Cathepsin X is secreted by human osteoblasts, digests CXCL-12 and impairs adhesion of hematopoietic stem and progenitor cells to osteoblasts

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ABSTRACT

Background
Hematopoietic stem cells are retained within discrete bone marrow niches through the effects of cell adhesion molecules and chemokine gradients. However, a small proportion of hematopoietic stem cells can also be found trafficking in the peripheral blood. During induced stem cell mobilization a proteolytic microenvironment is generated, but whether proteases are also involved in physiological trafficking of hematopoietic stem cells is not known. In the present study we examined the expression, secretion and function of the cysteine protease cathepsin X by cells of the human bone marrow.

Design and Methods
Human osteoblasts, bone marrow stromal cells and hematopoietic stem and progenitor cells were analyzed for the secretion of cathepsin X by western blotting, active site labeling, immunofluorescence staining and activity assays. A possible involvement of cathepsin X in cell adhesion and CXCL-12-mediated cell migration was studied in functional assays. Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) analysis revealed the digestion mechanism of CXCL-12 by cathepsin X.

Results
Osteoblasts and stromal cells secrete cathepsin X, whereas hematopoietic stem and progenitor cells do not. Using a cathepsin X-selective substrate, we detected the catalytic activity of cathepsin X in cell culture supernatants of osteoblasts. Activated cathepsin X is able to reduce cellular adhesive interactions between $CD34^+$ hematopoietic stem and progenitor cells and adherent osteoblasts. The chemokine CXCL-12, a highly potent chemoattractant for hematopoietic stem cells secreted by osteoblasts, is readily digested by cathepsin X.

Conclusions
The exo-peptidase cathepsin X has been identified as a new member of the group of CXCL-12-degrading enzymes secreted by non-hematopoietic bone marrow cells. Functional data indicate that cathepsin X can influence hematopoietic stem and progenitor cell trafficking in the bone marrow.

Key words: stem cell niche, proteolytic microenvironment, proteases, cell adhesion, cysteine cathepsins, active site labeling.

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The online version of this article has a Supplementary Appendix.

Introduction

Human cathepsin X (also known as cathepsin Z; Swiss-Prot: Q9UBR2) belongs to the CA clan of cysteine peptidases.[1,2] This class of enzymes, which includes other cysteine cathepsins such as cathepsin B, C, H, K or L, is part of the papain superfamily.[3,4] The cysteine cathepsin proteases are primarily involved in endolysosomal degradation, but have also been shown to function extracellularly and in the nucleus.[5] These proteolytic enzymes participate in various biological processes such as cell adhesion, proliferation and migration.[6]

Human cathepsin X shows structural and functional properties that clearly distinguish it from other cysteine cathepsins.[7] The prodomain contains an RGD motif in an exposed region which can interact with RGD-recognizing integrins such as $\alpha_v\beta3$.[8] Functionally mature cathepsin X shows only carboxy-terminal monopeptidase activity and cannot act as an endopeptidase, in contrast to other cysteine cathepsins such as cathepsin B.[9] Cathepsin X's lack of endopeptidase activity makes it unlikely that the secreted enzyme can take part in the degradation of the extracellular matrix, but it is a prime candidate for the inactivation of messenger molecules such as chemokines. However, a physiological extracellular substrate for cathepsin X has not yet been identified.

In contrast to earlier reports describing a ubiquitous expression pattern, cathepsin X expression seems to be restricted mainly to cells of the hematopoietic and immune system including monocytes, macrophages and dendritic cells.[10] T lymphocytes, which normally express small amounts of cathepsin X, use this protease in migration and invasion across cellular barriers.[11] Elevated levels of cathepsin X expression have been found in gastric and prostate carcinoma cells during tumor formation.[12,13]

In the bone marrow, where hematopoiesis takes place, the functional involvement of secreted cathepsins is less well defined. Hematopoietic stem cells reside in a specialized microenvironment called the endosteal stem cell niche which provides the conditions essential for stem cell quiescence and the maintenance of a constant number of stem cells.[14,15] The major cell type of the endosteal stem cell niche is the osteoblast.[16-18] These bone-lining cells synthesize and secrete a complex extracellular matrix and factors such as CXCL-12, which are necessary for stem cell maintenance.[19] CXCL-12, also known as stromal-derived factor-1 (SDF-1), occurs in two splice variants, SDF-1α (amino acids 1-68) and SDF-1β (amino acids 1-72) which have identical amino acid sequences except for four additional amino acids at the carboxy-terminal end of SDF-1β.[20] CXCL-12 binds to its receptor, CXCR-4, present on hematopoietic stem/progenitor cells (HSPC) and is a key regulator of stem cell motility and migration in the bone marrow.[21] Recently it was demonstrated that SDF-1α can be inactivated by the membrane-bound carboxypeptidase M, which is widely expressed in the hematopoietic system.[22] Other proteases that seem to be involved in hematopoietic stem cell migration include MT1-MMP[23] and the secreted neutrophil elastase, matrix metalloproteinase 9 and the serine protease cathepsin G. During cytokine-induced stem cell mobilization the latter three proteases are released by granulocytes creating a proteolytic bone marrow microenvironment.[24] However, mobilization of HSPC is only partially affected in mice lacking one or two of these proteases[25] indicating that additional secreted proteases are involved in normal or induced stem cell trafficking.

A previously conducted reverse transcriptase polymerase chain reaction (RT-PCR) screening analysis of human osteoblasts by our group (*unpublished data*) showed that cathepsin X is synthesized by osteoblasts. We, therefore, investigated in the present study whether: (i) osteoblasts and bone marrow stromal cells secrete cathepsin X into the extracellular milieu, (ii) cathepsin X modulates adhesive interactions of osteoblasts and HSPC, and (iii) CXCL-12 is a physiological substrate for secreted cathepsin X.

