Processing of CXCL12 by Different Osteoblast-Secreted Cathepsins

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Hematopoietic stem and progenitor cells (HSPCs) are known to reside in specialized niches at the endosteum in the trabecular bone. Osteoblasts are the major cell type of the endosteal niche. It is well established that secreted proteases are involved in cytokine-induced mobilization processes that release stem cell from their niches. However, migratory processes such as the regular trafficking of HSPCs between their niches and the periphery are not fully understood. In the current study we analyzed whether osteoblast-secreted cysteine cathepsins are able to reduce the direct interaction of HSPCs with bone-forming osteoblasts. Isolated human osteoblasts were shown to secrete proteolytically active cysteine cathepsins, such as cathepsins B, K, L, and X. All of these cathepsins were able to digest, although with different efficacy, the chemokine CXCL12, which is known to be important for retaining HSPCs in their niches. Of the 4 identified cathepsins, only cathepsin X was able to reduce binding of HSPCs to osteoblasts. Interestingly, nonactivated pro-cathepsin X and mature cathepsin X did not interfere with HSPC–osteoblast interactions. Only pro-cathepsin X treated with dithiothreitol, which unfolds but does not lead to full maturation of cathepsin X, significantly reduced HSPC adhesion to osteoblasts. These observations argue for a role of the accessible cathepsin X prodomain in diminishing cell binding. Our findings strongly suggest that the cysteine cathepsins B, K, and L constitutively secreted by osteoblasts are part of the fine-tuned regulation of CXCL12 in the bone marrow, whereas pro-cathepsin X with its prodomain can affect HSPC trafficking in the niche.

Introduction

CYSTEINE CATHEPSINS ARE PROTEOLYTIC enzymes belonging to the papain subfamily of cysteine proteases [1–3]. The 11 human cysteine cathepsins are predominantly, but not exclusively, endopeptidases that are mainly found in the endolysosomal compartment [4]. However, in the hematopoietic system, secreted cathepsins can also be found in the extracellular compartment. Osteoclasts secrete the cysteine cathepsin K that is essential for bone remodeling [5]. Osteoblasts, on the other hand, secrete cathepsin X, which impairs adhesive interactions of hematopoietic stem cells with osteoblasts [6]. In addition, human osteoblasts were shown to secrete the cysteine cathepsins B and K [7,8], but the effect of these proteases on hematopoietic stem cells is poorly characterized.

In the bone marrow hematopoietic stem cells are localized in specialized microenvironments referred to as niches [9].

Two types of niches, the endosteal or osteoblastic niche and the vascular niche, have been identified and characterized [10–12]. Hematopoietic stem cells are entrapped in their niches, but they can also migrate to distant locations. Recently it has been shown that about 5% of the niches are not constantly occupied, indicating a physiological trafficking of hematopoietic stem cells to the peripheral blood [13]. However, the relevance of this trafficking is not yet fully understood.

The chemokine CXCL12 (also known as stromal-derived factor, SDF-1) together with its cellular receptor CXCR4 are important regulators of hematopoietic stem cell retention to their niches [14]. CXCL12 is produced by various cell types associated with the niches, including osteoblasts and CXCL12-producing adventitial reticular cells [15,16]. These cells can produce a CXCL12 gradient, but CXCL12 is also a target for many bone marrow proteases capable of

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abolishing the chemoattractive activity of CXCL12 including the membrane-bound carboxypeptidase M and MT1-matrix metalloproteinase (MMP), the secreted MMP-9, and different secreted cathepsins [6,17–20]. Therefore, proteases of various families and origins seem to be responsible for the fine tuning of this important chemokine gradient [21].

Recently, cathepsin X has been added to the growing list of chemoattractant-degrading enzymes. In the current study, we analyzed whether human osteoblasts, a major cell type of the endosteal stem cell niche, can secrete more members of the cysteine cathepsin family in a proteolytically active form. The identified secreted cysteine cathepsins were tested for their abilities to degrade CXCL12, to impair hematopoietic stem cell adhesion to osteoblasts, and to proteolytically activate other secreted proteases in a paracrine fashion. Our data suggest that the proteolytic network in the extracellular milieu of the endosteal stem cell niche is far more complex than previously assumed.

Materials and Methods

Antibodies, proteases, and recombinant proteins

The human cathepsin-specific polyclonal antisera against cathepsin K and cathepsin L were purchased from Abcam (Cambridge, UK). These antisera target the catalytic domains whereas an antiserum against cathepsin X binds to the pro-domain of cathepsin X (Abcam). For the detection of cathepsin B a monoclonal antibody was used (clone CA10; Abcam).

All recombinant cysteine cathepsin proteases, except for cathepsin K purchased from Biomol (Hamburg, Germany), were obtained from R&D Systems (Köln, Germany). Human chemokine CXCL12 was ordered from Peprotech (Hamburg, Germany).

Human primary cells and cell lines, collection of conditioned media

Hematopoietic stem and progenitor cells (HSPCs) were isolated from human cord blood in accordance with the local ethics committee. After Percoll (1.077 g/mL) density gradient centrifugation, HSPCs were enriched using CD34-binding MACS microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturers' instructions. Freshly isolated HSPCs were immediately used for migration assays or stored overnight in StemSpan® SFEM serum-free expansion medium supplemented with StemSpan® CC100-cytokine cocktail prior to HSPC adhesion assays (StemCell Technologies, Cologne, Germany).

