Induction of cell death by the lysosomotropic detergent MSDH

Wei Li[a], XiMing Yuan[a], Gunnar Nordgren[a], Helge Dalen[b], Gene M. Dubowchik[c], Raymond A. Firestone[d], Ulf T. Brunk[a,*]

[a] *Division of Pathology II, Faculty of Health Sciences, University of Linköping, S-581 85 Linköping, Sweden*
[b] *Department of Pathology, The Gade Institute, University of Bergen, Bergen, Norway*
[c] *Pharmaceutical Research Institute, Bristol-Myers Squibb, Wallingford, CT, USA*
[d] *Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, USA*

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Abstract Controlled lysosomal rupture was initiated in lysosome-rich, macrophage-like cells by the synthetic lysosomotropic detergent, *O*-methyl-serine dodecylamide hydrochloride (MSDH). When MSDH was applied at low concentrations, resulting in partial lysosomal rupture, activation of pro-caspase-3-like proteases and apoptosis followed after some hours. Early during apoptosis, but clearly secondary to lysosomal destabilization, the mitochondrial transmembrane potential declined. At high concentrations, MSDH caused extensive lysosomal rupture and necrosis. It is suggested that lysosomal proteases, if released to the cytosol, may cause apoptosis directly by pro-caspase activation and/or indirectly by mitochondrial attack with ensuing discharge of pro-apoptotic factors.

Key words: Apoptosis; Lysosome; Lysosomotropic detergent; Macrophage; Mitochondrion; Necrosis

1. Introduction

Partial lysosomal rupture was recently suggested to play an important, perhaps even initiating, role in apoptosis based on observations following exposure to oxidative stress, FAS ligation, and growth factor starvation [1–7]. Lysosomes, which together with late endosomes constitute the cellular acidic vacuolar apparatus, are the major compartment for cellular catabolism and contain a wide spectrum of hydrolytic enzymes mostly active at an acidic pH, e.g. proteases, nucleases, and so on [8]. Christian de Duve, soon after his discovery of these organelles, nicknamed them 'suicide bags' and suggested that an uncontrolled leakage of their enzymes to the cytosol might be dangerous, or even lethal, for cells [9].

Major findings in support of the 'lysosomal' apoptosis hypothesis are that apoptosis can be induced by photo-oxidative processes that cause lysosomal rupture [1,10,11] and, especially, that oxidative stress-induced apoptosis is almost completely prevented by an initial exposure to the potent iron chelator desferrioxamine, which results in prevention of the type of lysosomal rupture that is a consequence of iron-catalyzed, intralysosomal oxidative reactions [2,4,6]. Desferrioxamine is taken up only by endocytosis, and it then largely remains within the lysosomal compartment [12–14] and, thus, should have little effect on potential cytosolic or mitochondrial Fenton-type reactions. Moreover, it has recently been reported that digitonin-treated lysosomes, and a purified lysosomal cysteine protease, cathepsin B, have the capacity to activate several cytosolic caspases [15,16], and that the lysosomotropic agents, L-leucyl-L-leucine methyl ester and C1311, an imidazoacridinone analogue, induce lysosomal destabilization and caspase-3-like protease activation, and apoptosis as well [17,18].

The objective of this study was to further elucidate the role of lysosomal labilization as an initiator of apoptosis by inducing controlled lysosomal rupture utilizing a synthetic lysosomotropic detergent, *O*-methyl-serine dodecylamine hydrochloride (MSDH). This drug belongs to a group of imidazole- and morpholine-type lysosomotropic detergents, initially designed as anticancer drugs [19–21].

2. Materials and methods

2.1. Cell cultures and MSDH exposure

J-774 cells (a murine macrophage cell line) were cultured in complete F-10 medium, supplemented with 10% fetal bovine serum, at 37°C in humidified air with 5% CO_2. The cells were subcultivated once a week, and used for experiments 24 h later.

The human monocyte-like cell lines U937 and THP-1 were grown in a RPMI 1640-based medium as above, but subcultivated twice a week.

The lysosomotropic detergent MSDH was prepared as already described [19]. Cells were exposed to 0–75 μM MSDH for 0–24 h in complete culture medium. Cell membrane permeability was assayed by the trypan blue (0.05%) dye exclusion test.