Design and Methods

Antibodies, proteases and recombinant proteins

Details are given in the *Online Supplementary Design and Methods*.

Human primary cells, cell lines and collection of conditioned media

Details on the primary cell isolation, cultivation and characterization of the various cell lines used in this study and on the collection of conditioned media are given in the *Online Supplementary Design and Methods*.

Reverse transcriptase polymerase chain reaction analysis and targeted knock-down of cathepsin X

Details on the reverse transcriptase polymerase chain reaction (RT-PCR) method and small inhibitory RNA (siRNA) transfection are provided in the *Online Supplementary Design and Methods*.

Active site labeling of cathepsins in conditioned media

Cysteine cathepsins in conditioned media can be labeled with the biotinylated activity-based probe DCG-04,[26] which is an analog of the E-64 broad spectrum inhibitor of cysteine cathepsins. Ten microliters of 10x concentrated conditioned media were incubated in 23 μL of 50 μM DCG-04 in 25 mM sodium acetate/2 mM EDTA buffer containing 20 μM dithiothreitol. Ten microliters of the samples were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis in 15% linear gels. Proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore). After a blocking step with Roti®Block solution (Roth, Karlsruhe, Germany), the blots were incubated with streptavidin-horse radish peroxidase (BD Pharmingen). After washing, the labeled cathepsins were detected by enhanced chemiluminescence (Millipore).

A pull-down assay was performed to identify the DCG-04 labeled proteases in the conditioned media. Twenty microliters of the concentrated conditioned media were labeled with 2 μL of 2 mM DCG-04 in 70 μL 25 mM NaOAc/20 mM dithiothreitol. The biotinylated proteases in the conditioned media were incubated with 80 μL streptavidin-sepharose beads (GE Healthcare) and 200 μL NaOAc (pH 4.4) overnight. For the pull-down of cathepsin B 10 mM citrate (pH 5.0) were used instead of NaOAc (pH 4.4) buffer. The beads were then washed with 10 mM citrate buffer. The last washing step was performed with 25 mM NaOAc (pH 4.4). The biotinylated cathepsins were separated by gel electrophoresis, transferred to polyvinylidene fluoride membranes and immunoblotted with antibodies against cathepsin X or B.

Immunofluorescence, immunoblotting and in vitro biotinylation of cell surface proteins

The immunofluorescence staining, immunoblotting and cell surface biotinylation techniques are described in the *Online Supplementary Design and Methods*.

Activity assays with fluorescence-quenched substrates

To analyze whether functionally active cathepsin X is present in conditioned media or cell lysates, activity assays with two different fluorescence-quenched substrates were performed. As a positive control 20 ng of the recombinant cathepsin X were incubated with 10 μM of the cathepsin X/A-selective substrate Mca-RPPGFSAFK(Dnp)-OH (Mca=7-amino-4-methyl-coumarin; Dnp=2,4-dinitrophenyl; R&D Systems). All measurements with the Mca-RPPGFSAFK(Dnp)-OH substrate were performed in 25 mM sodium acetate/5 mM DTT. Abz-FEK(Dnp)-OH (Abz=*ortho*-aminobenzoic acid) was employed as a more specific, but not very sensitive substrate for cathepsin X.[9] All measurements were performed in triplicate. The excitation/emission wavelengths of Mca-RPPGFSAFK(Dnp)-OH and Abz-FEK(Dnp)-OH were $340_{ex}/405_{em}$ and $320_{ex}/405_{em}$ nm, respectively. The increase in fluorescent product was measured in a time-dependent manner.

Cell-cell adhesion and cell migration assays

Details on the cell adhesion and cell migration assays used in this study are provided in the *Online Supplementary Design and Methods*.

Mass spectrometry analysis of cathepsin X-induced CXCL-12 digestion

To determine the carboxypeptidase activity of cathepsin X on the chemokine CXCL-12, the digested isoforms, SDF-1α and SDF-1β, were analyzed by matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) analysis. Recombinant procathepsin X was activated in 25 mM sodium acetate buffer (pH 3.5) containing 5 mM dithiothreitol. Different amounts of activated cathepsin X were then incubated with 3 μM of both CXCL-12 isoforms. Mass spectrometry analysis was performed with a Bruker Daltonics Ultraflex MALDI-TOF mass spectrometer (Bremen, Germany). Calibration was performed with a protein standard from Bruker.

Statistical analysis

The statistical analysis is described in the *Online Supplementary Design and Methods*.

Results

Expression and secretion of cathepsin X by non-hematopoietic bone marrow cells

The expression of cathepsin X mRNA in bone marrow-derived cells was monitored by RT-PCR analysis using a specific primer pair for human cathepsin X. Primary osteoblasts and the osteoblastic cell lines CAL72 and MG63, but not G292 cells, expressed cathepsin X mRNA (Figure 1A). The bone marrow stromal cell lines L87/4, L88/5 and HS-5 also strongly expressed cathepsin X mRNA, whereas cord blood-derived $CD34^+$ HSPC did not (Figure 1A).

To determine whether the non-hematopoietic bone marrow cells were able to secrete cathepsin X, conditioned media of bone marrow stromal and osteoblastic cells were concentrated ten-fold and analyzed by western blotting. An antiserum against human cathepsin X which recognizes both the mature and the immature forms detected mainly the immature proform of cathepsin X at 40 kDa in cell culture supernatants of primary osteoblasts, CAL72 and MG63 (Figure 1B). As expected from the RT-PCR results, G292 cells did not secrete cathepsin X in cell culture. A weaker band at 34 kDa could be detected in MG63 cell culture supernatants representing the mature form of human cathepsin X. Mature cathepsin X was also found in conditioned media of the bone marrow stromal cell lines L87/4 and L88/5 although these cells predominantly secreted the proform of cathepsin X (Figure 1B).