Human primary osteoblasts were isolated from bone waste of hip joint femoral heads as recently described [22]. Bone was mechanically shredded into small pieces and incubated in dispase II (Roche Diagnostics, Mannheim, Germany) and collagenase type XI (Sigma, Taufkirchen, Germany). After 1 h of enzymatic digestion, the supernatant was discarded. The bone fragments were washed in phosphate-buffered saline, and then incubated again with collagenase for 3 h before they were transferred into cell culture flasks. Adherent primary osteoblasts were cultured in glucose-enriched Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum (FCS), minimal essential medium vitamin solution, Fungizone (Invitrogen), β -glycerophosphate (Sigma), and

ascorbic acid (Sigma) according to an established protocol [23]. The obtained primary osteoblasts were routinely tested for osteogenic markers such as osteopontin, alkaline phosphatase, or RunX2 via quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and/or immunofluorescence staining. The primary cells were used between passages 1 and 5, with a maximum of 5 passages. All studies were approved by the local ethics committee.

The human osteoblastic cell lines CAL72 (DSMZ, Braunschweig, Germany), MG63 (ATCC, Manassas, VA), and G292 (ECACC, Salisbury, UK) were grown in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS. Culture medium for CAL72 cells also contained an insulin-transferrin-selenium supplement (Invitrogen).

The stromal cell lines L87/4 and L88/5 [24] were cultured in RPMI 1640/10% FCS supplemented with hydrocortisone, whereas HS-5 cells [25] were cultured in RPMI 1640/20% FCS without hydrocortisone.

To obtain osteoblast- or stromal cell-conditioned media, 3.5×10^6 cells were seeded overnight in tissue flasks with corresponding FCS-containing medium. On the following day, cells were washed twice with serum-free media, and then only 10 mL basic DMEM was added to the cells for 24 h. To concentrate the media 10-fold, ultrafiltration in Amicon columns with a cutoff of 10 kDa (Millipore Amicon, Schwalbach, Germany) was performed according to the manufacturers' recommendations.

RT-PCR analysis

Total RNA was isolated from cell pellets using the RNeasy® total RNA kit (Qiagen, Hilden, Germany). Two micrograms of RNA of each sample was first transcribed into cDNA using the SuperScript™ III First-Strand Synthesis System (Invitrogen), and then the DNA was amplified by the PCR using Taq DNA Polymerase (Roche Diagnostics) and primers specific for the different cathepsins. The primer sequences are given in Table 1. After an initial denaturation step at 94°C for 120 s, PCR was performed for 30 cycles (denaturation at 94°C, 30 s; primer annealing at 56°C, 40 s; primer extension at 72°C, 60 s). After 2% agarose gel electrophoresis of the amplified PCR products, they were stained with ethidium bromide and visualized through UV light exposure. The pGOLD 100-bp DNA ladder (peqlab, Erlangen, Germany) served as a size control.

Immunoblotting

Equal volumes of 10-fold concentrated conditioned cell culture media were loaded on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and run under reducing conditions. After electrophoresis proteins were blotted onto polyvinylidene fluoride (PVDF) membranes, which were then blocked with 3% bovine serum albumin/0.1% Tween 20 in Tris-buffered saline. The PVDF membranes were incubated overnight with the anti-human cathepsin X, B, K, or L antisera, respectively (Abcam). Then, the membranes were probed with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit antiserum (Abcam). Chemiluminescent detection of the bound antibodies was performed using the Millipore electrochemiluminescence detection system (Millipore).

TABLE 1. CATHEPSIN PRIMERS FOR RT-PCR ANALYSIS

	Primer sequence (5' → 3')	Product [bp]	NCBI number
CatB	For: AGA ATG GCA CAC CCT ACT GG Rev: TGG CCT GTC TGC ACT GTA AC	350	NM_001908.3; NM_147780.2; ... 781.2; NM_147782.2; ... 783.2
CatC	For: CGG CTT CCT GGT AAT TCT TC Rev: GAT GGT GAA ATG GCC AGA AT	303	NM_001814.2 NM_148170.2
CatD	For: GCT ACA AGC TGT CCC CAG AG Rev: CTC TAC CCC CAC CAA ACA GA	437	NM_001909.3
CatF	For: TCT CTG TCC CAA AAC CAT CC Rev: TGG CTT GCT TCA TCT TGT TG	346	NM_003793.3
CatG	For: TCC TGG TGC GAG AAG ACT TT Rev: TGC CTA TCC CTC TGC ACT CT	358	NM_001911.2
CatH	For: ACT GGC TGT TGG GTA TGG AG Rev: ATT CGT GGT CCA TGT GGT TT	420	NM_004390.2 NM_148979.1
CatK	For: TTC TGC TGC TAC CTG TGG TG Rev: CCA GGT GGT TCA TAG CCA GT	219	NM_000396.2
CatL	For: ACA GTG GAC CAA GTG GAA GG Rev: AAG CCC AAC AAG AAC CAC AC	341	NM_001912.2 NM_145918.1
CatO	For: AAG GAA GGA AAA GGG CAA AA Rev: CAG AGA CAA AGG GAG CCA AC	356	NM_001334.2
CatS	For: GGA TCA CCA CTG GCA TCT CT Rev: GTT GAG CAA TCC ACC AGG TT	444	NM_004079.3
CatW	For: ACT TGG GCA CAG CTG AGT TT Rev: TCT GCA GCA TGA TGA AGT CC	495	NM_001335.2
CatX	For: AAG GGG GTA ATG ACC TGT CC Rev: CCT CGA TGG CAA GGT TGT AT	483	NM_001336.2
β-Actin	For: CAG AAG GAT TCC TAT GTG GGC Rev: CCA TCA CGA TGC CAG TGG TA	316	NM_001101