2.2. Determination of lysosomal membrane stability

MSDH-treated and control cells (growing on coverslips) were stained with 5 μg/ml acridine orange (AO) in complete medium for 15 min at 37°C. The AO-induced granular red (lysosomal) fluorescence from 50 cells in each group was measured on a Nikon microphote-SA fluorescence microscope (Nikon, Tokyo, Japan) using green excitation light (Nikon G-cube) in combination with an extra 630 nm barrier filter [1–4,6].

2.3. Lysosomal enzyme cathepsin D immunocytochemistry

For assessment of the distribution of the lysosomal marker enzyme cathepsin D, cells (grown on coverslips) were treated with MSDH and fixed for 20 min at 4°C in 4% paraformaldehyde in phosphate buffered saline (PBS). The cells were then labeled with polyclonal rabbit anti-human cathepsin D (Dakopatts, Älvsjö, Sweden) and, finally, with Texas red-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA), as described [5].

2.4. Assessment of apoptosis

Nuclear condensation or fragmentation was examined by light microscopy following Wright–Giemsa staining. The percentage of apoptotic nuclei (condensed or fragmented) was quantified by randomly counting at least 200 cells in each group. Apoptotic cells also were detected by the TUNEL technique, using the ApopTag in situ apop-

*Corresponding author. Fax: (46)-13-221529.
E-mail: ulf.brunk@pat.liu.se

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tosis detection kit according to the manufacturer's instruction (Oncor Inc., Gaithersburg, MD, USA).

Control and MSDH-exposed cells were also examined by electron microscopy, as previously described [22].

2.5. Evaluation of mitochondrial membrane integrity

Cells, grown on coverslips, were incubated for 20 min in 2 ml complete culture medium with 5 μg/ml rhodamine-123 (Rh-123, Sigma, St. Louis, MO, USA) at 37°C. They were then rinsed in PBS and exposed to MSDH (50 μM) in ordinary culture medium for different periods of times. Fluorescence, reflecting mitochondrial uptake, was measured from 50 cells in each sample using the above Nikon fluorescence microscope and blue activation light (Nikon B-2A filter cube).

2.6. Measurement of caspase-3-like proteases

Cells were collected and lysed in 100 μl lysis buffer containing 10 mM Tris–HCl, pH 7.5, 130 mM NaCl, 1% Triton and 10 mM NaP_i. The cell lysate was incubated at 37°C for 2 h with 20 μM Ac-DEVD-AMC (Pharmingen, San Diego, CA, USA) in the reaction buffer (20 mM HEPES, 10% glycerol, 2 mM dithiothreitol). The intensity of fluorescence of the liberated 7-amino-4-methylcoumarin was measured using spectrofluorometry (Ex 380; Em 435 nm), and values were normalized to protein contents.

2.7. Statistical analysis

Results are given as means ± S.E.M. Statistical comparisons were made using the Mann–Whitney *U*-test. Results were considered significant at values for $P \leq 0.05$.

3. Results

3.1. Lysosomal membrane stability

In all three cell lines employed, MSDH affected the lysosomal stability in a dose- and time-dependent way. In moderate doses (≤50 μM), MSDH caused a slow and incomplete lysosomal destabilization, while high doses resulted in a rapid and extensive such process. In a parallel fashion, the intracellular distribution of immunoreactive cathepsin D (a lysosomal protease marker enzyme) shifted from a normal granular (lysosomal) distribution to a more diffuse pattern. The data for J-774 cells (Fig. 1A,B) are typical.