Secreted cysteine cathepsins were identified by active site labeling using the biotinylated activity-based probe DCG-04 which targets several cysteine proteases including cathepsins B, H, L, S and X. Strong signals could be observed at 40 kDa in conditioned media of primary osteoblasts and the osteoblastic cell lines CAL72, MG63 and G292. The lower, but weaker signal at 31 kDa represents the mature cathepsin B (Figure 2A). The position of the prominent 40 kDa signal corresponds to both procathepsin X and the immature form of cathepsin B (Figure 2B). This would explain why G292 cell culture supernatants also showed a signal even though these cells do not secrete cathepsin X. Precipitation of the biotinylated probe after binding to the secreted proteases and subsequent immunoblotting revealed that both cathepsin X and cathepsin B are hidden behind the strong 40 kDa signal in primary osteoblasts, CAL72 and MG63 cell culture supernatants (Figure 2C). Consistently weaker active site labeling signals at 40 kDa were obtained in cell culture supernatants of the bone marrow stromal cell lines L87/4, L88/5

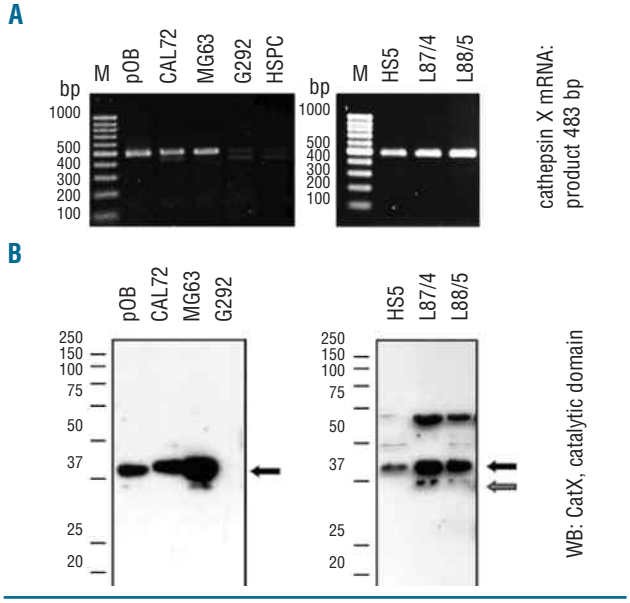

Figure 1. Cathepsin X is secreted by primary osteoblasts, osteoblastic cell lines and bone marrow stromal cell lines. (A) Primary osteoblasts (pOB) and the osteosarcoma cell lines CAL72 and MG63 express cathepsin X mRNA, as shown here by RT-PCR analysis. A 483 bp amplification product could be identified in these samples. Only trace amounts of cathepsin X mRNA were expressed by HSPC and the osteoblastic cell line G292, whereas cathepsin X mRNA could be clearly detected in the bone marrow stromal cell lines HS-5, L87/4 and L88/5. (B) Western blotting using a polyclonal antibody targeting the catalytic domain of cathepsin X revealed that pOB, CAL72 and MG63, but not G292, express and secrete immature cathepsin X (black arrow) into the cell culture supernatants. Cathepsin X could also be detected in the bone marrow stromal cell line-conditioned media of HS-5, L87/4 and L88/5 cells. These cells secrete mainly the immature procathepsin X, but also the mature form (gray arrow).

and HS-5 (Figure 2A). The origin of the strong signal found at 70 kDa in bone marrow stromal supernatants has not yet been identified.

Cathepsin X is localized on the cell surface of primary osteoblasts

The expression of cathepsin X by primary osteoblasts was confirmed by immunofluorescence staining. Paraformaldehyde-fixed osteoblasts which had been permeabilized by Triton X-100 treatment showed strong labeling signals with the monoclonal antibody detecting both the mature and immature cathepsin X (Figure 3A). To identify cathepsin X at the cell surface the osteoblasts were fixed with freshly prepared 4% paraformaldehyde and the whole staining procedure was performed at 4°C. This procedure prevents the penetration of the antibody into the cytosolic part of the cells. Fluorescence microscopy of optical sections then showed that both the monoclonal antibody targeting the catalytic domain and the polyclonal antiserum targeting the prodomain recognized their respective antigens at the cell surface (Figure 3B, C). This result indicates that cathepsin X is not only secreted into the cell culture supernatant, but can also be detected at the cell surface of primary osteoblasts. This finding was corroborated by precipitation of biotinylated cell surface proteins of primary osteoblasts and the osteosarcoma cell lines CAL72 and MG63 followed by immunoblotting with anti-cathepsin X antibodies (Figure 3D). The strong signals obtained by the western blot confirm that the immature and mature forms of cathepsin X are located on the cell surface of osteoblasts.