Cathepsin activities in conditioned media studied with peptide substrates

Activity assays with recombinant cathepsins and cathepsins in conditioned media were performed in triplicate. The peptide substrates Z-RR-AMC (AMC=7-amido-4-methyl-coumarin; to determine cathepsin B activity) and Z-GPR-AMC (to determine cathepsin K/L activities) were applied at a concentration of 10 μ M (Peptides International, Louisville, Kentucky). Ten microliters of 10-fold-concentrated culture supernatant and 10 μ M of each peptide substrate were combined in a total volume of 100 μ L 25 mM MES/5 mM dithiothreitol (DTT), pH 5.0. A range of 10–30 ng of the recombinant cathepsin proteases was utilized in the assays. To exclude cathepsin B activity in cathepsin K activity measurements with Z-GPR-AMC, these measurements were performed with 1 μ M of the inhibitor CA074 (Sigma-Aldrich, Hamburg, Germany). Cathepsin L activity could not be excluded as N-[(1S)-3-methyl-1-[[[(1S,2E)-1-(2-phenylethyl)-3-(phenylsulfonyl)-2-propen-1-yl]amino]-carbonyl]butyl]-4-morpholinecarboxamide (LHVS) blocked not only cathepsin L activity, but also cathepsin K activity.

The excitation/emission wavelengths of Z-RR-AMC and Z-GPR-AMC were 340_{ex}/460_{em}. The respective wavelengths of the FRET substrate Mca-RPPGFSAFK(Dnp)-OH [Mca=(7-methoxycoumarin-4-yl) acetyl] were 340_{ex}/405_{em}. The time-dependent increase of the fluorescent product over time was measured in a Tecan fluorescence plate reader (Tecan, Crailsheim, Germany).

Digestion of CXCL12 analyzed by matrix-assisted laser desorption/ionization-time of flight analysis

About 3 μ M of recombinant CXCL12 (Peprotech) was incubated with cathepsin B (51 nM), cathepsin L (5.1 nM), or

cathepsin K (51 nM). Digestion was stopped at different time points by adding 1% TFA. All digests were performed in 25 mM MES buffer pH 5.0 containing 5 mM DTT. The unprocessed and processed chemokine CXCL12 α were analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis using a Bruker Daltonics Reflex IV MALDI-TOF mass spectrometer (Bremen, Germany). Calibration was performed with a protein standard from Bruker. Samples were prepared according to the “dried droplet method.” The 2,5-dihydroxybenzoic acid (DHB) matrix was added to the anchor chip target, and 1.5 μ L of each sample was crystallized on top. MALDI measurements were performed using the Flex control and Flex analysis softwares.

CXCL12 enzyme-linked immunosorbent assay

CXCL12 enzyme-linked immunosorbent assay (ELISA) measurements characterized the processing of CXCL12 by cathepsins since the capture antibody recognizes and binds the C-terminal region of CXCL12 (R&D Systems, Wiesbaden, Germany). About 3 μ M CXCL12 (Peprotech) was incubated in MES/DTT buffer with or without 50 nM of the different cathepsins. Nonprocessed and processed CXCL12 can be recognized by the capture antibody. Bound C-terminal noncleaved CXCL12 could be detected by enzymatic HRP activity after antibody binding, according to the manufacturers' instruction (R&D Systems).

Chemotaxis assay

Migration assays were performed in ChemoTx[®] plates with a pore size of 3 μ m (Neuroprobe, Gaithersburg, MD). The membranes were precoated with 100 ng/ μ L laminin-511/521

(Sigma) and gently washed with PBS. About 1.7×10^4 CD34⁺ HSPCs derived from umbilical cord blood were added on top of the membranes. The lower chambers contained no stimulus, CXCL12 α (100 ng/mL), or protease-digested CXCL12 α (100 ng/mL). After 16 h of incubation, the number of cells present in each lower chamber was determined by manual counting. All experiments were performed in triplicates.

