

Granzyme H Is a Novel Protease Expressed by Human Mast Cells

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Key Words

Granzyme H · Mast cells · Proteases

Abstract

Background: Many of the functions attributed to mast cells depend on the various pro-inflammatory mediators that are secreted upon mast cell activation. These include a panel of mast cell-specific proteases. In addition, recent studies have indicated that murine mast cells also express granzyme D, a protease previously thought to be confined to cytotoxic lymphocytes. Here, we address the human relevance of the latter findings by investigating whether human mast cells express granzyme H, the granzyme that may represent the functional counterpart to murine granzyme D. **Methods:** Cord blood-derived mast cells, LAD2 cells and skin mast cells in situ were evaluated for their expression of granzymes using quantitative PCR, Western blot analysis and immunostaining. Mast cells were activated by either calcium ionophore stimulation or IgE receptor cross-linking. **Results:** Cord blood-derived mast cells and LAD2 cells were shown to express granzyme H and B mRNA, while granzyme A, K and M expression was undetectable. Mast cell activation by either calcium ionophore or IgE receptor cross-linking caused down-regulated expression of granzyme H. In contrast, granzyme B expression was up-regulated by the same stimuli. Granzyme H expression was also confirmed at the pro-

tein level, as shown by both Western blot analysis and confocal microscopy. Further, we show that granzyme H is expressed by human skin mast cells in situ. **Conclusions:** The present findings implicate granzyme H as a novel protease expressed by human mast cells and support earlier findings obtained in natural killer cells suggesting that granzymes B and H are reciprocally regulated.

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Introduction

Mast cells are major effector cells of the immune system, with important roles both in adaptive as well as innate immunity [1, 2]. In particular, mast cells are well known for their detrimental impact on various allergic conditions, but it is also widely recognized that mast cells play a harmful role in other types of disorders, including arthritis, cancer and atherosclerosis [1, 2]. On the other hand, mast cells are also known to have functions that are beneficial to their host, most notably in the protection towards bacterial infection but also in the defence towards viruses and parasites [3].

Elin Rönnerberg and Gabriela Calounova contributed equally to this work.

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It has been known for many years that various mast cell-specific proteases, e.g. tryptase, chymase and CPA3, are major products of mast cells being stored in large quantities within mast cell granules [4, 5], and it has been shown that these mast cell-specific proteases account to a large extent for the impact of mast cells on various pathological conditions [5]. In addition to their cell-specific proteases, mast cells have been shown to express a number of proteases whose expression is not limited to mast cells, e.g. renin [6], cathepsin G [7] and various metalloproteases [8, 9]. Moreover, it has been shown that murine and human mast cells express granzyme B [10–12], a proapoptotic protease that previously was thought to be predominantly a product of cytotoxic lymphocytes [13]. In line with the latter findings, we noted that one of the genes whose expression was affected to the highest extent by activation of murine mast cells by multiple stimuli was the gene encoding another member of the granzyme family – granzyme D [14, 15]. Hence, these studies implicate granzyme D as a novel protease expressed by murine mast cells, and the present study aimed to investigate if these findings can be translated into a human setting. Humans do not express a direct orthologue to murine granzyme D. However, examining the substrate specificity of the human granzymes (granzymes A, B, H, K and M), it is notable that granzyme H is the only human granzyme family member that has similar activity as granzyme D, i.e. chymotrypsin-like cleavage specificity [16–18]. Therefore, granzyme H may represent the closest functional homologue to murine granzyme D. Here, we show that granzyme H is a novel protease expressed by human mast cells.

Materials and Methods

LAD2 Cells

The LAD2 line isolated from the bone marrow of a patient with mast cell leukaemia [19] was a gift from A. Kirshenbaum and Dean D. Metcalfe (National Institutes of Health). LAD2 cells were cultured in StemPro-34 serum-free medium (Gibco, Life Technologies) supplemented with 2 mM L-glutamine (SVA, Sweden), 100 IU/ml penicillin-streptomycin (SVA) and 100 ng/ml stem cell factor (PeproTech). Half of the cell culture medium was replaced every week with fresh medium supplemented with 100 ng/ml stem cell factor.