Determination of proteolytically active cathepsin X in conditioned media and cell lysates

The fluorogenic peptide substrate Mca-RPPGFSAFK(Dnp)-OH, which is recommended by the manufacturers as a cathepsin X/cathepsin A-selective substrate, was readily processed by cell culture conditioned media of primary osteoblasts (Figure 4A) suggesting cathepsin X activity. However, G292 conditioned media also displayed a measurable activity with the substrate, even though the osteosarcoma cell line G292 does not secrete cathepsin X. The fluorogenic peptide substrate was, therefore, incubated with 20 ng of recombinant cathepsin X and cathepsin B. Notably, recombinant cathepsin B digested the substrate even better than cathepsin X, which can explain the activity observed in G292-conditioned media. The strong activity of primary osteoblast-conditioned media might therefore be the

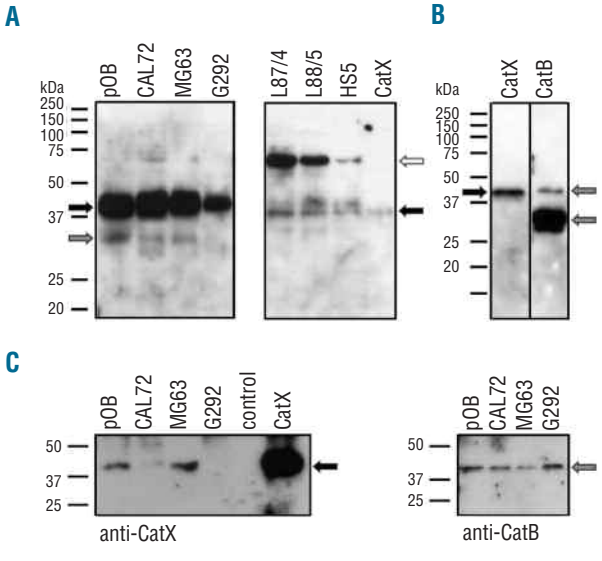

Figure 2. Active site labeling identifies cathepsin X in conditioned cell culture supernatants. (A) DCG-04 was used to identify secreted cysteine cathepsins in cell culture supernatants of primary osteoblasts (pOB), the osteosarcoma cell lines CAL72, MG63 and G292 and the bone marrow stromal cell lines L87/4, L88/5 and HS-5. Strong signals at 40 kDa corresponding to cathepsin X could be observed in the osteoblastic conditioned media, whereas only faint signals at 40 could can be detected in the supernatants of the bone marrow stromal cells (black arrows). Fainter bands at 31 kDa corresponding to cathepsin B were obtained in the conditioned media of pOB, CAL72 and MG63 (gray arrow). The strong signals at 70 kDa in the bone marrow stromal cell culture supernatants (white arrow) have not yet been identified. (B) Labeling recombinant cathepsin X and cathepsin B with DCG-04 revealed a strong band at 40 kDa for cathepsin X (black arrow), and a fainter band at 41 kDa and a strong band at 31 kDa (gray arrows) for cathepsin B. (C) A pull-down assay revealed that both cathepsin X and cathepsin B were responsible for the strong 40 kDa signal shown in (A). DCG-04 was allowed to bind to secreted cathepsins and was subsequently precipitated by streptavidin-sepharose. The precipitates were separated by SDS-PAGE followed by immunoblotting with cathepsin X- or cathepsin B-specific antibodies. Both cathepsin X and B were found in supernatants of pOB, CAL72 and MG63, whereas only cathepsin B could be detected in the supernatant of G292 cells. Recombinant cathepsin X was used as a positive control (CatX). For the negative control, the probe DCG-04 was omitted (control).

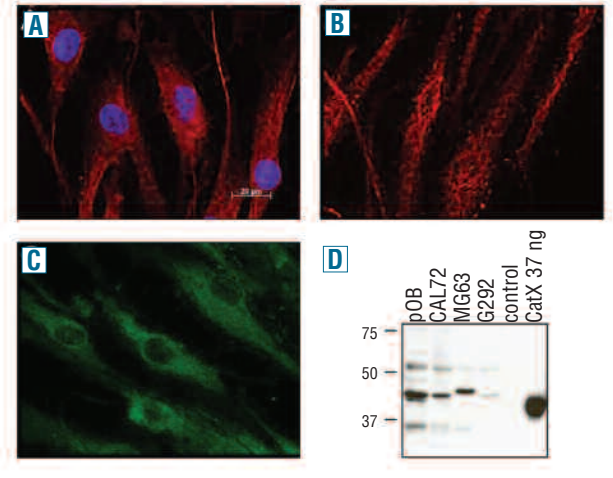

Figure 3. Detection of cathepsin X secretion by primary osteoblasts (pOB) using immunofluorescence staining and cell surface biotinylation. (A-C) pOB were cultured for 2 days on glass slides and then fixed with 4% paraformaldehyde. Immunofluorescence staining was performed using either a monoclonal antibody binding to the catalytic domain of cathepsin X and Cy3-labeled secondary antibodies (A, B) or a polyclonal antiserum targeting the cathepsin X prodomain and secondary Cy2-labeled anti-rabbit antibodies (C). For both intracellular and extracellular staining the cells were permeabilized with Triton X after fixation and nuclei were counterstained with DAPI (A). Fluorescence microscopy of optical sections revealed that procathepsin X was mainly located on the cell surface (C). (D) Cell surface proteins of pOB and osteosarcoma cell lines were biotinylated and precipitated after cell lysis by streptavidin beads. After electrophoresis the precipitated cell surface proteins were analyzed by immunoblotting using the anti-cathepsin X antiserum. Prominent signals for the immature form, but also weaker signals for the mature form of cathepsin X were detected for pOB, CAL72 and MG63, whereas almost no signal was found for G292 cells. Recombinant cathepsin X and the precipitate of a non-biotinylated cell medium served as positive and negative controls, respectively.

result of a mixture of cathepsin X and cathepsin B (Figure 4A). In contrast, the substrate Abz-FEK(Dnp)-OH is a cathepsin X-specific substrate which was not processed by cathepsin B[9] (Figure 4B). Although specific, the substrate is not very sensitive (Figure 4B-D; note the low differences in RFU over time). Activated recombinant cathepsin X and conditioned media of primary osteoblasts proteolytically processed the substrate during a 50 min incubation period, indicating that the osteoblasts can secrete proteolytically active cathepsin X in the cell culture supernatants (Figure 4D). The activity of the activated cathepsin X was dose-dependently blocked by the active site labeling reagent DCG-04 (Figure 4C). DCG-04 also completely blocked the proteolytic activity of primary osteoblast-conditioned media (Figure 4D).

