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Lysosomal Membrane Permeabilization Induces Cell Death in Human Mast Cells

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Abstract

Mast cells (MC) have pathogenic roles in numerous disorders, and strategies that stabilize MC or induce MC apoptosis are therefore emerging as possible therapeutic regimens. A typical feature of MC is their high content of secretory lysosomes (granules), containing numerous components such as biogenic amines, cytokines, serglycin proteoglycan and proteases. Damage to the secretory lysosomes will thus lead to leakage of these compounds, including the proteases, into the cytosol, and this could potentially trigger apoptosis. Here, we evaluated whether MC are sensitive to cell death induced by secretory lysosome destabilization, induced by the lysosomotropic agent Leu-Leu-OMe (LLME). Human MC were sensitive to LLME-induced cell death. In contrast, fibroblasts and HEK-293 cells were largely resistant. As judged by Annexin V/propidium iodide staining, LLME caused apoptotic cell death, and this was supported by induction of caspase-3-like activity, detection of activated caspase-3 by immunoblot analysis and reduced cell death in the presence of a caspase inhibitor. In support of a role for serglycin in regulating LLME-induced cell death, the survival rate of various cell types correlated negatively with the level of serglycin expression. In summary, this study introduces the concept of using lysosomotropic agents to induce cell death of human MC.

Introduction

Mast cells (MC) are widely recognized as harmful players in numerous disorders, including allergic conditions such as atopic asthma [1, 2]. Hence, intervention with MC functions can be used as a possible regimen for treatment of allergic disease. MC contain large amounts of secretory lysosomes (granules), filled with preformed inflammatory mediators such as histamine, cytokines, proteases and serglycin proteoglycans [3, 4]. These mediators are released when MC degranulate, and agents that interfere with MC degranulation [5, 6] will thus cause a global blockade of the effects mediated by the preformed granule components. As an alternative, compounds that block the action of individual MC granule compounds, such as histamine receptor antagonists and MC protease inhibitors, can be of therapeutic value [7–9].

Importantly, it has become clear that MC can be activated and release numerous compounds also under circumstances when degranulation is not evident [10]. Hence, blockade of MC degranulation will not affect actions of MC that are unrelated to the preformed MC mediators. To block degranulation-independent events related to MC, alternative strategies are thus required. One such strategy would be to induce MC apoptosis, a notion that is currently discussed [11].

Apoptosis is a tightly controlled process in which pro-apoptotic cytosolic compounds are proteolytically activated, largely because of the actions of caspases [12, 13]. MC of all species are exceptionally rich in secretory lysosomes (granules), and these contain vast amounts of proteases stored in complex with serglycin proteoglycan [14]. Rupture of the granules will thus lead to the release of large amounts of serglycin and proteases into the cytosol, and we hypothesized that this may trigger apoptosis. Indeed, we showed recently that murine MC are highly sensitive to apoptosis induced by secretory granule membrane permeabilization [15]. Moreover, the extent of apoptosis was markedly reduced in MC lacking serglycin. Here, we extended those findings by showing that human MC are sensitive to apoptosis induced by secretory lysosome/granule permeabilization.

Materials and methods

Reagents. Leu-Leu-OMe (LLME) and Ac-DEVD-AFC (Ac-Asp-Glu-Val-Asp-AFC) were from Bachem (Bubendorf, Switzerland). E64d (membrane permeable), Z-DEVD-

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FMK (Z-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me) fluoromethyl ketone), Z-VAD-FMK (N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone), Pefabloc® SC, pepstatin A and acridine orange (AO) were from Sigma-Aldrich (Steinheim, Germany). Polyclonal antibodies and anti-procaspase-3/CPP32 were from Invitrogen (Carlsbad, CA, USA); anti-active caspase-3 was from Abcam (Cambridge, UK; no ab2302), and anti-β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. LAD2 cells isolated from the bone marrow of a patient with MC leukaemia was a gift from A. Kirshenbaum and Dean D. Metcalfe (National Institutes of Health). LAD2 cells were cultured in StemPro-34 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin–streptomycin and 100 ng/ml stem cell factor (SCF). Cell culture medium was hemidepleted every week with fresh medium supplemented with 100 ng/ml SCF. HMC-1 cells (HMC-1.2: kind gift from the Mayo Foundation for Medical Education and Research), a cell line derived from a MC leukaemia [16, 17], were cultured in Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin and 1.2 mM α-thioglycerol. The cell culture medium was replaced when the cell density reached 1.0–1.5 $\times 10^6$ cells/ml. Human peripheral blood-derived MC (primMC) were derived from peripheral blood mononuclear cells isolated by density gradient centrifugation on Ficoll-Paque Plus. Cells were maintained in StemPro-34 supplemented with SCF (100 ng/ml), IL-6 (50 ng/ml) and passed once a week. The primMC were used when more than 95% of the cells stained positively with toluidine blue (typically 6 weeks). Human Embryonic Kidney 293 cells (HEK-293) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin and 50 μg/ml streptomycin. Cells were split 2–3 times a week, and the cell count was kept between 1×10^5 and 3×10^5 cells/ml. Primary human dermal fibroblast neonatal cells (HDFn) were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 50 μg/ml streptomycin. The cell culture medium was replaced once a week. The U-937 line isolated from histiocytic lymphoma [18] were cultured in RPMI 1640 – GlutaMAX™-1 supplemented with 5% heat-inactivated FBS, 100 IU/ml penicillin and 50 μg/ml streptomycin. Cells were split 2–3 times a week, and the cell count was kept at 0.5×10^6 cells/ml.

