Biochimica et Biophysica Acta 1794 (2009) 1372–1377

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Cysteine cathepsins are not critical for TNF-α-induced cell death in T98G and U937 cells

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ARTICLE INFO

Article history:
Received 30 January 2009
Received in revised form 24 March 2009
Accepted 6 April 2009
Available online 3 May 2009

Keywords:
TNF-α
Apoptosis
Cathepsin B
Cathepsin
Caspase
Cysteine protease

ABSTRACT

The tumor necrosis factor (TNF) is a cytokine known to be an important mediator of apoptosis and inflammation. It has been implicated in the pathogenesis of a number of diseases, including cancer and rheumatoid arthritis. TNF apoptosis has been known for a number of years to be critically dependent on caspases; however, recently it has been suggested that cysteine cathepsins might also be involved in the pathway. In the present work the hypothesis that cathepsins can act as an essential downstream mediator of TNF-α-triggered apoptosis was tested. The TNF-α apoptosis was investigated in two tumor-cell lines: U937 and T98G. Based on the use of pharmacological caspase inhibitors, the TNF-α induced caspase-dependent apoptotic cell death in both cell lines, which was accompanied by lysosomal destabilization and the release of cathepsins in the cytosol. However, blocking cysteine cathepsins with a broad-spectrum inhibitor, E64d, or a more specific cathepsin B inhibitor, CA-074Me, had no effect on the progression of the apoptosis in both cell lines, suggesting that the TNF-α apoptosis is not critically dependent on the cathepsins in these two cellular models.

1. Introduction

The tumor necrosis factor-alpha (TNF-α) is a cytokine produced mainly by activated macrophages, and in minor quantities by several other types of cells. It is capable of inducing different biological responses and it plays a role in inflammation, stress response and apoptosis, where it can induce both pro- and anti-apoptotic signaling. The TNF-α binds to two cell-surface receptors, TNF-R1 and TNF-R2, which in turn oligomerize and bind the adaptor protein TNF receptor-associated death domain (TRADD), which recruits additional adaptor proteins: receptor-interacting protein (RIP), TNF-R-associated factor 2 (TRAF2) and Fas-associated death domain (FADD). In the apoptotic pathway this complex recruits caspase-8 molecules, which bind to FADD [1,2] and are activated in the complex by dimerization, due to their high local concentration [3]. Active caspase-8 then activates the effector caspases, primarily through the cleavage of the Bcl-2 family member Bid and the subsequent activation of the mitochondrial pathway, which serves as an amplifier of the signal [4,5]. The apoptotic pathway can, however, be attenuated through the activation of the NF-κB signaling pathway, which critically depends on RIP binding to TRADD [1,2].

In the past decade, several studies have suggested that in addition to caspases, the lysosomal cysteine cathepsins also play an important role in TNF-α-induced cell death. Among these cathepsins, an important role was assigned primarily to cathepsin B and, to a lesser extent, to cathepsin L [6,7]. Cathepsin B was thus suggested to be a major factor in both TNF-α-induced liver damage [8] and tumor-cell apoptosis [9,10]. Moreover, isolated hepatocytes from cathepsin B-deficient mice were shown to confer significant protection against TNF-α-induced apoptosis. In addition, in cultured hepatocytes, a selective cathepsin B inhibitor, CA-074, which is an epoxysuccinyl derivative of E64, attenuated significantly the TNF-α-induced apoptosis [11,12]. Similar results were also observed in a number of tumor-cell lines and in transformed cathepsin B-deficient mouse embryonic fibroblasts [9,10]. In contrast to these findings, transient transfections of the cathepsins B and L, resulting in expression levels comparable to those found in many tumors, did not sensitize HeLa or McA RH 7777 tumor cells to TNF-α-mediated apoptosis [13]. In addition, primary tumor cells isolated from mammary tumors of wild-type or cathepsin B-deficient PyMT mouse have not shown any difference in terms of their sensitivity toward TNF-α-mediated apoptosis [14], so arguing against any essential role of cathepsins in this cell-death pathway.

Abbreviations: CA-074Me, [(2S,3S)-3-Propylcarbamoyloxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester; CHX, cycloheximide; E-64d, (2*S*,3*S*)-trans-epoxysuccinyl-leucylamido-3-methyl-butane ethyl ester; TNF-α, tumor necrosis factor-alpha; Z-DEVD-FMK, Z-Asp(OMe)-Glu(OMe)-Val-DL-Asp(OMe)-fluoromethylketone; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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doi:10.1016/j.bbapap.2009.04.011

In order to address these discrepancies, we decided to characterize TNF-α-induced cell death in two different cell lines: the promonocytic U937 and the glioblastoma T98G. Although lysosomes were found to be destabilized following the TNF-α treatment, resulting in the release of cysteine cathepsins into the cytosol, the broad-spectrum cysteine cathepsin inhibitor E-64d was not able to prevent apoptosis progression, suggesting that cysteine cathepsins are not critical for TNF-α-induced cell death in the cellular models used.