Activated cathepsin X can impair hematopoietic stem/progenitor cell adhesion to primary osteoblasts

A cell adhesion assay using confluent adherent primary osteoblasts and cord blood-derived CD34⁺-MACS-sorted HSPC was performed to analyze whether immature or mature cathepsin X can influence adhesive cellular interactions. Primary osteoblasts were seeded at a density of $4x10^4$ cells per well 1 day before the assay was performed. Fluorescently labeled CD34⁺ HSPC were allowed to adhere to the osteoblasts for 40 min followed by 20 min incubation with immature or mature cathepsin X. Each experiment was performed in triplicate. In five different experiments with active mature cathepsin X we observed diminished adhesion after adding cathepsin X to the attached CD34⁺ HSPC (Figure 5A). When we combined the results of all five experiments, we observed a significant decrease of 24%. The decrease ranged from 15% to 35% in the experiments performed (Figure 5B). In contrast, the immature form of cathepsin X did not alter the adhesion of CD34⁺ HSPC to primary osteoblasts significantly (Figure 5B). The ability of activated cathepsin X to decrease CD34⁺ HSPC adhesion to osteoblasts was abrogated by adding the cysteine cathepsin inhibitor DCG-04 to the protease (Figure 5C), confirming the proteolytic activity of cathepsin X in this assay. Targeted knock-down of cathepsin X in primary osteoblasts enhanced the attachment of CD34⁺ HSPC to siRNA-treated osteoblasts. A significant increase of about 25% was observed for the siRNA with the low GC content, whereas the other siRNA with the medium GC content only yielded a (non-significant) trend (Figure 5D). The knock-down efficiencies in the cell supernatants and cell lysates are shown by the inserted western blot (Figure 5D).

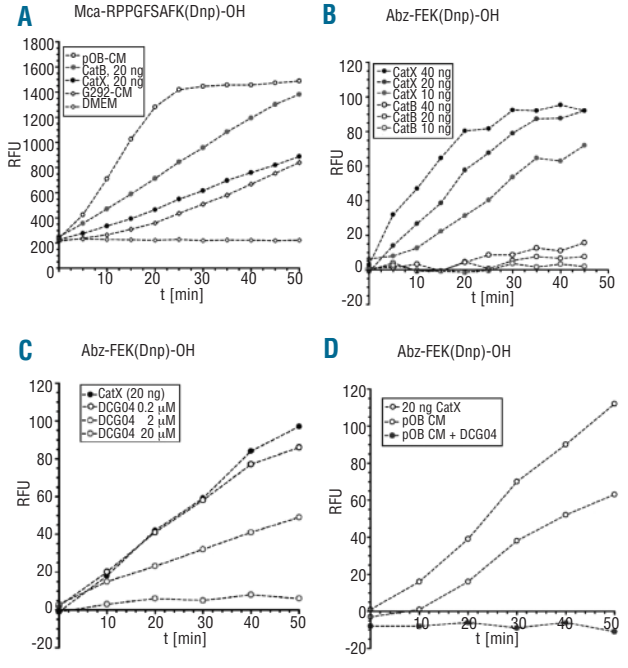

Figure 4. Determination of cathepsin X activity in osteoblast conditioned media. The fluorogenic substrates Mca-RPPGFSAFK(Dnp)-OH or Abz-FEK(Dnp)-OH were used to evaluate cathepsin X catalytic activity in conditioned media of primary osteoblasts (pOB). (A) Using pOB-conditioned medium, Mca-RPPGFSAFK(Dnp)-OH was converted to its fluorescent unquenched product; 20 ng cathepsin X also hydrolysed the substrate, but not as efficiently as the pOB-conditioned medium. G292-conditioned medium also induced substrate hydrolysis, although not to the same extent as pOB-conditioned medium. Pre-activated cathepsin B (20 ng) is also able to process the Mca-RPPGFSAFK(Dnp)-OH substrate. No activity was found in the control DMEM medium. (B) In contrast to the substrate Mca-RPPGFSAFK(Dnp)-OH, the substrate Abz-FEK(Dnp)-OH is highly specific for cathepsin X but not very sensitive, and cannot be converted by cathepsin B. Increasing amounts of cathepsin X (10, 20 and 40 ng) correlated with increasing hydrolytic activities. (C) Hydrolysis of Abz-FEK(Dnp)-OH by recombinant cathepsin X (CatX) can be dose-dependently inhibited by addition of DCG-04 (0.2, 2 and 20 µM). (D) The substrate Abz-FEK(Dnp)-OH was applied in the activity assay with osteoblast-conditioned medium (pOB CM). The activity observed in the pOB CM could be completely blocked by addition of 20 µM DCG-04.

Degradation of both isoforms of CXCL-12 by activated cathepsin X

To address the question of whether cathepsin X is capable of efficiently degrading the important chemoattractant chemokine CXCL-12 and might, therefore, play a role in HSPC trafficking, pre-activated cathepsin X was incubated with both CXCL-12 isoforms, SDF-1α and SDF-1β.