Cord Blood-Derived Mast Cells

Fresh cord blood was diluted 4 times with PBS and carefully overlaid on Ficoll-Paque Plus (GE Healthcare). After centrifugation (400 g, 30 min), the interface containing mononuclear cells was transferred to new 50-ml Falcon tubes and washed with PBS. CD34+ cells were selected from the mononuclear cell fraction us-

ing MACS separation columns (LD Columns) and the CD34 human MicroBead kit (Miltenyi Biotec, Gladbach, Germany). The CD34+ cells selected were cultured for 4 weeks in StemPro-34 serum-free medium supplemented with L-glutamine (2 mM), 6 mg/ml penicillin G, 5 mg/ml streptomycin sulphate, rhIL3 (1 ng/ml; PeproTech), stem cell factor (100 ng/ml) and rhIL6 (10 ng/ml; PeproTech). After the 1st week, rhIL3 was excluded. After 4 weeks of culture, cord blood-derived mast cells (CBMCs) were stained for tryptase-like enzymatic activity as a marker of maturity. For tryptase staining, cytopsin slides were incubated for 30 min with a solution of 10 mM Z-Gly-Pro-Arg 4-methoxy-2-naphthylamine (Sigma) in 0.5 M Tris-HCl (pH 7.5) and 5 mg/ml Fast Garnet GBC sulphate salt (Sigma).

Quantitative Real-Time RT-PCR

Total RNA was isolated and analyzed by real-time quantitative RT-PCR (qPCR) as previously described [9] using the primers specified below. Granzyme H: forward 5'–3' TTCAAGGGGG ACTCCGGGGG; reverse 5'–3' TGCCTGCTGTAGAGGCGCT; granzyme B: forward 5'–3' AGGAAACAACAGCAGCTCCAA CCA, reverse 5'–3' TCCCCTGCATCTGCCCTGGG; GAPDH: forward: 5'–3' TCGACAGTCAGCCGCATCTTCTTT, reverse: 5'–3' ACCAAATCCGTTGACTCCGACCTT; TPSAB1: forward 5'–3' CACCCGTGTACCTACTACTTG, reverse: 5'–3' TTGGA CAGCAGGGGTTGGTC; TPSB2: forward 5'–3' TATGTCCCC AAAAGCCGTGA, reverse: 5'–3' CACCTGGGTAGGAAGCA GTG, and CMA1: forward 5'–3' GGGCAATCCCAGGAAGA CAA, reverse: 5'–3' CCGTAATGGGAGATTCGGG. In addition, 3 primer pairs each for granzymes A, K and M (not shown) were evaluated.