Influence of cathepsins on HSPC adhesion to osteoblasts

To test the different cathepsins' abilities to abate HSPC adhesion to osteoblasts, MACS-sorted CD34⁺ HSPCs, which were fluorescently labeled with BCECF-AM [2', 7'-bis (2-carboxyethyl)-5 (and 6)-carboxyfluorescein acetoxymethyl-ester; Sigma-Aldrich], were incubated for 60 min on an adherent layer of osteoblasts. About 4×10^4 primary osteoblasts were seeded in 48-well plate chambers 24 h prior to the assay. After binding of HSPCs to osteoblasts, the different proforms of cathepsin B, K, L (185 ng per well), and X (92.5 ng per well) were activated by incubation in 25 mM MES pH 5.0 and 5 mM DTT for 5 min. The activated cathepsins were then added to the different wells.

To determine whether proteolytically activated CatX is more capable of influencing HSPC binding to osteoblasts,

CatX (92.5 ng per well) was preincubated with CatL in a ratio of 5:1 for 40 min prior to addition to cells.

After 20 min, unbound cells were removed. The amount of cell adhesion between HSPCs and osteoblasts was determined by measurement of the remaining fluorescence.

Activation of pro-cathepsin X

To analyze the proteolytic activation of cathepsin X, pro-cathepsin X was preincubated with DTT-activated cathepsin L for 1 h at RT. The absolute DTT concentration per well was 0.1% DTT in 25 mM NaOAc, pH 3.5. Since cathepsin L is also able to convert the fluorogenic substrate Mca-RPPGFSAFK(Dnp)-OH that is known to be converted by cathepsin X/A, cathepsin L was specifically blocked for 15 min with 10 μ M LHVS. Then, the pro-cathepsin X substrate was added and the enzymatic activity was measured by a Tecan reader.

Peptide mass fingerprinting of pro-cathepsin X and mature cathepsin X

To investigate whether any of the cathepsins present in osteoblast-conditioned medium might be capable of proteolytically activating cathepsin X, 100 ng pro-cathepsin X

FIG. 1. Expression and secretion of cathepsins by cells of the bone marrow microenvironment. (A, C) RT-PCR analyses with cathepsin-specific primers show the transcription of the cathepsins B, C, D, F, H, K, L, O, and S by the osteoblastic cell lines CAL72, MG-63, and G292 (A) and by primary osteoblasts (C: pOB). Cathepsin X is also expressed by all osteoblastic cells except G292 (A). HSPCs do not express cathepsin X, but these cells additionally express the cathepsins G and W (C). (B) A similar transcription profile as seen in osteoblasts was observed for the bone marrow stromal cell lines HS-5, L87/4, and L88/5. (D) Primary osteoblasts secrete the pro-cathepsins B, K, L, and X, whereas the stromal cell lines HS-5, L87/4, and L88/5 secrete only small amounts of these cathepsins compared with primary osteoblasts, as shown by western blotting. The osteoblastic cell lines CAL72, MG-63, and G292 secrete cathepsins B and L and, with the exception of G292, cathepsin X, whereas cathepsin K is not secreted by these cell lines. RT-PCR, reverse transcriptase-polymerase chain reaction.

(219.3 nM) was incubated overnight with 20 ng of the cathepsins B, K, or L. The processed forms were examined by tryptic digest and peptide mass fingerprinting (PMF). Aliquots of 30 ng pro-cathepsin X that had been processed by the different cathepsins were run on SDS gels. After zinc staining with Roti[®] White (Carl Roth, Karlsruhe, Germany), the processed CatX forms were excised from the gels. Each gel piece was dehydrated with 100 μ L acetonitrile (ACN) for 10 min. The gel pieces containing the proteins were reduced in the dark during a 30-min incubation at 56°C with 10 mM DTT/50 mM ammonium bicarbonate (AB). The proteins

were then alkylated with 100 μ L of 55 mM IAA/50 mM AB. Afterward, the gel pieces were washed with 50 mM AB for 15 min. They were washed again with 70% 50 mM AB/30% ACN for 10 min before they were allowed to completely dehydrate with CAN. Twenty microliters of trypsin (sequencing grade; Promega, Mannheim, Germany) was added to the dehydrated samples. After 90 min of digestion, 0.75 μ L of the samples was deposited on a MALDI gold target plate with 0.75 μ L of a DHB matrix for measurements. Mass comparison was performed with MASCOT (www.matrixscience.com/).

FIG. 2. Activities of cathepsins B, K, and L in osteoblast-conditioned media and cathepsin X activity in the presence of DTT. **(A)** Cathepsin B in a 10-fold concentration of osteoblast-conditioned medium (pOB 10 $\times$ CM) or G292-conditioned medium (G292 10 $\times$ CM) converted the CatB-specific substrate Z-RR-AMC. Recombinant CatB (10 ng) led to a similar substrate conversion whereas only moderate activity was observed using the same amount of recombinant cathepsin X compared with cathepsin B activity. **(B)** Several inhibitors were used to specifically analyze cathepsin K activity, since the cathepsin K-specific substrate Z-GPR-AMC is not only cleaved by cathepsin K but also by cathepsin B, and to a lesser extent, by cathepsin L. However, in the presence of LHSV (2 μ M) and CA074 (1 μ M), not only were cathepsins L and B completely inhibited but also cathepsin K activity was totally abolished. **(C)** The cathepsin B-specific inhibitor CA074 (1 μ M) was preincubated with osteoblast-conditioned media before adding the substrate Z-GPR-AMC. Cathepsin K activity in pOB-conditioned medium could be confirmed, although cathepsin L activity could not be completely excluded. **(D)** Using the Mca-RPPGFSAFK(Dnp)-OH substrate recombinant pro-cathepsin X only showed activity in the presence of DTT. The inset shows active-site labeling of recombinant cathepsin X with DCG-04 in the presence or absence of DTT. All data are given as means of triplicate measurements. DTT, dithiothreitol; LHSV, N-[(1S,2E)-1-(2-phenylethyl)-3-(phenylsulfonyl)-2-propen-1-yl]amino]carbonyl]butyl]-4-morpholinecarboxamide.