Induction of secretory lysosome/granule permeabilization. Triplicates of cells (0.5×10^6 cells/ml) were transferred into individual wells of 24-well flat-bottomed plates and were either left untreated or induced to undergo apoptosis with different concentrations of LLME in complete culture medium, followed by incubation for different time periods (as specified in the figure legends). Inhibitory assays were performed using the same methodology. However, before LLME treatment, cells were preincubated for 30 min with the caspase-3, -6, -7, -8 and -10 inhibitor Z-DEVD-FMK (20 μM), the caspase-1 and -3 inhibitor Z-VAD-FMK (20 μM), the broad-spectrum cysteine cathepsin inhibitor E64d (20 μM), the serine protease inhibitor Pefabloc® SC (0.1 mM) or the aspartic acid protease inhibitor pepstatin A (50 μM).

Viability assay. Live and dead cells were distinguished using 1 μM calcein AM and 2 μM ethidium homodimer-1 (LIVE/DEAD® Viability/Cytotoxicity kit – Molecular Probes®, Eugene, OR, USA). After staining, the results were monitored using a microtitre plate reader (Infinite M200 - TECAN, Männedorf, Switzerland).

Flow cytometry. Flow cytometry was performed using an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich) according to the protocol provided by the manufacturer. After staining, the cells were analysed using a FACScan® flow cytometer and the CELLQUEST™ 3.3 software (Becton Dickinson, San Jose, CA, USA). Data from 10,000 events/sample were collected.

Measurement of caspase-3 activity and processing. Caspase-3-like activity was measured using the fluorogenic substrate Ac-DEVD-AFC as described [15]. Immunoblot analysis to detect pro-caspase-3 processing and appearance of activated caspase-3 was performed as previously described [15].

Acridine orange staining. For staining of acidic compartments, cells were stained with AO as described previously [15].

RNA preparation and quantitative real-time PCR. Total RNA preparation and quantitative real-time PCR (qPCR) were preformed as previously described [19]. The following primers were used. Serglycin (fw): 5′-TGG GAG TGG CTT CCT AAC GGA AAT-3′; Serglycin (rev): 5′-TGTCTGAGGGCAGATTCCTGTCAA-3′; GAPDH (fw): 5′-TCGACAGTCAGCCGCATCTTCTTT-3′; GAPDH (rev): 5′-ACCAAATCCGTTGACTCCGACCTT-3′.

Statistical analyses. All data shown are from individual experiments, representative of several (up to 5) independent experiments. Statistical significance was tested using one-way analysis of variance (ANOVA) performed by using ORIGIN 7.5 (OriginLab Corporation, Northampton, MA, USA). Correlation analysis was performed using the Spearman correlation test and the GRAPHPAD PRISM 4.0 software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Human MC undergo LLME-induced cell death

To assess whether human MC are sensitive to cell death induced by secretory lysosome/granule permeabilization, we used the lysosomotropic agent Leu-Leu-OMe (LLME). LLME has been widely used for inducing lysosomal