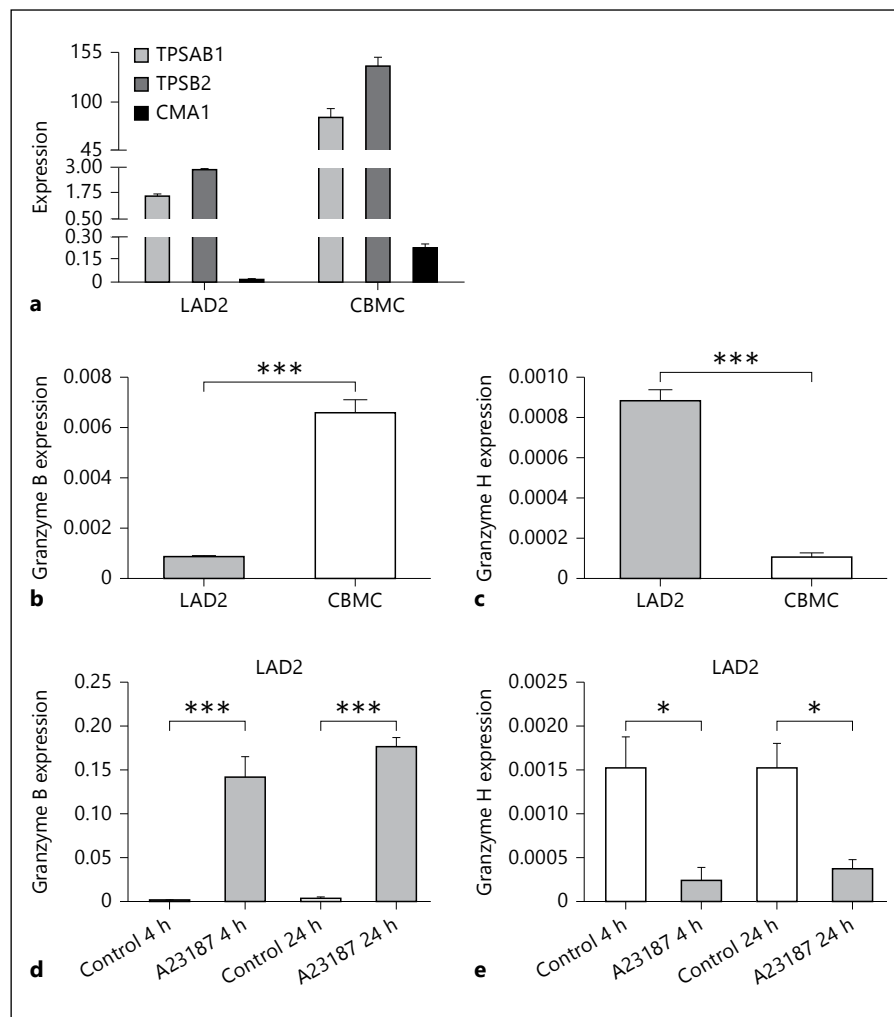
Western Blot Analysis and ELISA

Cell lysates were separated on 12% SDS-PAGE gels and electrophoretically transferred onto polyvinylidene difluoride membranes. Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences; Cambridge, UK) and incubated with primary monoclonal antibody towards human granzyme H [20] (diluted 1:500 in blocking buffer) overnight at 4°C, followed by washing and incubation with donkey anti-mouse IR dye 680 (1:1,000; LI-COR Biosciences). Antibody to β -actin [(I-19): sc-1616; Santa Cruz Biotechnology; 1:1,000] was used as a loading control and detected after incubation of membranes with secondary donkey anti-goat IR dye 800 antibody (1:1,000 dilution). Visualization was performed using the Odyssey IR imaging system (LI-COR Biosciences). Granzyme H was also detected by ELISA, using the SEL575Hu kit (USCN Life Science, Aachen, Germany) according to the procedure described by the manufacturer.

Immunostaining of Skin Biopsies

Punch biopsies (3 mm) of lesional skin of a patient with chronic plaque psoriasis were fixed in formalin for 24 h and embedded in paraffin. Sections of 5 μ m were boiled in a pressure cooker in Reveal Decloaker (Biocare Medical, Concord, Calif., USA), blocked in Background Sniper (Biocare Medical) and then incubated with murine monoclonal anti-granzyme H antibody [20] (dilution 1:40 in PBS with 2% BSA). Adjacent sections were stained with anti-tryptase antibody (monoclonal mouse antibody; MAB1222; Chemicon/Millipore Corporation, Billerica, Mass., USA). The MACH3 mouse AP polymer detection kit (Biocare Medical) and

Fig. 1. Human mast cells express granzymes H and B. **a** Expression of mast cell markers, tryptase (TPSAB1 and TPSB2) and chymase (CMA1) by LAD2 cells and CBMCs. **b, c** Comparison of the levels of mRNA encoding granzymes B and H in LAD2 cells and CBMCs. **d, e** LAD2 mast cells (1×10^6 cells) were cultured either in the absence (control) or presence of $1 \mu\text{M}$ of calcium ionophore A23187 for the time periods indicated. Cells were thereafter harvested, followed by isolation of total RNA and analysis by qPCR for levels of mRNA encoding granzymes B (**d**) and H (**e**). Expression levels were normalized to the expression of *GAPDH*. Data are shown as means $\pm$ SEM. $n = 4$. Student's *t* test: * $p < 0.05$; *** $p < 0.001$.



Vulcan Fast Red Chromogen Kit 2 (Biocare Medical) were used to visualize staining, and counterstaining was done with Mayer's haematoxylin.

Mast Cell Activation

Cells (2×10^5 CBMCs or 1×10^6 LAD2 cells/sample) were stimulated for 0.5 or 4 h with $1 \mu\text{M}$ calcium ionophore A23187. In addition, CBMCs were passively sensitized with $2 \mu\text{g/ml}$ of human myeloma IgE (Millipore, AG30P-K) overnight, washed, resuspended in medium (without IgE) and then stimulated with $1 \mu\text{g/ml}$ of goat anti-human IgE (AP175; Millipore) [21].