Statistical analysis

Statistical analysis was performed with unpaired, 2-tailed *t*-tests with Welch correction. A *P* value of 0.05 was considered as statistically significant.

Results

Expression and secretion of cathepsins by osteoblasts, stromal cells, and CD34⁺ HSPCs

The expression of several cathepsins was examined by RT-PCR analyses with human cathepsin-specific primers (Table 1). For the 3 investigated osteosarcoma cell lines, CAL72, MG-63, and G292, the expression profile of their cathepsin mRNAs is comparable with each other, with the exception of cathepsin

X, which is expressed by the osteosarcoma cell lines CAL72 and MG63, but not by G292 cells (Fig. 1A). Among the cultured bone marrow stromal cell lines, cathepsin S was barely detectable in HS-5 cells; however, it was strongly detected in L87/4 and L88/5 cells (Fig. 1B). Primary osteoblasts exhibit a similar cathepsin mRNA expression profile compared with the osteosarcoma cell lines. On the other hand, CD34-sorted HSPCs also show cathepsin G and cathepsin W expression, but no cathepsin X expression (Fig. 1C).

Functional cathepsins are mainly found in the endolysosomal compartment, but we were interested in the cathepsins secreted by the nonhematopoietic bone marrow cells. Conditioned media of primary osteoblasts, the osteoblastic cell lines, and the bone marrow stromal cell lines were analyzed by western blotting. Strong signals for the cysteine

FIG. 3. Time-dependent processing of CXCL12 by recombinant cathepsins. **(A)** Recombinant CXCL12 (3 μ M) was incubated in MES/DTT buffer in the absence (*upper panel*) or presence of recombinant cathepsins B, K, and L for several periods (10, 20, and 40 min) and the processing of CXCL12 was investigated by MALDI-TOF analysis. After 40 min of incubation, intact CXCL12 could only be detected with cathepsin K. Processing of CXCL12 was detected for all these cathepsins **(A)**, and after an extended incubation, it was also detected with cathepsin X **(B)**. MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight.

cathepsins B, K, L, and X were found in the supernatants of primary osteoblasts (Fig. 1D). Only weak or negligible signals for these proteases were found in the cell culture supernatants of the bone marrow stromal cell lines. The osteoblastic cell lines did not secrete cathepsin K, but they strongly expressed and secreted cathepsins B and L (Fig. 1D). Cathepsin X is expressed by CAL72 and MG63, but not by G292 cells (Fig. 1A, D), as expected from the RT-PCR data (Fig. 1A).

Activities of the secreted cathepsins B, K, L, and X in osteoblast-conditioned cell culture media

Proteolytic activities of the identified cathepsins secreted by osteoblasts were examined with different fluorogenic peptide substrates. The specificities of these substrates were verified by simultaneous measurements in which purified recombinant human cathepsins were used.

Initial measurements with the fluorogenic peptide Z-FR-AMC, which was recommended as a cysteine cathepsin-specific substrate, did not clearly distinguish between cathepsin B- and cathepsin X-specific activities (data not shown). Since cathepsin X activity is present in the osteoblast conditioned media [6], cathepsin B measurements were performed with the substrate Z-RR-AMC that was rapidly converted into its fluorescent product by cathepsin B (Fig. 2A). Recombinant cathepsin X, on the other hand, showed only a weak proteolytic activity against Z-RR-AMC. Using the peptide Z-RR-AMC, cathepsin B activity in conditioned media of pOB and G292 cells could be verified (Fig. 2A).

Cathepsin K activity in osteoblast-conditioned medium was investigated by applying the peptide Z-GPR-AMC (Fig. 2B). When using this substrate, however, we observed that both cathepsin K and B are able to cleave Z-GPR-AMC, cathepsin B even better than cathepsin K. The cathepsin L-specific inhib-

itor LVHS (1 μ M) also blocked cathepsin K activity (Fig. 2B). As a result, we only applied the cathepsin B-specific inhibitor CA074 in pOB-conditioned media and performed further cathepsin K activity measurements. The observed activity is mainly mediated by cathepsin K, although cathepsin L activity cannot be completely excluded (Fig. 2C).

Cathepsin X activity in pOB-conditioned media has been shown in an earlier study [6]. But since no proteolytic self-activation of CatX has ever been shown, we further analyzed whether pro-cathepsin X is also able to convert the fluorogenic substrate Mca-RPPGFSAFK(Dnp)-OH. Surprisingly pro-cathepsin X converts this substrate in the presence of DTT, whereas in the absence of DTT, this cysteine protease is barely active (Fig. 2D). This result is in accordance with the finding that pro-cathepsin X can only be labeled with the active site label reagent DCG-04 if DTT is present (small box in Fig. 2D).