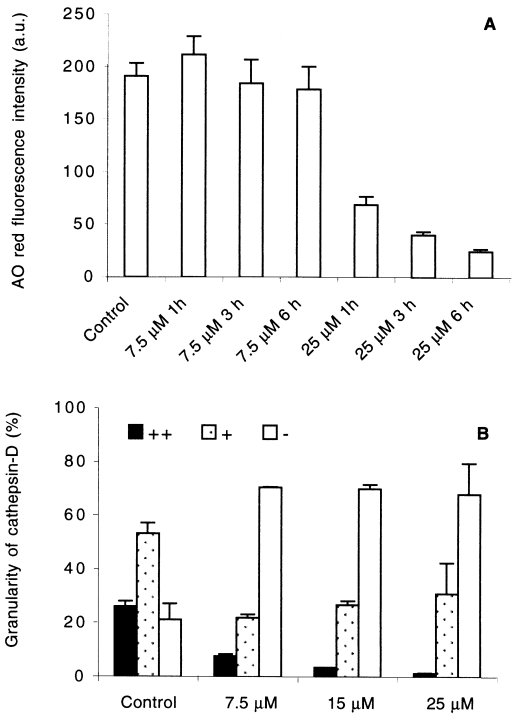

Fig. 1. Lysosomal MSDH-induced destabilization of J-774 cells. A: Uptake of the lysosomotropic, weakly basic, fluorochrome AO following 1–6 h exposure to MSDH at concentrations between 7.5 and 25 μM. The data are mean values, based on measurements of 50 individual cells from each group. The slight increase following exposure to 7.5 μM MSDH for 1 h reflects reparative autophagocytosis with an expanded lysosomal vacuome. B: Granularity scoring of the reaction product of cathepsin D immunocytochemistry. Cells were initially exposed to different concentrations of MSDH for 24 h. Quantification of the immunoreactivity was scored according to the presence of strong (++), moderate (+), or absent (−) granularity. Note decreasing granularity and increasing diffuse cytosolic 'staining' pattern with increasing doses, indicative of lysosomal leak. Means ± S.E.M., $n = 2$–3.

3.2. Plasma membrane permeability

MSDH induced a dose- and time dependent cytotoxicity in all three cell lines. There was a slight difference in sensitivity, J-774 cells being the most sensitive and THP-1 cells the least. Upon exposure to MSDH, the cells clearly died as a result of both apoptotic and post-apoptotic necrotic processes. For example, as assessed by trypan blue exclusion, about 15% and 25% of the J-774 cells no longer excluded trypan blue after 3 and 5 h, respectively, of exposure to 50 μM MSDH. However, at those time points the frequency of cells with pycnotic (apoptotic) nuclei was about 25 and 35%, respectively, indicating the presence of a mixture of apoptotic and post-apoptotic necrotic cells. This would be expected in a cell culture system where almost all cells are simultaneously affected. In this situation, apoptotic cells are not phagocytosed, due to lack of viable phagocytic cells, but instead proceed to post-apoptotic necrosis. It is likely that most, if not all, cells went through apoptosis before they turned post-apoptotic necrotic; only a few cells with swollen nuclei, indicative of primary necrosis, were seen as long as the cells were exposed to MSDH in doses ≤50 μM.

3.3. Assessment of apoptosis

Apoptosis, as evaluated by Wright–Giemsa staining and the TUNEL technique, was clearly evident after exposure for 24 h to lower concentrations of MSDH (7.5–25 μM), as shown in Fig. 2A–C, or for 3–5 h to 50 μM MSDH (data not shown). When cells were exposed to higher concentrations of MSDH (≥75 μM), they rapidly underwent necrosis without intervening apoptosis.

Control cells showed a normal ultrastructure, depicted for J-774 cells in Fig. 3A. Following exposure to MSDH (50 μM, 5 h), obvious alterations occurred in the acidic vacuolar apparatus, indicative of so-called reparative autophagocytosis (Fig. 3B). At this time, lysosomes were plentiful, enlarged, and filled with membranous, myelin-type inclusions and many cells showed chromatin margination, nuclear pycnosis, and fragmentation (Fig. 3B,C).

3.4. Mitochondrial destabilization and caspase-3-like protease activation

To determine whether lysosomal destabilization accompanied or preceded mitochondrial alterations, lysosomes and mitochondria were labeled with AO or Rh-123, respectively. As previously mentioned, AO is concentrated within intact lysosomes, while Rh-123, being a weakly acidic fluorophore, accumulates in the mildly alkaline matrix of viable mitochondria with normal transmembrane potential.