2. Materials and methods

2.1. Materials

All the cell lines used – the leukemic monocyte lymphoma cell line U937, the glioblastoma T98G cell line, the monocytic cell line THP-1 and the human immortalized keratinocytes HaCaT – were purchased from LGC Standards GmbH (Wesel, Germany). The human TNF-α was from ProSpec-Tany TechnoGene LTD (Rehovot, Israel). The CHX, the protein inhibitor cocktail, the propidium iodide, the phosphate buffer saline (PBS), and the culture media RPMI 1640 and DMEM were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibiotics penicillin/streptomycin, the L-glutamine, the HEPES buffer, the fetal calf serum and the fluorescent probes Mitotracker Red CMXRos and Lysotracker Green DND-26 were purchased from Invitrogen (Carlsbad, CA, USA). The substrates 7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) and benzyloxycarbonyl-Phe-Arg-7-amino-4-methyl-coumarin (Z-Phe-Arg-AMC) and the caspase inhibitors benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) and Z-Asp (OMe)-Glu(OMe)-Val-DL-Asp(OMe)-fluoromethylketone (Z-DEVD-FMK) were from Bachem AG (Bubendorf Switzerland), whereas the cathepsin inhibitors (2*S*,3*S*)-trans-epoxysuccinyl-leucylamido-3-methyl-butane ethyl ester (E-64d) and [(2S,3S)-3-Propylcarbamoyloxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester (CA-074Me) were from the Peptide Institute (Osaka, Japan). Stock solutions of the substrate and the inhibitors were prepared in dimethyl sulfoxide and stored at −20 °C until use. Annexin V-PE and 7-amino-actinomycin D were from BD Biosciences Inc. (San Jose, CA, USA). The monoclonal antibodies against cathepsins B and the polyclonal antibodies against cathepsin L were prepared as previously described [15–17]. All the other reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell cultures

All the cell lines were grown at 37 °C in a humidified air atmosphere with 5% CO_2. The U937 and THP-1 cells were cultured in the RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES buffer, whereas the T98G and the HaCaT cells were grown in the DMEM medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Induction of apoptosis

The cells were seeded in 6-well plates at 1×10^6 cells/well in the appropriate complete medium at least 16 h prior to the induction of apoptosis. The cathepsin or caspase inhibitors, E-64d, CA-074Me, Z-VAD-FMK or Z-DEVD-FMK, were added at a final concentration of 10–25 μM, 1 h prior to the addition of the TNF-α and CHX. Following 3–16 h of incubation, the cells were observed using light microscopy and then harvested. The cell viability was checked using the CellTiter-Blue™ assay, following the manufacturer's instructions (Promega, Madison, WI, USA). Whole-cell, cell-free or cytosolic extracts were prepared as described previously [18,19]. Inhibitors were omitted in the control experiment.

2.4. Determination of the protein concentration and the caspase activity

The total protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). A total of 50 μg of cellular proteins was used to determine the activity of the caspases (DEVD-ase) within the extracts. The assay was carried out as previously described [20]. The cleavage of the fluorogenic substrate Ac-DEVD-AFC was measured continuously in a 96-well plate reader (Tecan Safire, Männedorf, Switzerland) at excitation and emission wavelengths of 400 and 505 nm, respectively.

2.5. Quantification of cell death

To measure the phosphatidylserine exposure, 10^6 cells were labeled with annexin V-PE and 7-amino-actinomycin D or propidium iodide according to the manufacturer's instructions. The percentages of viable and dead cells were determined from 20,000 cells per sample, using the FL2 channel for annexin V-PE. The cells were subjected to flow-cytometry analysis using a FACScalibur flow cytometer (Becton Dickinson, USA) and the CellQuest software.

2.6. Determination of the mitochondrial and lysosomal stability

The integrity of the mitochondria and the lysosomes following the TNF-α treatment was monitored by flow cytometry using the fluorescent probes Mitotracker Red CMXRos and Lysotracker Green DND-26, respectively, following the manufacturer's instructions. Briefly, 1×10^6 cells/ml of treated cells were incubated for a maximum of 30 min with 50 nM of the fluorescent probe in a pre-warmed medium. The red mitochondrial or green lysosomal fluorescence of 20,000 cells per sample was quantified by flow cytometry using the FL3 or FL1 channel, respectively, as described previously [18,19].

2.7. Immunoblotting

A total of 70 μg of protein was loaded and resolved in 15% SDS-PAGE gels and electro-transferred to nitrocellulose membranes. The membranes were probed with specific antibodies against cathepsin B or L at 1:500–1:1000 dilutions. After the incubation with horseradish peroxidase-conjugated secondary antibodies (at 1:3000 dilution), the membrane was incubated with the ECL Plus Western blotting reagents according to the manufacturer's instructions (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

2.8. Statistical analysis

Data are expressed as means ± S.D. of at least three independent experiments. Statistical comparisons between the different groups were made using one-way ANOVA test. A probability value (P) below 0.05 was considered statistically significant.