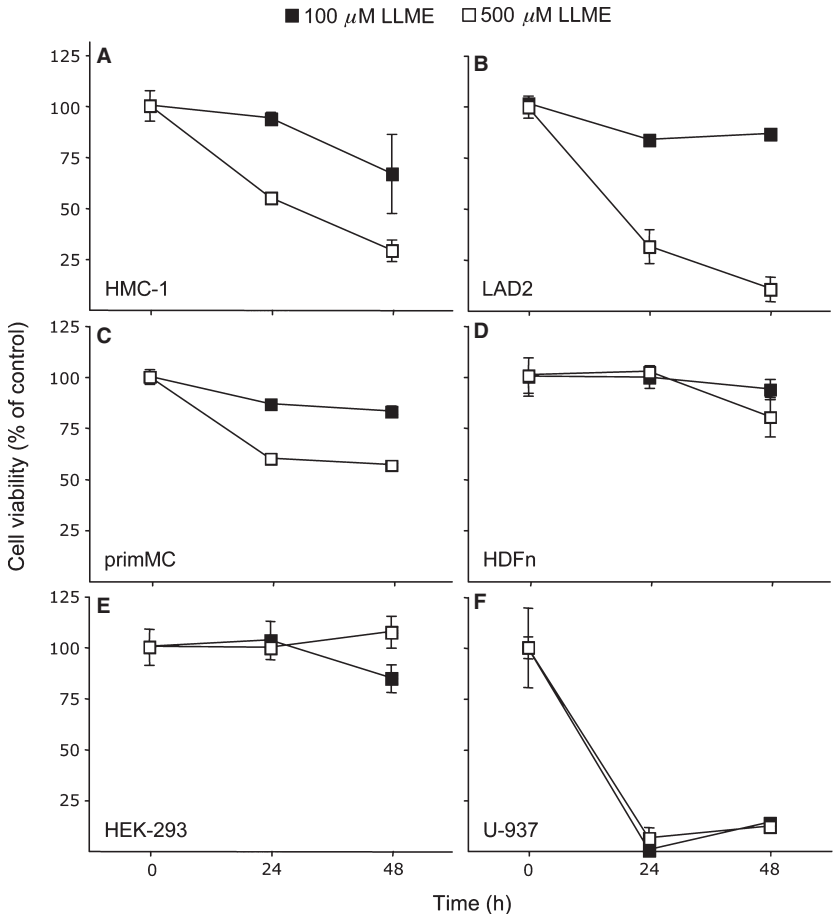

Figure 1 Leu-Leu-OMe (LLME) causes cell death of human mast cells (MC). Human MC (HMC-1, LAD2, primMC; A–C), macrophage-like cells (U-937; F), fibroblasts (HDFn; D), and HEK-293 (E) cells were cultured in the presence of 100 or 500 μM LLME. At the time points indicated, cells were assessed for viability as described in Materials and methods. Results are expressed as mean ± SEM (n = 3).

damage in various cell types [20–23] and has been assessed as a potential therapeutic agent for treatment of graft versus host disease [24, 25]. However, its effect on human MC has not been tested previously. Addition of LLME to MC leukaemia cells, HMC-1, caused extensive cell death (Fig. 1A). In comparison with HMC-1 cells, LAD2 cells represent MC of a more differentiated state, expressing typical MC markers such as FcεRI [26]. Moreover, similar to primary MC, LAD2 cells express the SCF receptor (c-kit) and are dependent on SCF for cell viability and proliferation [26]. As seen in Fig. 1B, LAD2 cells were also susceptible to cell death induced by LLME. We also examined the effect of LLME on primary human peripheral blood-derived MC (primMC). Similar to LAD2 and HMC-1 cells, primMC were susceptible to LLME-induced cell death (Fig. 1C). In contrast, HEK-293 cells (Fig. 1D) and fibroblasts (HDFn; Fig. 1E) were relatively resistant to LLME. On the other hand, but in agreement with a previous report [20], human macrophage-like cells (U-937) were highly sensitive to cell death induced by LLME (Fig. 1E).

Cell death of human MC in response to LLME involves apoptosis

To examine whether LLME-induced cell death was because of apoptotic events, LLME-treated cells were stained with Annexin V (detects cell surface-exposed phosphatidyl serine on apoptotic cells) and propidium iodide (PI; localizes in nucleus of late stage apoptotic/necrotic cells). As shown in Fig. 2A–D, incubation of HMC-1, LAD2, primMC and U-937 cells for 24 h with LLME resulted in positive Annexin V staining, suggesting apoptosis. However, substantial portions of the cells were double Annexin V/PI positive, indicating late-stage apoptosis or necrosis (gates were set as shown in Fig. 2G, H). To assess whether the double Annexin V/PI positivity was a late event preceded by single Annexin V positivity characteristic of apoptotic cell death, a time course experiment was conducted. As shown in Fig. 2E, F, at early stages after LLME addition, HMC-1 cells were predominantly Annexin V single positive, thus indicating apoptosis rather than necrosis.