Immunocytochemistry and Confocal Microscopy

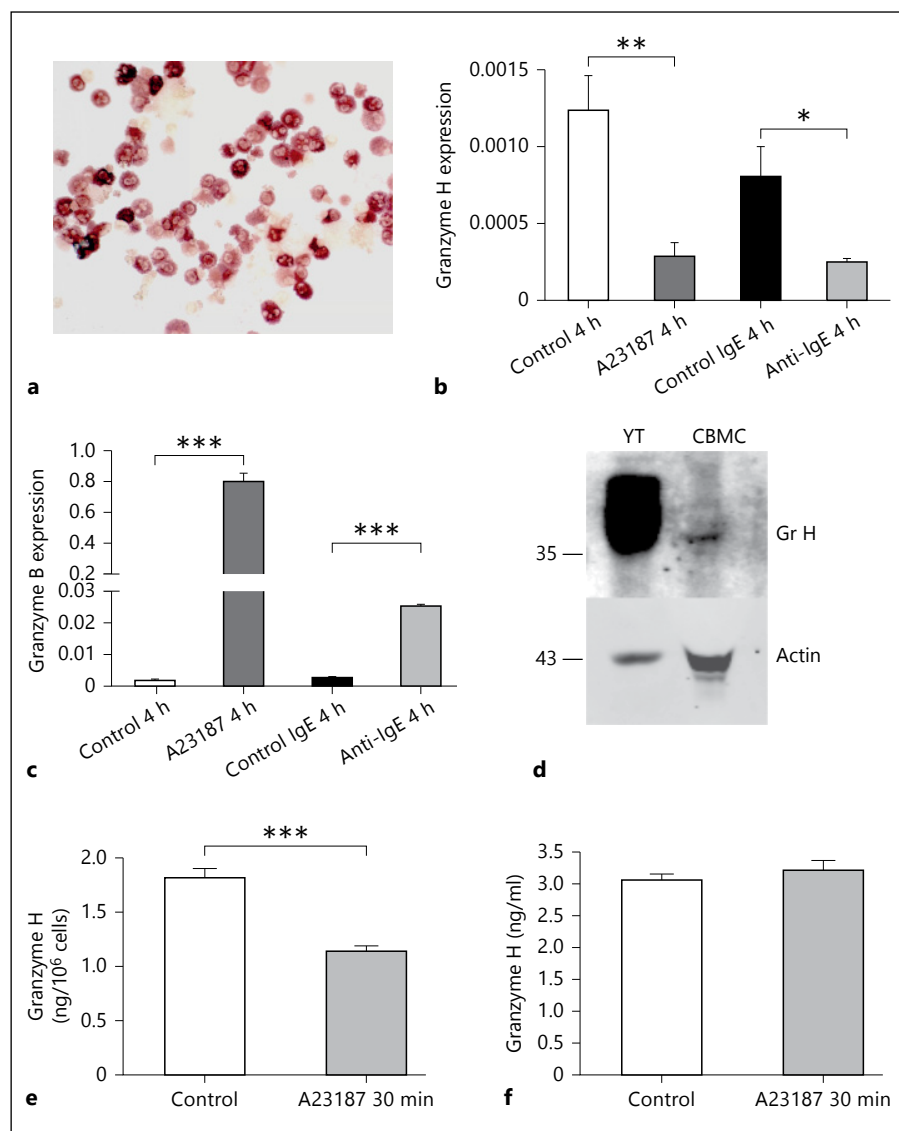
Cytospin slides were dried for 1 h at room temperature, fixed for 10 min with 4% paraformaldehyde and dried, followed by permeabilization with $50 \mu\text{g/ml}$ digitonin (Sigma) in PBS for 10 min. Next, slides were washed in TBS-Tween, followed by blocking with goat serum (1:50 in TBS) for 1 h and incubation with primary monoclonal antibody towards human granzyme H [20] (1:500) or isotype control IgG overnight at 4°C . Cytospin slides were then washed and incubated with secondary antibody (Alexa Fluor 555

goat anti-mouse IgG2a; Invitrogen Life Technologies) for 1 h protected from light. After washing, nuclei were visualized with DAPI. Finally, cells were washed and mounted with fluorescent mounting medium S3023 (Dako). The images were generated using a confocal microscope (Zeiss LSM700) and ZEN 2009 software (Zeiss).