Molecular masses of 7,960 Da for undigested SDF-1α or 8,475 Da for undigested SDF-1β were observed; both were incubated in 25 mM NaOAc buffer/5mM dithiothreitol at pH 3.5. The addition of 0.135 µM activated cathepsin X to SDF-1α for 2 h resulted in peaks exactly matching the calculated molecular weights of 7,832 Da for SDF-1αΔK and 7,719 Da for SDF-1αΔNK, indicating that the two C-terminal residues are cleaved off the protein sequentially by the exo-peptidase (Figure 6A). A three-fold amount of pre-activated cathepsin X (0.35 µM) was applied in further experiments. MALDI-TOF analysis of both CXCL-12 isoforms incubated overnight with activated cathepsin X revealed that neither intact SDF-1α nor SDF-1β was detectable any more. Instead, peaks matching a 15 amino acid-truncated SDF-1α or a 19 amino acid-truncated SDF-1β were detected. As no SDF1α was detectable after overnight incubation, the degradation of CXCL-12 was analyzed in a time-dependent manner. The kinetics of this degradation were explored by arresting CXCL-12 degradation at 2 h, 4 h, 6 h and 8 h as shown in Figure 6B. The exo-peptidase cathepsin X gradually cleaves 15 amino acids until proline P^{74} is present at the P2 position. Several intermediates with the carboxy-terminal amino acids lycine (K), leucine (L) or glutamine (Q) accumulate during the degradation process (Figure 6B). A similar degradation profile can be observed for SDF-1β, although it is not as efficient as for SDF-1α (*Online Supplementary Figure S1A*).

We confirmed that the chemotactic response of CD34+ HSPC to intact SDF-1α is different from that to SDF-1α treated with activated cathepsin X. For the digestion of the chemokine, 3 μM SDF-1α were incubated in the presence or absence of 50 nM cathepsin X overnight. After 15 h of cell migration, chemotaxis of CD34+ HSPC was reduced by 60% when the cathepsin X-treated SDF-1α was used (*Online Supplementary Figure S1B*). As a negative control, cell migration was determined in the absence of a chemotactic stimulus or when the intact SDF-1α was only added to the CD34+ HSPC-containing upper chamber. No effective cell migration could be observed in either control (*Online Supplementary Figure S1B*).

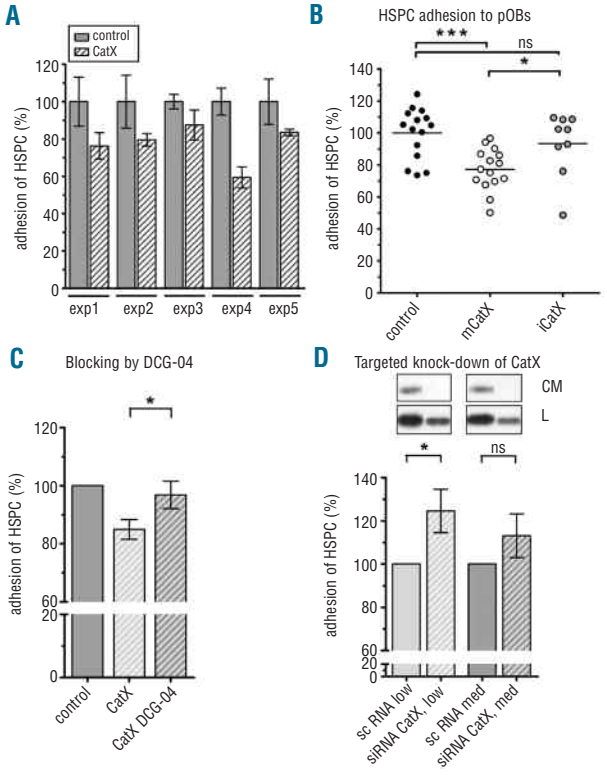

Figure 5. Mature cathepsin X modulates adhesion of hematopoietic progenitor cells to osteoblasts. (A) BCECF-labeled, cord blood-derived CD34+ HSPC were incubated on a confluent layer of primary osteoblasts (pOB) to allow cell attachment. The cells were then incubated either with activated cathepsin X in activation buffer or with activation buffer only (control). In five independent experiments a significant decrease in cell adhesion was observed. The results of the five independent experiments are shown as means and SEM. (B) When the results of these experiments (n=5) were combined, a decrease of 24% in cell adhesion (mean average) could be observed. Inactive procathepsin X (n=3) did not significantly modulate CD34+ HSPC adhesion to osteoblasts. (*$P \leq 0.05$; ***$P \leq 0.005$; ns: not significant). (C) The adhesion-modulating effect of cathepsin X could be abolished by blocking the activity of cathepsin X with DCG-04. The results of three experiments performed in triplicate are shown as means and SEM. (*$P \leq 0.05$) (D) The expression of cathepsin X in primary osteoblasts was down-regulated by targeted knock-down with siRNA. Two different siRNA (siRNA CatX, low and med) were used, together with the respective scrambled control oligonucleotides. Three days after transfection the decreased expression of cathepsin X in the conditioned media (CM) or cell lysates (L) of the treated primary osteoblasts was checked by western blotting. CD34+ HSPC adhere significantly better to osteoblasts treated with the low GC siRNA. Although the medium GC siRNA also down-regulated cathepsin X expression in the primary osteoblasts, only a non-significant increase in cell adhesion was observed.