Potential of the different osteoblast-secreted cathepsins to digest the chemokine CXCL12

The chemokine CXCL12 is of pivotal importance for retaining stem cells in the human bone marrow, but its actual concentration is under the control of a delicate proteolytic network. To check whether the osteoblast-secreted cathepsins are also part of this network we incubated 3 μ M CXCL12 with 51 nM recombinant cathepsin B or K or 5.1 nM cathepsin L for different time periods. All analyzed cathepsins were capable of digesting CXCL12 in a time-dependent manner (Fig. 3). After 40 min of incubation, no intact CXCL12 is detectable anymore, as seen by MALDI-TOF analysis (Fig. 3A). Cathepsin L seems to be the most effective enzyme regarding CXCL12 digestion. After 10 min, no intact CXCL12 is detectable by MALDI-TOF analysis. In comparison, complete digestion of CXCL12 with cathepsin X takes much longer (Fig. 3B).

FIG. 4. Inactivation of C-terminally processed CXCL12 by cathepsins B, K, L, and X. **(A)** Nontruncated CXCL12 (SDF-1) was measured in a CXCL12 ELISA. After 2 h of incubating the chemokine with different recombinant cathepsins, only small amounts of the intact CXCL12 could still be detected. The amount of the intact chemokine further decreased after 16 h. **(B)** To investigate the activity of the chemokine, 2 h-preincubated processed and nonprocessed CXCL12 were used in cell migration assays with MACS-sorted CD34⁺ HSPCs. Significant reduction of CD34⁺ cell migration compared with unprocessed CXCL12 was only detected for cathepsin B-cleaved CXCL12 after 2-h incubation although a strong trend of decreased cell migration was also observed for cathepsin L. **Highly significant, P -value < 0.005. ELISA, enzyme-linked immunosorbent assay.

Functional inactivation of the chemokine CXCL12 by cathepsin B

CXCL12 was incubated in 25 mM MES or NaOAc buffer containing 5 mM DTT in the presence or absence of 50 nM cathepsin B, K, L, or X for 2 or 16 h. A CXCL12 ELISA directed against the C-terminal end of the intact chemokine showed that after 2 h of incubation with the individual cathepsins most of the intact chemokine is lost (Fig. 4A). The digested chemokine was tested in migration assays performed in ChemoTx® plates. Intact CXCL12 or cathepsin-processed CXCL12 was inserted in the lower chambers of the plate as migration-inducing stimuli. The MACS-sorted CD34⁺ HSPCs were added to the upper chambers. After 16 h of incubation, only CXCL12 that had been processed by activated cathepsin B led to a highly significant decrease of HSPC migration compared with nonprocessed CXCL12 (Fig. 4B). Incubation of CXCL12 with cathepsin K or X did not lead to a substantial reduction of HSPC migration. A strong trend of functional inactivation of CXCL12 was also seen for cathepsin L treatment in the migration assay, although this effect did not turn out to be significant compared with the unprocessed chemokine using an unpaired *t*-test with Welch correction (Fig. 4B).

Activated cathepsins B, K, and L, in contrast to DTT-treated pro-CatX, have no influence on CD34⁺ HSPC adhesion to primary osteoblasts

Since cathepsin X had been shown to modulate pre-existing adhesive HSPC–osteoblast interactions [6], we tested whether the other osteoblast-secreted cathepsins also could influence HSPC adhesion to osteoblasts. However, neither cathepsin B nor K nor L alone was able to interfere with the adhesive interactions of HSPCs to primary osteoblasts (Fig. 5). Interestingly only pro-cathepsin X pretreated with DTT showed a significant reduction of cell adhesion. However, mature cathepsin X, which was proteolytically activated by cathepsin L, showed no reduction of HSPC binding, suggesting that the prodomain of cathepsin X is directly involved in the modulation of HSPC–osteoblast interactions (Fig. 5).

Pro-cathepsin X can be processed by cathepsins B, K, and L, but only cathepsin L activity leads to cathepsin X maturation and proteolytic activation

Since cathepsin X cannot be activated autocatalytically, we tested whether the other osteoblast-secreted cysteine cathepsins were able to proteolytically activate cathepsin X by removing its prodomain. Since the molecular masses of the different cysteine cathepsins are not drastically different in a silver-stained SDS gel (Fig. 6A) we also performed western blot analysis with a cathepsin X-specific antibody that can detect the 40 kDa immature form and the 37 kDa mature form of cathepsin X. Only the treatment of pro-cathepsin X with cathepsin L led to a complete conversion of the immature form to the activated mature form of cathepsin X (Fig. 6B). An activity assay of cathepsin X that was activated by cathepsin L and then treated with LHVS to specifically inactivate cathepsin L activity showed an enhanced activity of cathepsin X with the fluorogenic peptide Mca-RPPGFSAFK(Dnp)-OH