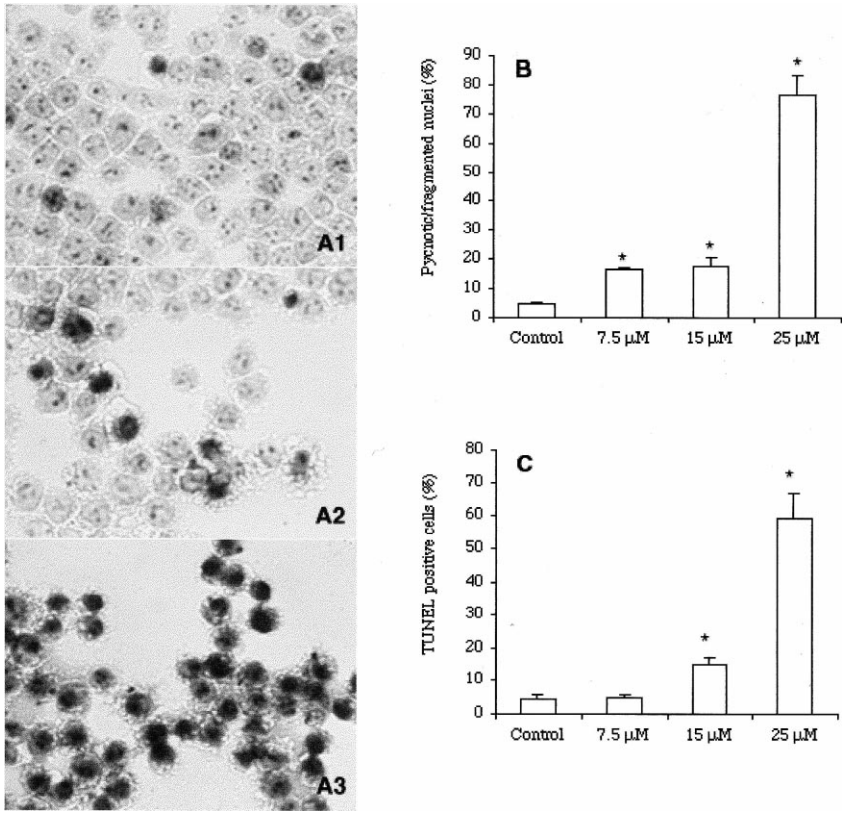

Fig. 2. A: Light microscopy of J-774 cells exposed or not to MSDH. Compared to control cells (A1) many MSDH-exposed cells (24 h) are shrunken with condensed nuclei (A2, 15 μM; A3, 25 μM). B: The quantitative results of Wright–Giemsa staining of J-774 cells, exposed to MSDH for 24 h at indicated concentrations. C: TUNEL positivity of MSDH-exposed J-774 cells (7.5, 15, or 25 μM) for 24 h. Means ± S.E.M., $n = 3$–5. *Significantly different from the control, $P < 0.05$.

Following exposure to 50 μM MSDH for different periods of time, no noticeable decrease of the Rh-123-induced fluorescence was observed until significant lysosomal rupture had occurred (Fig. 4A). Furthermore, no increase in apoptotic cells and no mitochondrial alterations were observed during the first hour of exposure to MSDH in moderate doses (≤ 50 μM). However, obvious lysosomal destabilization was then already apparent. Only when greatly increased numbers of apoptotic cells were found (3 h and 5 h after start of a 50 μM MSDH treatment, resulting in about 25 and 35% apoptotic cells, respectively) were mitochondrial effects observed as indicated by decreased Rh-123 fluorescence. All three cell lines behaved similarly, although the THP-1 and U937 cells were somewhat less sensitive than the J-774 cells.

As indicated in Fig. 4B, 50 μM MSDH induced a time-dependent activation of caspase-3-like proteases. Supporting the possible importance of mitochondrial destabilization – perhaps caused by released lysosomal enzymes – in the apoptotic process, pronounced increases in caspase activity did not appear until after 3 h of MSDH exposure. It paralleled the appearance of morphologic signs of apoptosis and mitochondrial destabilization and occurred much later than the initial partial lysosomal rupture.