Cell death in response to LLME is caspase-dependent

Caspase activation is a hallmark of apoptosis, but caspase-independent apoptosis has also been reported [27]. To evaluate the role of caspases in LLME-induced MC cell death, we assessed the effect of the broad-spectrum caspase inhibitor Z-DEVD-FMK (inhibitor of caspase-3, -6, -7, -8 and -10) and Z-VAD (inhibitor of caspase-1

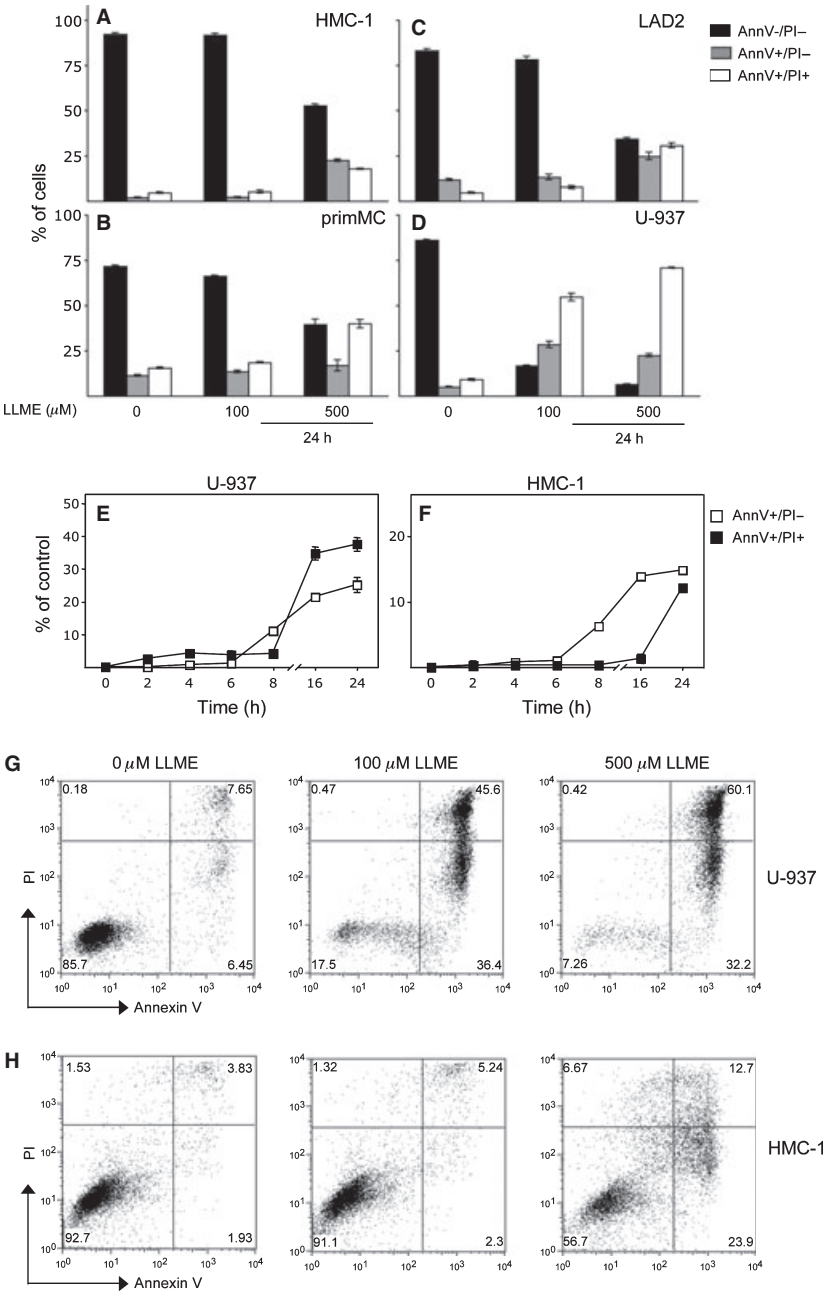

Figure 2 Human mast cells (MC) undergo apoptosis in response to lysosome membrane permeabilization. (A–D) Human MC (HMC-1, LAD2, primMC) and macrophage-like cells (U-937) were cultured in the presence of 100 or 500 μM Leu-Leu-OMe (LLME). After 24 h, cells were stained with Annexin V and propidium iodide (PI). Results are expressed as mean ± SEM (n = 3). (E and F) Macrophage-like cells (U-937) and human MC (HMC-1) were treated with 500 μM LLME for different time periods, followed by Annexin V and PI staining. Results are expressed as mean ± SEM (n = 3) normalized against untreated cells. (G and H) Dot plots for representative samples (U-937; HMC-1) showing Annexin V-FITC (FL-1) and PI (FL-3) staining. The percentage of cells is indicated within each quadrant.

and -3) on the extent of apoptosis. As shown in Fig. 3A, B, the addition of Z-DEVD-FMK caused a marked reduction in the extent of cell death induced by LLME, both in HMC-1 MC and in U-937 cells, whereas Z-VAD had a minor effect. These results thus indicate a role of caspases in the LLME-induced cell death, and this was also supported by an induction of caspase-3-like activity in LLME-treated HMC-1 cells (Fig. 4A), whereas no detectable induction was seen in cells (HEK-293) that were non-sensitive to LLME-induced cell death (Fig. 4B). Moreover, immunoblot analysis showed the appearance of activated caspase-3, accompanied by processing of pro-caspase-3, after the addition of LLME to HMC-1 and U-937 cells (Fig. 4C).