FIG. 5. Influence of cathepsins B, K, L, and X on HSPC adhesion to osteoblasts. After binding of HSPCs to osteoblasts recombinant preactivated cathepsins were added and incubated with the cells for 20 min. After washing, HSPC adhesion was measured. Only recombinant cathepsin X (104 ng per well) significantly decreased HSPC adhesion to osteoblasts whereas twice the amount of cathepsin B, K, or L (185 ng per well) did not result in a significant reduction of HSPC binding. However, preincubation of cathepsin X with cathepsin L in a 5:1 ratio completely abolished the cathepsin X-mediated decrease of cell–cell interactions. Results are presented as mean and range of the single values per well of 3 independent experiments and cord blood donor samples ($n=3$). Statistical analysis was performed using unpaired, 2-tailed *t*-test with Welch correction and *P* values ≤ 0.01 were considered as highly significant and indicated with *.

indicating a proteolytic activation of cathepsin X (Fig. 6C). To prove that only cathepsin L removed the prodomain of cathepsin X, we performed PMF. Cathepsin X was analyzed after treatment with the different cysteine cathepsins and tryptic digestion. The loss of the prodomain of cathepsin X indicated by the loss of the 1971 kDa peak was only seen in the presence of cathepsin L, whereas cathepsins B and K were not capable to cleave off the prodomain of pro-cathepsin X (Fig. 6D, E).

Discussion

In the current study, we have demonstrated that human osteoblasts, which are an essential component of the endosteal hematopoietic stem cell niche, actively secrete several members of the cysteine cathepsin family capable of different activities. The chemokine CXCL12, part of the crucial CXCR4/CXCL12 axis in the niche, can be proteolytically processed by virtually all osteoblast-secreted cysteine cathepsins, although with different kinetics and efficacy. The most effective digestion was observed for cathepsin B since this protease almost completely abolished the migratory activity of the

FIG. 6. Pro-cathepsin X is proteolytically activated after incubation with cathepsin L. (**A**, **B**) Pro-cathepsin X was either incubated in NaOAc/DTT buffer or incubated in the same buffer in the presence of cathepsins B, K, and L in a ratio of 5:1. The stained SDS gel indicated processing of pro-cathepsin X by the other cathepsins (**A**), which could be further confirmed by western blotting (**B**) with a cathepsin X-specific antibody. Incubation with cathepsins B and K showed only partial processing whereas incubation with cathepsin L led to complete processing of pro-cathepsin X. (**C**) Activity assays of cathepsin X preincubated with cathepsin L in the presence of the cathepsin L-specific inhibitor LHVS demonstrated higher cathepsin X activity after proteolytic activation. (**D**, **E**) To analyze whether the prodomain is lost after incubation and thereby if the processing occurs at the C-terminal end, peptide mass fingerprinting was performed after band excision of a zinc-stained gel. MALDI-TOF analysis of the peptide mass fingerprinting revealed the specific peak of 1971 Da, indicating the prodomain is only lost after incubation with cathepsin L.

chemokine for HSPCs after a short incubation period. Interestingly, all proteolytically mature forms of the secreted cathepsins did not influence adhesive interactions between HSPCs and osteoblasts, whereas only partially activated pro-cathepsin X did. Therefore we propose that during homeostasis bone marrow osteoblasts constitutively secrete cysteine cathepsins that can differentially modulate the communication between HSPCs and their endosteal niche.

This study aimed to investigate which of the cysteine cathepsins secreted by hematopoietic stem cell niche cells can influence HSPCs. Our RT-PCR analyses of primary osteoblasts, the 3 osteoblastic cell lines, and the 3 bone marrow stromal cell lines indicated that these cell types transcribe the mRNA of most members of the cysteine cathepsin family. On

the contrary, the serine cathepsin G, which is an active component of the proteolytic microenvironment of the bone marrow during stem cell mobilization [26], is not synthesized by osteoblasts or bone marrow stromal cells. Secretion of the translated cysteine cathepsins by primary osteoblasts was detected for cathepsin X as reported earlier [6], for cathepsins B and K—which is in agreement with recent studies [7,8]—and for cathepsin L, whereas bone marrow stromal cells secreted much less of these cysteine cathepsins, if at all. The molecular weight of the osteoblast-secreted cysteine cathepsins as observed by western blotting suggested that these cathepsins are secreted as proteolytically inactive zymogens.

To determine whether the secreted cysteine cathepsins were proteolytically active the osteoblast-conditioned media

FIG. 7. Suggested model of different cathepsin X activities. Pro-cathepsin X with its disulfide-bound prodomain can be proteolytically activated by cathepsin L. The mature cathepsin X without its prodomain has a higher proteolytic activity but cannot interfere with HSPC–osteoblast adhesive interactions. However, if the disulfide bond in pro-cathepsin X is reduced by a reducing agent such as DTT, the unfolded pro-cathepsin X can interfere with adhesive interactions between HSPCs and osteoblasts. This indicates that pro-cathepsin X unfolds but the prodomain is still covalently linked to the catalytic domain as shown in the western blot (lower part, on the right). Only reduced pro-cathepsin X is proteolytically active as shown by active site labeling of pro-cathepsin X in the presence and absence of DTT.