4. Discussion

The involvement of mitochondria in the apoptotic process has been the subject of much research during the past few years, and relocation of cytochrome *c* and apoptotic inducing factor (AIF) to the cytosol is now often considered to be an initiating and basic event in apoptosis [23–25]. However, another cellular organelle, the lysosome, has recently been suggested to be involved in the apoptotic process as well. We have observed lysosomal rupture to be an early and consistent event preceding apoptosis caused by a variety of agonists, such as oxidative stress, FAS receptor ligation, growth factor withdrawal, exposure to oxidized low-density lipoprotein, and photo-oxidative damage to lysosomal membranes [1–7]. However, the idea that lysosomes might participate in apoptosis has met with some skepticism, perhaps because many seemingly intact lysosomes are found even late during apoptosis.

In order to further evaluate the hypothesis that lysosomal leakage may cause apoptosis, a method to induce lysosomal rupture in a regulated and specific manner would be an important tool. Lysosomotropic agents are able to diffuse across cellular membranes and become protonated, and thus charged, within the acidic compartment, where they become trapped and accumulate [26]. In the present study, we have taken advantage of such a compound, MSDH, which is both relatively lysosomotropic ($pK_a = 5.8$–5.9) and, when protonated, a detergent. Thus, only the acidic apparatus will be exposed to its detergent effect [19]. Upon diffusing into the acidic vacuolar compartment, MSDH becomes protonated and thus accumulates via the mechanism of 'proton trapping', since the free base diffuses through membranes much more freely than the protonated form.

When MSDH was applied to the three cell lines (J-774, U937 and THP-1) it caused lysosomal destabilization and

rupture, as well as cytotoxicity. These phenomena were shown by increased numbers of cells taking up trypan blue, decreased uptake of the lysosomotropic fluorescent weak base AO, and relocation to the cytosol of the lysosomal marker enzyme cathepsin D. Although the J-774 cells proved to be the most sensitive, all three cell types basically showed the same behavior. At moderate concentrations, where lysosomal rupture was initially incomplete, MSDH caused typical apoptosis, including caspase activation, and only few cells stained with trypan blue during the early phase of the process, although they subsequently underwent post-apoptotic necrosis. In contrast, following exposure to high concentrations of MSDH, a primary necrotic type of cell death rapidly followed, and rupture of the acidic vacuolar apparatus was almost complete already early after exposure to MSDH.