LLME-induced cell death in certain cell types has previously been shown to involve the action of cysteine cathepsins and/or aspartic acid proteases that are stored in lysosomal/granular compartments [28, 29], and serine proteases have also been implicated [30]. To test whether these classes of proteases contribute to the death of LLME-treated cells, we used general inhibitors of cysteine cathepsins (E64d), aspartic acid proteases (pepstatin A) and serine proteases (Pefabloc SC). As shown in Fig. 3E–J, cysteine cathepsins play a role in cell death of U-937 cells, but contribute only modestly to LLME-induced cell death of HMC-1 cells. Further, inhibition of serine proteases reduced cell death of U-937 cells to some extent, but had

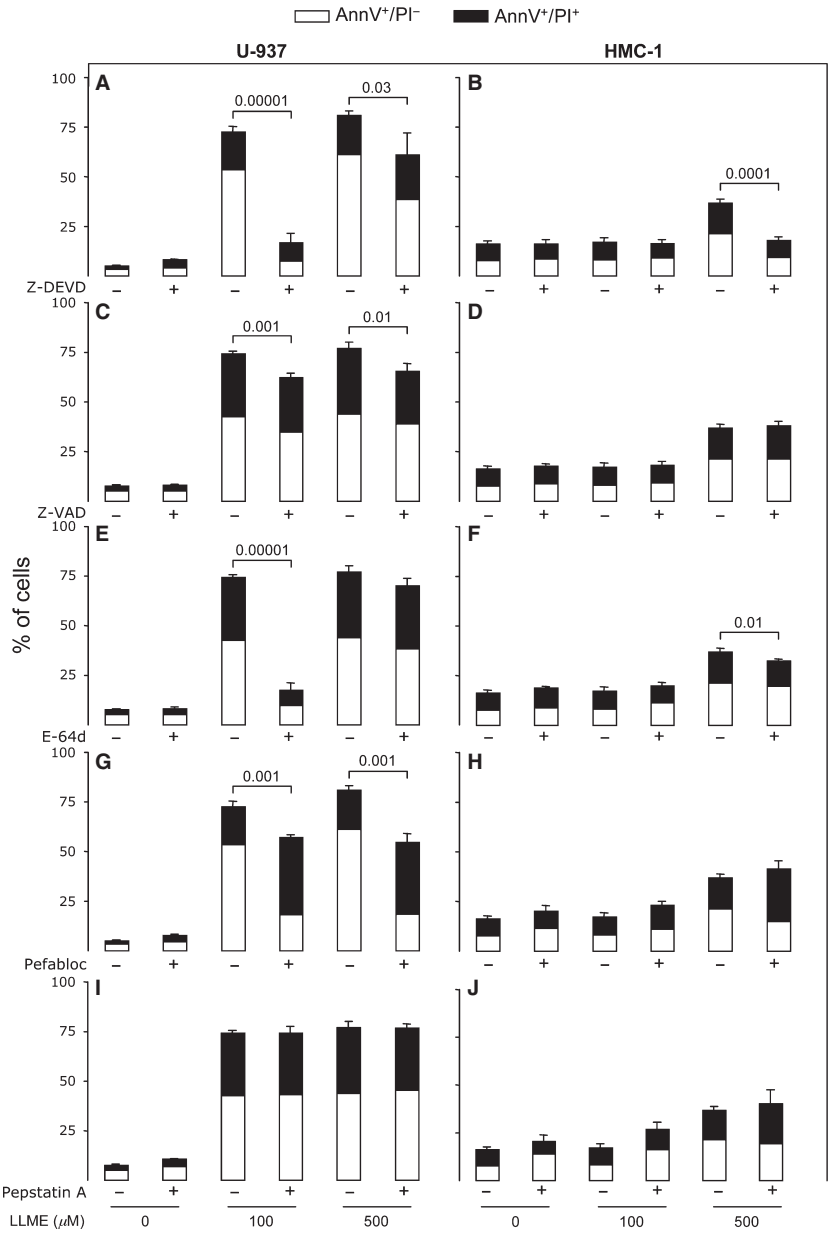

Figure 3 Apoptosis of human mast cells (MC) in response to Leu-Leu-OMe (LLME) is caspase dependent. Macrophage-like cells (U-937)(A, C, E, G, I) and HMC-1 cells (B, D, F, H, J) were cultured in the absence or presence of 100 or 500 μM LLME as indicated. Cells were incubated as indicated either without or with the following inhibitors: caspase inhibitors Z-DEVD-FMK (20 μM) and Z-VAD (20 μM), E64d (20 μM), Pefabloc SC (0.1 mM) or pepstatin A (50 μM). After 24 h, cells were stained with Annexin V and propidium iodide by flow cytometry. Results are expressed as mean ±SEM (n = 3).

no effect on cell death in HMC-1 cells. Pepstatin A did not prevent cell death in either of the cell types tested.