Discussion

In the present study we investigated the influence of cathepsin X on CD34+ HSPC cell-cell interactions which occur in the bone marrow. Cathepsin X can be secreted by non-hematopoietic bone marrow cells in an activated form. In the presence of mature cathepsin X we observed a significant decrease in the number of CD34+ HSPC attached to human osteoblasts. Since the immature proform of cathepsin X had no influence on the pre-existing cell-cell interactions these findings indicate a proteolytic role of mature cathepsin X. Accordingly, the targeted knock-down of cathepsin X resulted in an increase of CD34+ HSPC attached to osteoblasts. Mature cathepsin X, which can degrade the chemoattractant CXCL-12 *in vitro*, was determined to have carboxy-monopeptidase activity. Digestion of SDF-1α by activated cathepsin X abrogated a chemotactic response of HSPC. Since the CXCL-12/CXCR-4 signaling pathway plays an important role in CD34+ HSPC trafficking, cathepsin X constitutively secreted by osteoblasts could contribute to the regulation of effective CXCL-12 concentrations and to the controlled retention of stem cells in their niche.

Although cathepsin X is preferentially expressed by mature cells of the hematopoietic and immune system,[10] the protease does not seem to be synthesized by hematopoietic progenitor cells. In contrast, non-hematopoietic cells of the bone marrow, the osteoblasts and bone marrow stromal cells, express and secrete cathepsin X. Western blotting showed mainly the inactive form of cathepsin X in the conditioned media. Using an antibody against the prodomain of cathepsin X, confocal immunofluorescence microscopy revealed that the immature form of cathepsin X can be found on the cell surface of osteoblasts. Since the antibody against the catalytic domain of cathepsin X did not discriminate between immature and mature forms in immunofluorescence microscopy, it was not possible to determine by this method whether mature cathepsin X can also be found on the cell surface of the osteoblasts. However, cell surface biotinylation followed by streptavidin-mediated precipitation and western blotting clearly showed that although the immature form of cathepsin X was predominant on the cell surface of primary osteoblasts, the mature form was also present.

Although mainly immature cathepsin X was present in the conditioned media of primary osteoblasts and the osteosarcoma cell lines, strong signals of cathepsin X were obtained by active site labeling. This result is an indication that the prodomain of cathepsin X might be flexible, allowing access of the DCG-04 probe to the active site. Very recently it was shown that the proform of another cysteine cathepsin, cathepsin B, can also be labeled with DCG-04.[27] Here autocatalytic digestion of procathepsin B is achieved through the activity of the proenzyme which can act as an endopeptidase. However, an autocatalytic processing of procathepsin X into its active form, which was found in small amounts in the immunoblots, seems to be unlikely since cathepsin X only functions as a carboxypeptidase. Other still unidentified proteases are expected to be responsible for the extracellular activation of cathepsin X.

Activity assays with the two different fluorogenic substrates also revealed the presence of an active form of cathepsin X in the conditioned media of the osteoblasts.

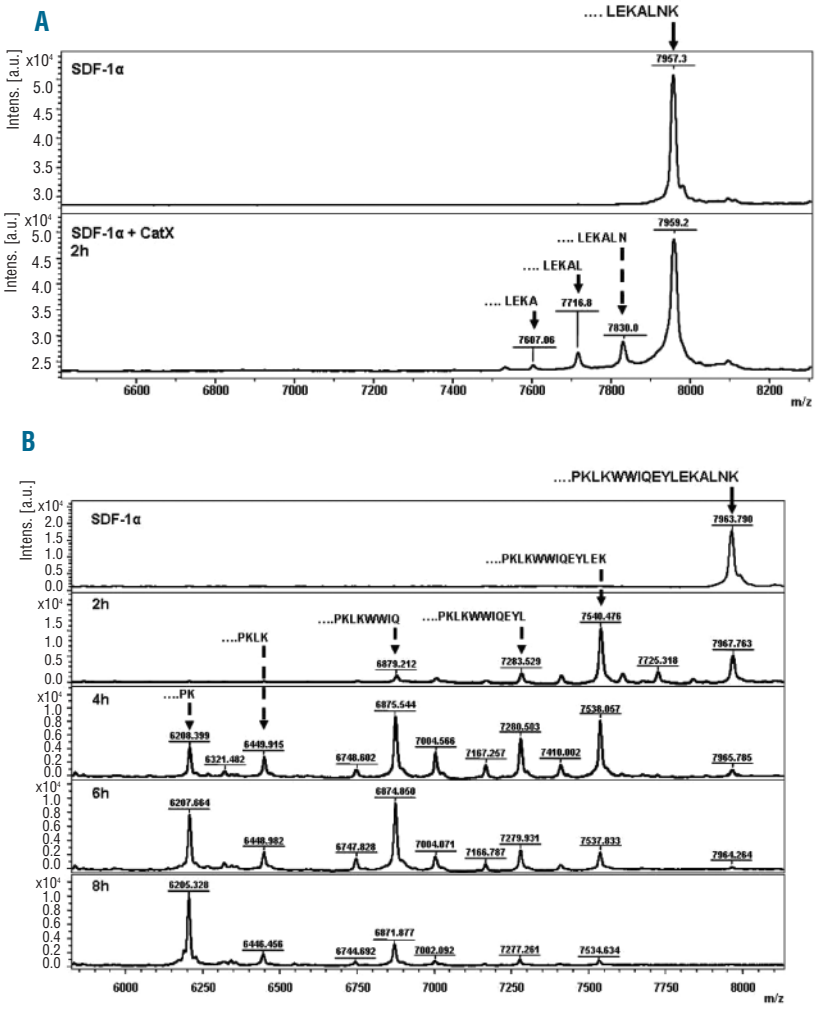

Figure 6. Cathepsin X cleaves SDF-1α showing monopeptidase activity. (A) 3 μM SDF-1α were incubated with 0.13 μM pre-activated cathepsin X for 2 h. The undigested and the digested chemokine were analyzed by MALDI-TOF analysis. After 2 h of proteolytic digestion, the intact chemokine and two prominent degradation products (SDF-1αΔK and SDF-1αΔNK) could be detected indicating monopeptidase activity of cathepsin X. (B) 3 μM of the CXCL-12 isoform SDF1α were incubated with 0.35 μM pre-activated cathepsin X for up to 8 h at 37°C. At 2 h, 4 h, 6 h and 8 h, aliquots of the digested chemokine were taken and, together with the undigested SDF1α, examined by MALDI-TOF analysis. The carboxy-monopeptidase cathepsin X is able to digest SDF1α in a time-dependent fashion. After 4 h of digestion, the 3 μM CXCL-12 were almost completely digested, and after 8 h of digestion, a product lacking the C-terminal 15 amino acids (…PK) had accumulated.