Results

To study the expression of granzymes in human mast cells, we first evaluated LAD2 cells, a well-established mast cell line that shows several features resembling those of mature primary mast cells [19]. To verify that the LAD2 cells have mast cell-like properties, we first analyzed the expression of mast cell markers, tryptase and chymase, in these cells. As shown in figure 1a, LAD2 cells expressed mRNA coding for tryptase (TPSAB1 and TPSB2) and low

Fig. 2. CBMCs express granzyme H. **a** Staining of CBMCs for tryptase, showing positivity as a sign of mast cell maturation. **b, c** CBMCs (2×10^5 cells) were either incubated alone (control) or with $1 \mu\text{M}$ of calcium ionophore A23187 for 4 h. Alternatively, cells were first passively sensitized by incubation overnight with IgE followed by either no additional treatment (control IgE) or addition of anti-IgE antibody for 4 h (anti-IgE). Cells were thereafter harvested, followed by total RNA isolation and analysis by qPCR for levels of mRNA encoding granzymes H (**b**) and B (**c**). Expression levels were normalized to the expression of *GAPDH*. **d** Western blot analysis showing granzyme (Gr) H protein in CBMCs, with YT cell as a positive control. β -Actin was used as loading control. **e** Quantification of cell-associated granzyme H in CBMCs before or after stimulation for 30 min with $1 \mu\text{M}$ calcium ionophore A23187 using ELISA. **f** Detection of granzyme H by ELISA in conditioned medium from non-stimulated and calcium ionophore-stimulated (30 min) CBMCs. Expression levels were normalized to the expression of *GAPDH*. Data are shown as means $\pm$ SEM. $n = 4$. Student's *t* test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



levels of chymase (CMA1), hence verifying that the cells indeed displayed mast cell-like characteristics. Next, mRNA extracted from LAD2 cells was analyzed by qPCR for levels of mRNA encoding all members of the human granzyme family, i.e. granzymes A, B, H, K and M. As shown in figure 1c, expression of granzyme H was detected in LAD2 cells and, in agreement with previous findings [10, 12], granzyme B expression was also detected (fig. 1b). In contrast, granzyme A, K and M mRNA levels were below the level of detection (not shown). As a positive control for granzyme expression, we used YT cells, a natural killer cell line. As expected, YT cells expressed both granzymes B and H, showing expression levels that were higher than in LAD2 cells (data not

shown). To evaluate whether granzyme expression was influenced by mast cell activation, we subjected LAD2 cells to calcium ionophore stimulation. As depicted in figure 1d, e, calcium ionophore stimulation induced a profound increase in the expression of granzyme B, whereas granzyme H expression was repressed.

Although LAD2 cells are widely used as a model to study human mast cells it was important to investigate whether granzyme H is also expressed by non-transformed human mast cells. For this purpose, we developed CBMCs. After a 4-week culture, we observed that CBMCs stained positively for tryptase-like enzymatic activity (fig. 2a), a sign that cells were mature. In agreement with this, qPCR analysis revealed high levels of tryptase

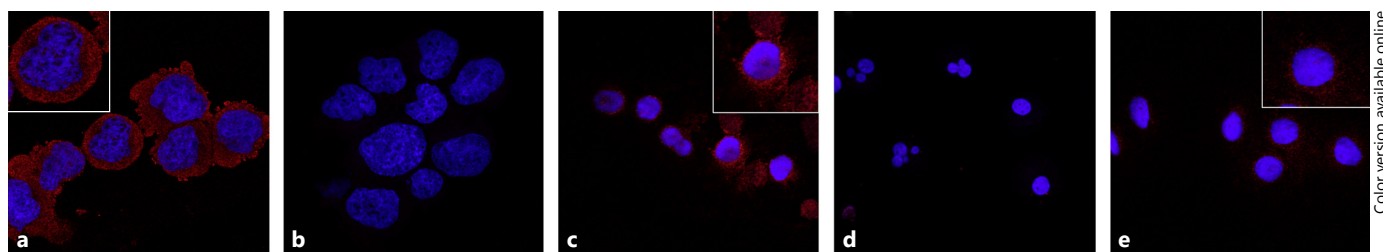


Fig. 3. CBMCs stain positively for granzyme H. YT cells (**a, b**) and CBMCs (**c–e**) were centrifuged onto cytospin slides. Slides were stained with anti-granzyme H antibody (**a, c, e**) using control IgG as negative control (**b, d**). CBMCs were stained either without

any prior treatment (**c**) or after treatment for 30 min with 1 μ M of calcium ionophore A23187 (**e**). DAPI was used as a marker for cell nuclei. Results shown are representative of 3 independent experiments.