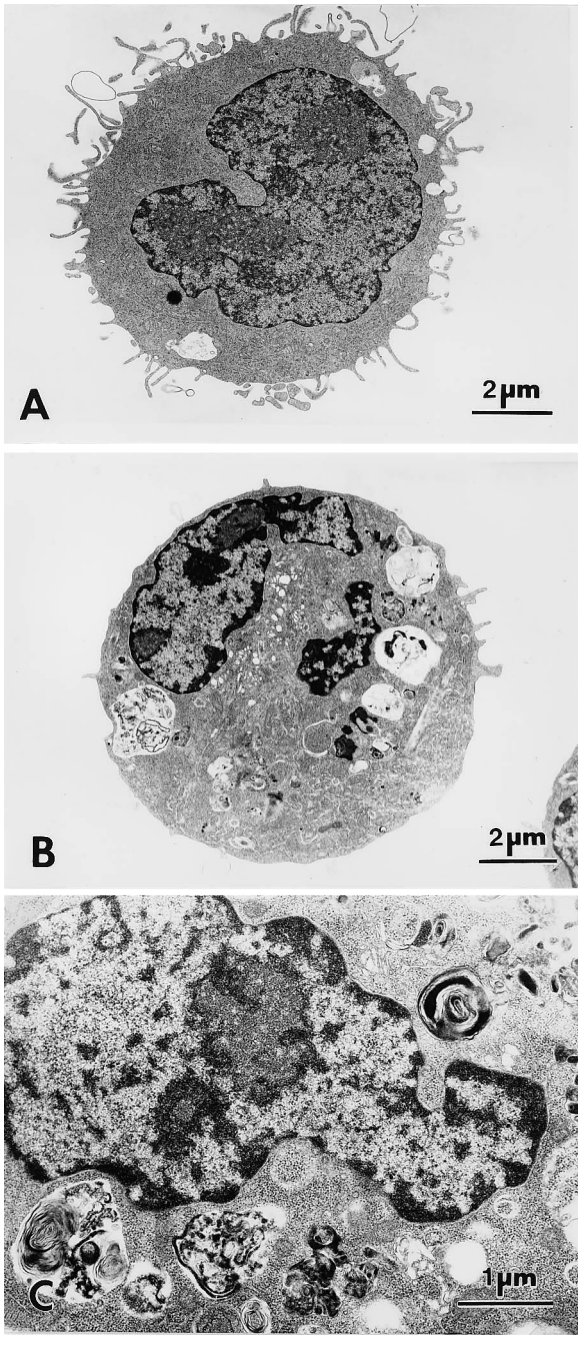

Fig. 3. Transmission electron micrographs of cultured J-774 cells. A: Control cell. B and C: MSDH-exposed (50 μM, 5 h) cells. Note typically apoptotic, partly fragmented, nuclei with heterochromatin margination and increased number of enlarged autophagic lysosomes containing whorled, myelin-type membranous material in the exposed cells.

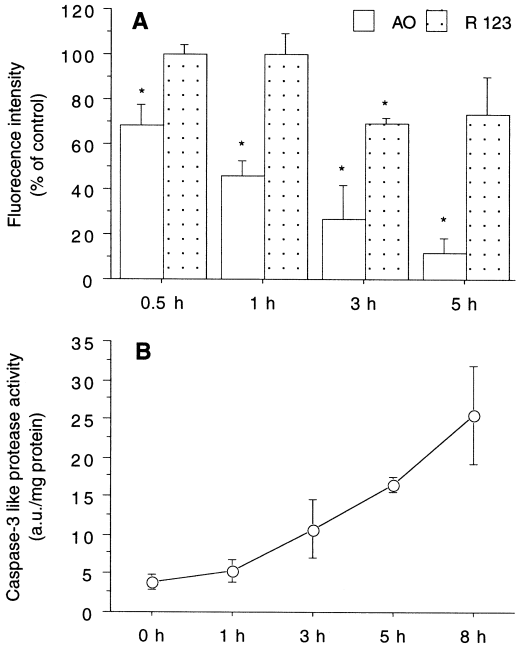

Fig. 4. Effect of MSDH on lysosomal and mitochondrial stability, and activation of caspase-3-like proteases. A: Lysosomal and mitochondrial stability of J-774 cells after MSDH exposure (50 μM, 0–5 h). The AO- or Rh-123-induced fluorescences were quantified by static cytofluorometry. Both the lysosomal and the mitochondrial fluorescence decreased over time, indicating impaired stability. B: Activation of caspase-3-like proteases of J-774 cells following exposure to MSDH. Note that lysosomal destabilization was a significantly earlier phenomenon than both mitochondrial alteration and activation of caspase-3-like proteases. Means ± S.E.M., $n = 3$. *Significantly different from the control, $P < 0.05$.

When MSDH was applied at low or moderate concentrations (≤50 μM), lysosomal rupture was clearly initiated before any morphologic signs of apoptosis took place. At that early stage, a few hours before the first apoptotic cells were found, there were no alterations in the mitochondrial Rh-123 fluorescence in any of the cell types. The mitochondrial destabilization that occurred later may have been caused by attack on the mitochondrial membranes by lysosomal enzymes, releasing pro-caspase activating factors, such as cytochrome *c* and AIF. The importance of the caspase family of proteases, having very defined substrate specificity and a complicated pattern of inducement involving a cascade-type self-activation, was pointed out some years ago [27].

In accord with a number of earlier observations, made with a variety of pro-apoptotic agonists, our present data indicate again that lysosomal leakage is the earliest detectable event during apoptosis induced by MSDH, followed by mitochondrial changes, caspase activation, and nuclear apoptotic alterations. Our results strengthen the very early and prescient suggestion by Christian de Duve [9] that lysosomes are, indeed, suicide bags. However, we are now beginning to appre-

ciate that, although the same bag is involved, the suicide may occur in either an orderly or a chaotic fashion.

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