The substrate Mca-RPPGFSAFK(Dnp)-OH was commercially obtained as a cathepsin X/cathepsin A-specific substrate. However, our results documented that cathepsin B, which can be secreted by osteoblasts,[28] also converts this substrate. This unexpected result explains why conditioned medium of the osteosarcoma G292 cells, which neither synthesize nor secrete cathepsin X, but do produce cathepsin B, can nevertheless convert the substrate Mca-RPPGFSAFK(Dnp)-OH. In contrast, as shown by this and other studies, the substrate Abz-FEK(Dnp)-OH cannot be hydrolysed by activated cathepsin B.[9] Recombinant cathepsin X and the conditioned medium of primary osteoblasts cleaved this substrate, clearly indicating the secretion of an active form of cathepsin X by osteoblasts.

Immunoblotting showed that the artificial *in vitro* activation of recombinant cathepsin X by dithiothreitol and a low pH always led to a partial, but never to a complete activation of cathepsin X (*data not shown*). Nevertheless activated cathepsin X was able to impair pre-existing adhesive interactions of $CD34^+$ HSPC with osteoblasts whereas the non-activated form of cathepsin X had no significant influence on the observed cell binding. The specificity of the impairment was shown by the complete abrogation of the impairment with the cysteine cathepsin-specific inhibitor DCG-04. This strongly indicates an extracellular proteolytic activity of cathepsin X. These results were corroborated by the targeted knock-down of cathepsin X in primary osteoblasts. However, the extracellular targets of the enzyme are still unknown. Since degradation of extracellular matrix components by the carboxypeptidase with functional consequences is not very likely, an involvement of an adhesive receptor-ligand pair, such as vascular cell adhesion molecule – α4β1 integrin, seems more reasonable. Cathepsin X has already been shown to interact intracellularly with the integrin β2 chain.[11] This interaction influences cellular migration, but also cell adhesion and activation of lymphocytes.[11,29] An extracellular role has only been identified for the prodomain of cathepsin X. The pro-peptide contains an RGD motif which mediates cell adhesive properties, and this region interacts with the integrin αvβ3.[8] This integrin can also be found on $CD34^+$ HSPC.[30] Whether it contributes to cell binding of HSPC to osteoblasts presenting immature procathepsin X on their cell surface still has to be clarified.

There is accumulating evidence that CXCL-12/ CXCR-4 signaling plays an essential role in HSPC trafficking in the bone marrow.[31,32] Bone marrow stromal cells and osteoblasts are an important source of CXCL-12 in the human bone marrow.[33,34] The isoform SDF-1α can be degraded by several secreted enzymes such as matrix metalloproteinases,[35] the CD26/dipeptidyl peptidase IV,[36] neutrophil elastase[37] and cathepsin G.[38] Recently it was shown that SDF-1α can be cleaved and inactivated by the mem-

brane-bound carboxypeptidase M.[22] In the present study we show that the osteoblast-derived carboxypeptidase cathepsin X processes and inactivates both SDF-1α and the four amino acid longer isoform SDF-1ß, by cleaving one amino acid after the other from the C-terminus. Cleavage of only one carboxy-terminal amino acid from SDF-1α has already been shown to abolish the migratory capacity of the chemokine.[22] Interestingly, several truncated products accumulated during an 8 h incubation, indicating different catalytic efficiencies of these newly generated substrates. Cleavage products of truncated SDF1-α with a C-terminal lysine in the P1 position (eg, ..EK, ..LK, ..PK) were converted with rather low catalytic efficiencies which is consistent with earlier observations.[9] Digestion of CXCL-12 by cathepsin X stopped after the elimination of 15 amino acids when a proline was found to be present at position P2. As expected, SDF-1α digested with activated cathepsin X lost its migratory capacity.

Secreted cathepsin X has been identified as a new member of the group of CXCL-12-degrading enzymes. The CXCL-12/CXCR-4 signaling axis is a delicate network regulating the homing and engraftment, the mobilization, and also the maintenance of hematopoietic stem cells in their niches. As the major cell type of the endosteal stem cell niche, osteoblasts secrete immature procathepsin X which is then processed to its mature form, although the physiological extracellular activator of cathepsin X still remains to be elucidated. Glycosaminoglycans have been shown to facilitate the activation of procathepsin B,[39] and membrane-associated heparan sulfate proteoglycans bind cathepsin X.[40] Whether glycosaminoglycans present on the cell surface of osteoblasts also facilitate procathepsin X activation must still be determined. The fact that mature cathepsin X interferes with $CD34^+$ HSPC-osteoblast adhesive interactions *in vitro* only partially, but not completely, indicates that cathepsin X might not be the key component, but that it plays a subtle role in hematopoietic stem cell trafficking.

Authorship and Disclosures

NDS: collection and assembly of data, data analysis and interpretation, manuscript writing; WKA, HK, AKC, and MB: provision of study materials, data analysis and interpretation; SS: data analysis and interpretation; GK: conception and design of the study, data analysis and interpretation, manuscript writing.

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