were analyzed with different fluorogenic peptide substrates in combination with cathepsin-specific inhibitors. The conditioned media of primary osteoblasts and of the osteoblastic cell line G292, which both secreted cathepsin B, but differed in the secretion of cathepsin X, strongly converted the substrate Z-RR-AMC indicating cathepsin B activities in both supernatants. The proteolytic activity of cathepsin B can be specifically blocked by the CA-074 inhibitor. Using a combination of this inhibitor with the peptide substrate Z-GPR-AMC, a strong activity of cathepsin K, most probably in combination with cathepsin L, could be observed. Our results indicate that human osteoblasts, in addition to secreting cathepsin X [6], can constitutively secrete further proteolytically active cysteine cathepsins. Interestingly, pro-cathepsin X can only convert a fluorogenic substrate in the presence of the reducing agent DTT. Whether naturally occurring reducing agents (e.g., glutathione) are also present at the endosteum has to be analyzed in future studies, and some recent reports suggest a role of the redox status in hematopoietic stem cell homing and mobilization [27,28].

The chemokine CXCL12 that is highly expressed by endosteal osteoblasts is a very potent chemoattractant for human hematopoietic stem cells [14]. Degradation of CXCL12 in the endosteal niche, together with an enhanced concentration of CXCL12 in the peripheral blood, leads to stem cell mobilization into the periphery [29]. Our MALDI-TOF analysis revealed that all cysteine cathepsins that are constitutively secreted by human osteoblasts are capable of degrading CXCL12 although with a different efficacy. Interestingly, all cathepsins (B, K, L, and X) seemed to degrade CXCL12 at the C-terminal end since the ELISA used for determination of intact CXCL12 after 2 h of incubation with the proteases was specifically directed against the C-terminal domain of CXCL12. However, when we applied a migration assay to determine the chemoattractant activity of CXCL12, only cathepsin B-treated CXCL12 was functionally inactivated. It is well documented that MMPs such as MMP-2 or -9, as well as the serine proteases neutrophil elastase or cathepsin G, can inactivate CXCL12 by cleaving off 3 or 4 amino acids from the N-terminus of the chemokine [30]. This N-terminal domain is essential for the interaction of the chemokine with its

receptor CXCR4 [31]. As recently reported for the carboxypeptidase M, a C-terminal truncation of CXCL12 might also lead to a functional inactivation of the chemokine [17]. Our MALDI-TOF analysis data combined with the functional migration analysis, however, suggest that a C-terminal truncation only does not seem to be sufficient for an effective inactivation of the chemoattractant nature of CXCL12.

Interestingly, neither activated cathepsin B nor cathepsin K nor cathepsin L was able to interfere with adhesive interactions of HSPCs with osteoblasts. We had recently reported that cathepsin X activated with 5 mM dithiotreitol at pH 5.0 can significantly reduce the binding of HSPCs to osteoblasts. Accordingly, an siRNA-mediated knockdown of endogenous cathepsin X in osteoblasts resulted in an enhanced HSPC binding to osteoblasts [6]. Now we show by PMF that only cathepsin L, but not cathepsin B or K, is capable of proteolytically activating cathepsin X by cleaving off the prodomain of this protease. However, this proteolytic cleavage and full activation of cathepsin X by cathepsin L abrogated the inhibitory function of cathepsin X for cell–cell interactions. The nonactivated zymogen of cathepsin X is also unable to interfere with these adhesive interactions [6]. Considering the proteolytic and biological activities and the activation of cathepsin X we therefore suggest the following model (Fig. 7).

The proteolytic activity of pro-cathepsin X is inhibited when its prodomain is tightly bound to the catalytic domain via a disulfide bond [32]. Removal of the prodomain by another protease such as cathepsin L can fully activate cathepsin X, but the activated enzyme cannot interfere with cell–cell interactions, indicating that the proform is needed for the inhibition of adhesive interactions. This prodomain has been shown to directly bind to integrins via its RGD sequence [33]. Thus we hypothesize that reducing agents such as DTT can open up the disulfide bond that makes the prodomain more accessible while still covalently linked to the catalytic domain. Within the niche, hypoxic conditions and naturally occurring redox regulators such as the tripeptide glutathione might fulfill this function [27,28]. This model can also explain why the active site label reagent DCG-04 can bind to DTT-treated pro-cathepsin X while the

untreated zymogen cannot be labeled with DCG-04. Thus the prodomain of cathepsin X is required for the observed inhibition of HSPC–osteoblast interactions but only in an open form with a reduced disulfide bond.

The current study showed that human osteoblasts can constitutively secrete several members of the cysteine cathepsin family that are capable of degrading CXCL12. Given the important function of the CXCL12/CXCR4 axis in the retention of hematopoietic stem cells to their niche(s), the actual concentration of extracellular CXCL12 concentration must be under tight control. Secreted cystatins are well-known natural inhibitors of cysteine cathepsins [34]. A detailed analysis of the expression, function, and secretion of different cystatins by human osteoblasts, but also by other bone marrow stromal cell types, will help to elucidate how the secreted cysteine cathepsins in the bone marrow stem cell niche(s) are regulated.

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Author Disclosure Statement

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