LLME causes lysosomal damage in human MC

Next, we evaluated the extent of lysosomal damage induced by LLME. To monitor lysosomal damage, we used a fluorescent dye, AO. When added to intact cells, AO will localize to acidic compartments such as lysosomes and secretory granules, in which the prevalent low pH will cause AO to produce intense fluorescence. When the integrity of the lysosomes/granules is disturbed, the pH of the lysosomes/granules will be elevated and this will cause a reduction in AO fluorescence. Thus, a reduction in AO staining can be used for monitoring the extent of lysosomal/granule damage. As displayed in Fig. 5, LLME caused a marked reduction in AO fluorescence in HMC-1 and LAD2 MC, and also U-937 cells underwent extensive lysosomal damage in response to LLME. In contrast, HDFn and HEK-293 cells showed minimal signs of lysosomal damage in response to LLME.

Levels of serglycin expression correlate with the sensitivity to LLME

We showed previously that serglycin proteoglycan has a key role in regulating lysosomal damage and apoptosis of murine bone marrow-derived MC in response

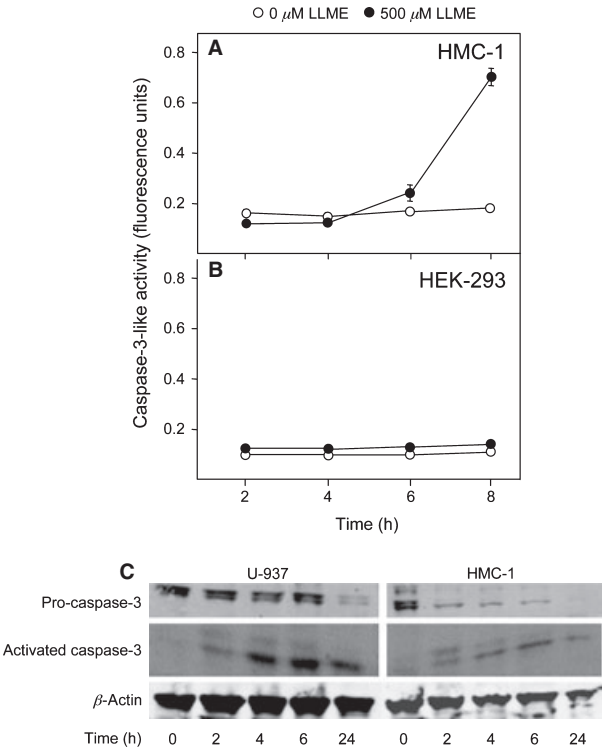

Figure 4 Induction of caspase-3 activity in response to lysosomal membrane permeabilization. (A) HMC-1 cells (10^6 cells/ml) or (B) HEK-293 cells (10^6 cells/ml) were either left untreated or treated with Leu-Leu-OMe (LLME) at the concentrations and time periods indicated. (A, B) Triplicates of each sample were transferred into individual wells of a 96-well flat-bottomed plate and incubated with Ac-DEVD-AFC for 60 min. Caspase-3-like activity was measured by AFC release as described under Material and methods. Data are expressed as mean ± SD (n = 3). (C) Western blot showing extensive processing of pro-caspase-3 in U-937 and HMC-1 cells and appearance of activated caspase-3 in response to LLME treatment.