(TPSAB1 and TPSB2) expression in CBMCs, whereas CMA1 expression was lower (fig. 1a). Noteworthy, the expression of tryptase and CMA1 was considerably higher in CBMCs than in LAD2 cells. Similar to LAD2 cells, CBMCs were found to express mRNA encoding both granzymes H and B (fig. 2b, c), although it was noted that the expression levels of both of these granzymes were lower than in YT cells (data not shown). It was also noted that, when comparing LAD2 cells and CBMCs, LAD2 cells expressed higher levels of granzyme H and lower levels of granzyme B than did CBMCs (fig. 1b, c).

To study granzyme regulation in CBMCs, we subjected the cells to either calcium ionophore stimulation or IgE receptor cross-linking, the latter representing the classical pathway for mast cell activation that is a hallmark event during allergic reactions for example [22]. As seen in figure 2b, c, similar to LAD2 cells, calcium ionophore induced a profound induction of granzyme B expression whereas granzyme H expression was repressed. When activating CBMCs by IgE receptor cross-linking, similar regulation patterns were seen as for calcium ionophore stimulation, i.e. elevated expression of granzyme B accompanied by down-regulated expression of granzyme H (fig. 2b, c). Hence, human mast cells express both granzymes H and B, and the expression of these granzyme family members is reciprocally regulated by mast cell activation. Notably, reciprocal regulation of granzymes B and H has also been observed in natural killer cells [20].

To extend these findings, we investigated whether granzyme H could be detected in CBMCs at the protein level. Indeed, Western blot analysis using a granzyme H monospecific antibody [20] showed the presence of a ~36-kDa band in extracts from CBMCs, in reasonable agreement with the reported size of recombinant granzyme H [16]. A granzyme H band with similar migration velocity was seen in protein extracts from positive control

YT cells (fig. 2d). Granzyme H protein was also detected in CBMCs using ELISA (fig. 2e; ~1.8 ng/ 10^6 cells).

To further evaluate the expression of granzyme H by CBMCs, we used confocal microscopy. As seen in figure 3, CBMCs showed positive staining for granzyme H. Staining was most pronounced in the perinuclear region, but positive staining was also seen in vesicular compartments close to the cell membrane (fig. 3b). Notably, this staining pattern was similar to that seen in positive control YT cells (fig. 3a) and also similar to the staining for granzyme D seen in murine mast cells [14]. When CBMCs were activated by calcium ionophore, a decrease in granzyme H staining was observed (fig. 3e). In apparent agreement with the latter finding, calcium ionophore activation of CBMCs resulted in a moderate reduction in intracellular levels of granzyme H judged by ELISA measurements (fig. 2e). Granzyme H was also detected in the culture medium by ELISA analysis (fig. 2f). However, similar levels of extracellular granzyme H were seen in medium from non-activated and activated cells, suggesting that granzyme H is constitutively released from CBMCs. Notably, the decrease in intracellular granzyme H levels seen after CBMC activation was not accompanied by a corresponding increase in granzyme H levels, arguing against the presence of granzyme H in secretory granules. Possibly, the reduction in intracellular granzyme H seen after CBMC activation could be a consequence of increased intracellular degradation or decreased expression. In contrast, calcium ionophore stimulation of mast cells produced a robust (~60%) release of the secretory granule marker β -hexosaminidase (not shown).

In order to establish whether granzyme H is expressed by human mast cells in vivo, we stained punch biopsies obtained from human skin for granzyme H. Indeed, granzyme H-positive cells were seen in the dermis region, i.e. the region in which mast cells are mostly abundant

