120 min with CXCR3-transfected B300-19 cells. Migrated cells were quantified by CyQUANT assay, and a chemotactic index was calculated, defined as the ratio of cells migrating in response to stimulus compared with the buffer control.
pH, due to the presence of occluding loop in its structure, and as an endopeptidase at neutral pH (51). The removal of two amino acid residues from the C terminus, which we observed in CXCL8–10, suggests that CatB may function as a carboxydi-peptidase also at neutral pH. Consistent with this observation, CatB processing of chemokines was almost completely abolished in the presence of GAGs, which bind chemokines via basic residues. In vivo chemokines bind to GAGs to form haptotactic gradients and oligomers (52). Similarly, GAGs are shown to facilitate autocatalytic activation of cathepsins (46, 53, 54), stabilize the structure of mature proteases (55), and potentiate their activity (47). We propose that GAGs sterically hinder the C-terminal interaction between CatB and chemokines. In addition, GAG binding was found to delay or even prevent further processing of the intermediates, suggesting that this binding either stabilized the structure of the intermediates and/or prevented secondary cleavages in the C-terminal region. In contrast to the effects of GAGs on Cat B activity, our results show that processing by CatL and CatS in the N-terminal part of chemokines was preserved in the presence of GAGs, suggesting that GAGs are less likely to interact with this region of chemokines. The collagenase activity of CatK depends on its association with GAGs, including C4S (56, 57). Herein, we show a profound effect of C4S and C6S on the activity of CatK, CatL, and CatS on small substrates at neutral and slightly acidic pH, presumably because of the increased cathepsin stability. Although cathepsin activity in the presence of GAGs was increased, the truncated forms of chemokines were generally more abundant, suggesting that GAGs restricted processing. This is different from processing by MMPs, which does not appear to be affected by the presence of GAGs (4, 10, 15).

CatK differed from CatL and CatS, as it was the only cathepsin with increased processing of chemokines CXCL1, -2, -3, -5, and -8 in the presence of GAGs. This seems to be a direct effect of GAGs on CatK proteolytic activity and not just on its structural stability. Similar to the acquisition of the collageneolytic activity by CatK, the increased processing of chemokines may be explained by the formation of the oligomeric CatK-GAG complex. This effect is mediated by both C4S and C6S, unlike the collagenolytic activity of the CatK-GAG oligomers, which is mediated primarily by C4S and to a lesser extent by some other GAGs including C6S (56, 57). However, at higher GAG concentrations, chemokine processing by CatL, CatS, and even CatK was further decreased, suggesting that cathepsin and chemokine molecules become spatially separated by association to different GAG chains. Because both chemokines and cathepsins associate with GAGs in vivo, these observations suggest that the availability of binding sites on GAG molecules can determine the extent of proteolysis.

Another factor that may influence chemokine processing by cathepsins is the pH. Although cysteine cathepsins as lysosomal enzymes have adapted to acidic pH, they remain active also at neutral pH but only for a limited amount of time because of structural instability and oxidative stress. CatS is an exception and, as the most stable cathepsin, remains active under these conditions for several hours (58). The pH in the pericellular and extracellular region of inflamed tissues and also in the tumor microenvironment can be slightly acidic (~pH 6.5), which would improve the structural stability of cysteine cathepsins and thereby prolong their activity (29). The combination of GAGs and decreased pH is therefore of physiological importance for the prolonged activity of cysteine cathepsins.

There is increasing evidence that cathepsins are linked with the physiological processing of chemokines. One such example are the N-terminally truncated forms of CXCL1–3 with increased chemotactic activity for neutrophil granulocytes, which have been isolated from cultured peripheral blood monocytes; however, the enzymes generating them have been only partially identified. DPPIV/CD26 can generate only (3–73)-fragments (8, 59), whereas MMP1, -9, -12, and -25 can generate CXCL2-(5–73) (13, 14). In contrast, MMP12 inactivates CXCL1–3 by cleaving these chemokines at the E↓LR and inactivates all other CXCL chemokines by cleavage C-terminal to the ELR motif (14). The functional consequence of the absence of inactivating cleavages by MMP12 in the Mmp12 knockout mouse is readily apparent in vivo as a highly exaggerated neutrophil influx in collagen-induced arthritis (60). Because CatK, CatL, and CatS can generate truncated CXCL1-(5–72), CXCL2-(5–73), and CXCL3-(5–73, 6–73) forms with increased chemotactic activity, we hypothesized that cathepsins are involved in in vivo processing of these chemokines. Although CatL was previously identified as a protease that processes CXCL8 at the N terminus to generate forms with elevated activity (33), we show here that CatK and CatS can also generate more active CXCL8-(6–77, 8–77) forms cleaving N-terminal to the ELR motif. Similarly, CatK, CatL, CatS, and CatB truncated CXCL5 to generate more active CXCL5-(5–78, 9–78) forms, as confirmed by calcium mobilization assay. All of these chemokines contain the ELR motif and can attract neutrophil granulocytes and promote angiogenesis (8, 61).

The four non-ELR CXC chemokines tested were all inactivated and/or degraded by cathepsins. In the presence of GAGs, CXCL10 was processed to the CXCL10-(5–77) form. We have demonstrated with calcium mobilization and chemotaxis assays that this cleavage inactivates the chemokine; this response is similar to that observed following N-terminal truncation of CXCL11 (4, 19). CD26/DPPIV-truncated CXCL9, CXCL10, and CXCL11, which lack the first two amino acids at the N terminus, are impaired in receptor signaling and lymphocyte chemotaxis but retain angiostatic activity (24). Further processing of the N terminus of CXCL11 was shown to abolish the angiostatic activity (19), which would also apply to CXCL10-(5–77) and CXCL11-(5–73, 6–73) generated by cysteine cathepsins. All ELR CXC chemokines are angiogenic, whereas non-ELR CXCL9, CXCL10, and CXCL11 inhibit angiogenesis. Differential processing of ELR and non-ELR CXC chemokines by cysteine cathepsins suggests different functional consequences (61). Moreover, ELR CXC chemokines and angiostatic non-ELR chemokines are classified as inflammatory chemokines, in which expression is inducible and up-regulated by inflammatory stimuli. In contrast, CXCL12 is a homeostatic chemokine and is expressed constitutively. Although a non-ELR chemokine, CXCL12 is unique here in that it has angiogenic activity; it acts through the CXCR4 receptor, whereas angiostatic CXCL9, CXCL10, and CXCL11 act through CXCR3 (1). Unlike inflammatory chemokines, homeostatic CXCL12
Cysteine cathepsins promote signaling by ELR CXC chemokines and terminate signaling by non-ELR CXC chemokines. Under inflammatory conditions, cysteine cathepsins are secreted extracellularly, where they encounter and process chemokines. The source of cysteine cathepsins can be tissue-resident cells, such as fibroblasts or smooth muscle cells, or/and tissue-infiltrating cells, such as macrophages and neutrophil granulocytes. Our results show that cysteine cathepsins N-terminally truncate ELR CXC chemokines to produce fragments with increased activity, whereas they inactivate or degrade non-ELR chemokines. By chemokine processing, cysteine cathepsins could promote recruitment of neutrophil granulocytes and angiogenesis.

Note Added in Proof—The legend to Fig. 6 was missing in the version of this article that was published as a Paper in Press on April 1, 2015. The correct version including the legend to Fig. 6 is now shown.

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Acknowledgments—We thank Gregor Kosec, Marko Mihelič, Matej Vizovišek, Mojca Prebanda, and Urska Požgan for preparing recombinant cysteine cathepsins.
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