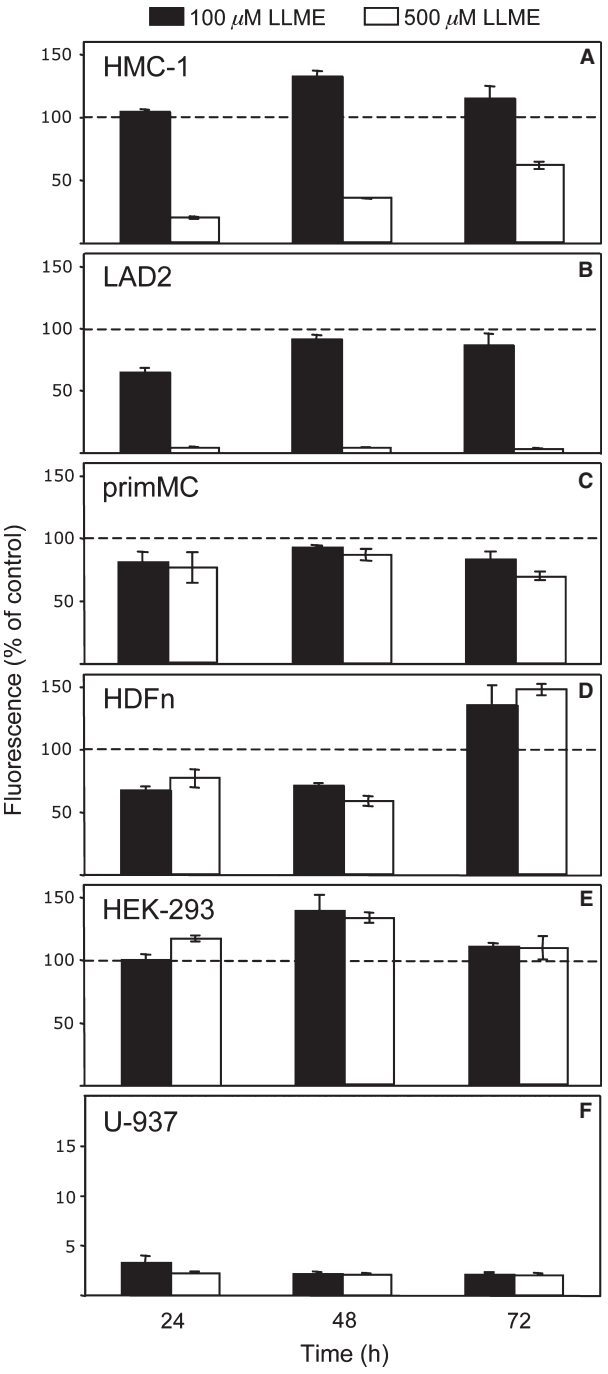

Figure 5 Lysosomal damage of human mast cells (MC) in response to Leu-Leu-OMe (LLME). Human MC (HMC-1, LAD2, and primMCs; A–C), macrophage-like cells (U-937; F), fibroblasts (HDFn; D) and HEK-293 cells (E) were cultured in the presence of 100 or 500 μM LLME as indicated for 24–72 h. Acridine orange (AO; 5 μg/ml) was then added and cells were incubated further for 15 min, followed by recovery of cells and quantification of AO fluorescence. The dashed line represents the AO staining in the absence of added LLME. Results are expressed as mean ± SEM (n = 3).

to LLME [15]. On the basis of that study, we hypothesized that the extent of cell death in response to lysosomotropic agents may correlate with the level of serglycin expression. To address this possibility, we analysed the levels of serglycin expression in the various cell types using qPCR (Fig. 6A). Interestingly, cell types resistant to LLME (HEK-293 and HDFn) expressed low levels of serglycin. It is also noteworthy that HMC-1 and primMC expressed approximately equal levels of serglycin (Fig. 6A) and showed similar susceptibility to LLME (see Fig. 1). In comparison with HMC-1 and primMC, LAD2 cells expressed higher levels of serglycin and were correspondingly more sensitive to LLME-induced cell death than were HMC-1 and primMC (see Fig. 1). Finally, of all tested cell types, U-937 expressed the highest levels of serglycin (Fig. 6A) and showed the highest sensitivity to LLME-induced cell death (see Fig. 1). Hence, the extent of survival in response to LLME correlates negatively with the level of serglycin expression, as also verified by statistical analysis (Fig. 6B).