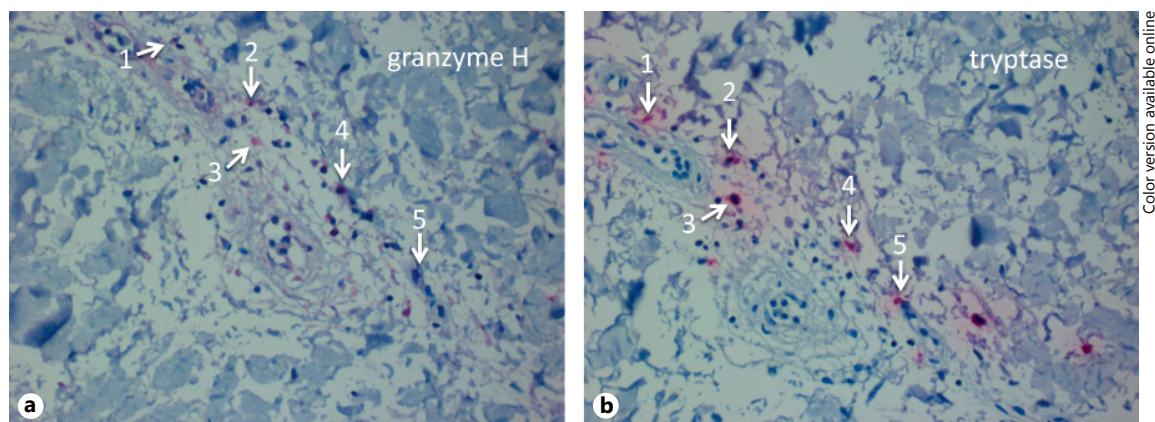


Fig. 4. Human skin mast cells are positive for granzyme H by immunohistochemistry. **a** Punch biopsies (3 mm) from human skin were stained for granzyme H and counterstained with Mayer's haematoxylin. **b** Adjacent sections were stained for tryptase. Note

the co-expression of granzyme H and tryptase in individual cells (marked by numbered arrows), suggesting that granzyme H is expressed by skin mast cells. Results shown are representative of 3 independent experiments.

(fig. 4a). Staining of adjacent sections revealed co-staining of multiple cells for granzyme H and tryptase (fig. 4b), tryptase being a well-established mast cell marker, supporting that skin mast cells can express granzyme H.

Discussion

In this study, we introduce the mast cell as a novel cell type expressing granzyme H and we extend previous observations showing that mast cells can express granzyme B. Granzyme H expression by human mast cells was supported both at the mRNA and protein level. Moreover, we demonstrate that granzyme H is expressed by a mast cell line isolated from a mast cell leukaemia patient, by non-transformed cultured mast cells and also by mast cells residing in the skin. Together, we may thus propose that granzyme H is a product of human mast cells at different stages of differentiation and at different tissue locations.

The function of granzymes in a mast cell context is intriguing. Considering that granzymes, including granzyme H [23, 24], in general are widely implicated as pro-apoptotic factors [13], it is conceivable that granzyme H expressed in a mast cell setting may possess cytotoxic activity. In line with this notion, there is limited evidence suggesting that mast cells can be cytotoxic for various target cells [25, 26], and the present study thus introduces the possibility that mast cell-derived granzyme H could contribute to such cytotoxic effects.

The general notion is that granzymes exert their pro-apoptotic activity after being delivered from cytolytic

granules of cytotoxic lymphocytes into the cytosol of respective target cells [13]. This process is known to be strictly dependent on perforin, a protein that induces pore formation in the plasma membrane of the target cell [27]. However, we and others failed to detect the expression of perforin in murine mast cells [11, 14] and, similarly, it has not been possible to detect perforin expression in differentiated human mast cells [10, 12]. These findings thus argue against a major role for mast cell granzymes in inducing target cell death after perforin-dependent delivery into the cytosolic compartment of target cells. On the other hand, it has been shown that mast cell-expressed granzyme B may induce apoptosis by perforin-independent mechanisms, possibly involving the cleavage of extracellular matrix components and thereby inducing detachment of cells from their substratum [11]. It is thus possible that mast cell-derived granzyme H could contribute to such perforin-independent cytotoxicity, but it is noteworthy that various granzymes have recently been ascribed functions beyond their role in cell death [13]. For example, it has been demonstrated that granzyme B may have a role in extracellular matrix remodelling [28] and granzyme A has been shown to cleave protease-activated cell surface receptors [29]. Moreover, recent evidence points to a unique, direct anti-viral effect of granzyme H, mediated by the cleavage of different virus-encoded proteins [30, 31]. It has also been demonstrated that granzyme H can interfere with viral translational activity through cleavage of La, a protein that is involved in RNA metabolism [32]. Considering that mast cells have been implicated in the defence

against viral infection [3], a plausible scenario is thus that mast cell-derived granzyme H may account, at least partly, for such effects. However, future studies are required to fully elucidate the role of granzyme H in a mast cell context.

Acknowledgements

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