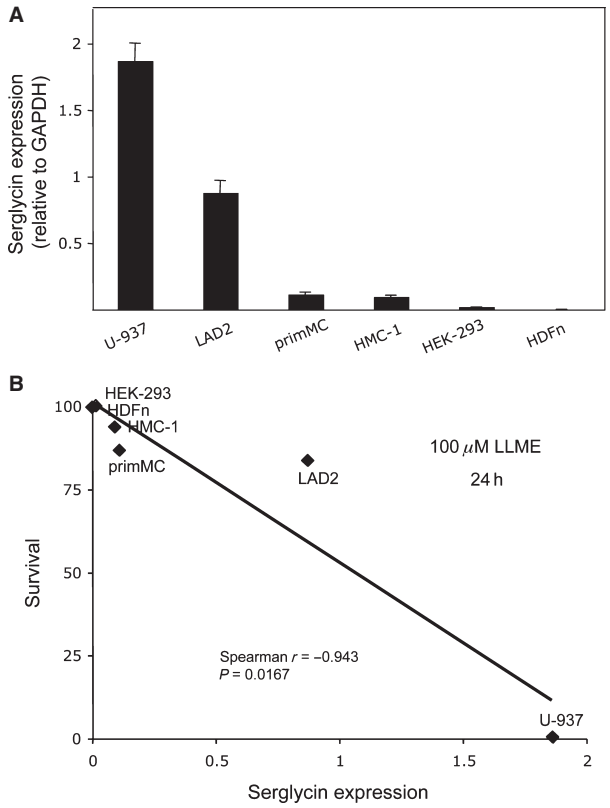

Figure 6 The expression of serglycin correlates with sensitivity to cell death and lysosomal damage in response to Leu-Leu-OMe (LLME). (A) Total RNA was prepared from human mast cells (HMC-1, LAD2, primMC), macrophage-like cells (U-937), fibroblasts (HDFn) and HEK-293 cells. The level of mRNA coding for serglycin was quantified using qPCR analysis. Results (mean of duplicate determinations) are expressed as relative values, compared to the expression of the house keeping gene (GAPDH). (B) Analysis of the correlation between levels of serglycin expression and survival in response to LLME (100 μM LLME; 24 h incubation). Correlation analysis was performed using the Spearman test, resulting in a correlation coefficient (r) of −0.943 and a two-tailed P-value of 0.0167.

Discussion

The concept of inducing MC apoptosis is emerging as a potential future strategy for treatment of allergic disorders. However, there is so far no agent that selectively induces apoptosis in MC. For example, although the BH3-mimetic ABT-737 shows selectivity for inducing apoptosis in MC over several other leucocyte populations [31], this drug is also known to induce apoptosis in a variety of other cell types, including neuronal cells and transformed cells of various origin [32, 33]. Another potential way of inducing MC death would be to activate death receptor pathways. In line with such a scenario, it has recently been shown that human MC express TRAIL receptor and undergo apoptosis in response to TRAIL [34]. However, although TRAIL receptor engagement could provide an efficient means of killing MC, the TRAIL receptor is widely expressed among many cell types and it is thus questionable whether TRAIL receptor engagement can be used for selective induction of MC apoptosis.

Here, we explored an alternative strategy for inducing MC apoptosis. A unique property of MC is their high abundance of electron dense secretory lysosomes (granules), containing large amounts of preformed compounds, including serglycin proteoglycan and various proteases [4]. Damage to the secretory lysosomes/granules may thus lead to the release of large amounts of active proteases into the cytosol, potentially leading to downstream activation of proteolytic cascades such as apoptosis. Indeed, we show here for the first time that human MC are susceptible to cell death induced by secretory lysosome/granule permeabilization.

Notably, despite being sensitive to cell death induced by LLME, primMC underwent relatively little lysosomal damage as assessed by AO staining in response to LLME. A likely reason for this apparent discrepancy could be that the sensitivity to cell death is related to the variable contents of lysosomes/granules in the different cell types tested. Hence, it is possible that primMC store larger amounts of cell death-inducing compounds in their lysosomes/granules than do, e.g., LAD2 and HMC-1 cells. Thus, even a relatively minor release of lysosomal/granular compounds (as indicated by a small reduction in AO staining) may induce significant cell death.

LLME-induced cell death showed some selectivity for MC, as indicated by the low sensitivity of various other cell types to the compound. It is also notable that the concentration of LLME needed for inducing MC cell death is lower than the effective concentrations reported for inducing cell death in numerous other cell types (this study and [20–22, 35]). However, LLME was also shown to induce apoptosis in macrophage-like cells (this study and [20]), and it has also been reported that LLME effectively induces apoptosis in cytotoxic T lymphocytes and NK cells [20, 35, 36]. Hence, lysosomal targeting effectively induces MC cell death but, importantly, this strategy of inducing apoptosis is only partially selective for MC. Therefore, LLME and similar (lysosomotropic) compounds may not be ideal for systemic administration, in which side effects because of the death of cells other than MC may be problematic. On the other hand, lysosomotropic compounds may prove useful in situations in which topical application is sufficient, such as treatment of MC-related disorders localized to skin or lung. For example, lysosomotropic agents may potentially be used for treating mastocytosis, in particular cutaneous mastocytosis, a group of diseases for which there is currently no efficient cure [37].

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Conflict of interest

Neither of the authors declares any conflict of interest.

Author contributions

Fabio R Melo planned experiments, performed most of the experiments, analysed and interpreted data, prepared the figures and contributed to the writing of the manuscript; Anders Lundequist contributed to the experiments and contributed to the writing of the manuscript; Gabriela Calounova contributed to the experiments; Sara Wernersson analysed data and contributed to the writing of the manuscript; Gunnar Pejler conceived the study, contributed to experimental set-ups, interpreted data and wrote the manuscript.

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