3. Results

3.1. TNF-α induces caspase-dependent apoptotic cell death in the U937 and T98G cells

In order to obtain a better idea about the role of cysteine cathepsins, including cathepsin B, in TNF-α-induced apoptosis, we selected two non-related cell lines – a promonocyte cell line U937, which is known to express high levels of cathepsins, including cathepsin B; and the glioblastoma T98G cell line – as the model systems. In the preliminary experiments, the levels of TNF-α and CHX were varied in order to obtain an optimal apoptotic response. The U937 cells were found to be substantially more sensitive: even a 3-h incubation with 10 ng/ml TNF-α and 0.2 μg/ml CHX had a

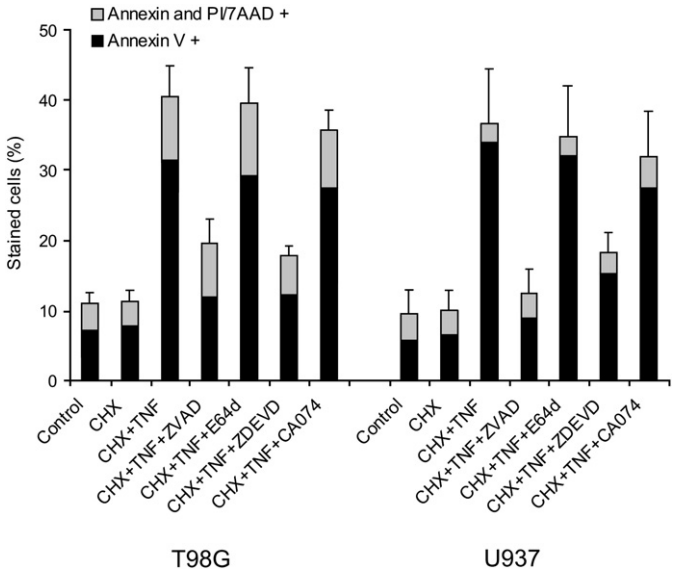

Fig. 1. Cytofluorometric assessment of cell death after TNF-α-induced apoptosis. The percentages of annexin V+ and 7-AAD+/PI+ cells resulting from the flow-cytometry analysis are indicated on each histogram. The results are means ± S.D. (S.D. from both annexin V and 7-AAD positive cells) of four independent experiments performed 3 h and 16 h after apoptosis induction. The *P* value was below 0.0001.

profound effect on cell survival, judged on the basis of the CellTiter-Blue™ cell viability assay and light microscopy. In contrast, 16-h incubation with 100 ng/ml TNF-α and 1 μg/ml CHX was required to obtain a similar response from the T98G cells (not shown). These conditions were then used in all the subsequent experiments. In the next step we quantified the apoptosis with measurements of the phosphatidylserine exposure and the caspase activation. As shown in Fig. 1, about 35% of the cells in each of the two cell lines tested were apoptotic, based on annexin V-PE and 7AAD staining. Pretreatment of the cells with 15 μM Z-VAD-FMK completely prevented phospatidylserine exposure. Since Z-VAD-FMK, as a broad-spectrum inhibitor, blocks all the caspases, including caspase-8, which is critical for the initiation of apoptosis, an additional experiment was performed in which both cell lines were pretreated with 15 μM Z-DEVD-FMK, an inhibitor that is more selective for the executioner caspase-3. The latter inhibitor also prevented phospatidylserine exposure, consistent with an essential role for caspases in this model of cell death. In contrast, pretreatment of cells with 15-μM E64d, which is known to efficiently block cysteine cathepsin activity in cells at that concentration [21], did not significantly prevented phosphatidylserine exposure. Similar results were obtained with CA-074Me, a more selective cathepsin B inhibitor (Fig. 1), suggesting that cysteine cathepsins are not critical for the progression of TNF-α-induced apoptosis in both cell lines examined. In the next series of experiments, apoptotic cell death was also confirmed by the measurements of DEVD-ase activity, indicative of caspase activation. As can be seen in Fig. 2, a 4–8-fold increase in DEVD-ase activity was observed in the two cell lines, which could be prevented by both Z-VAD-FMK and Z-DEVD-FMK, confirming the critical role of caspases in this model of cell death. Again, E64d and CA-074Me had no significant effect on the caspase activity in both cell lines, in agreement with the above results, and consistent with the idea that cathepsins are not the major players in these cellular models.

3.2. TNF-α-induced apoptosis is accompanied by the disruption of mitochondria and lysosomes

As the integrity of mitochondria is normally compromised in TNF-α-induced cell death [22], we next examined mitochondria integrity using Mitotracker Red. As can be seen in Fig. 3A, about 30% of the U937 cells, but only ~15% of the T98G cells, had damaged mitochondria after the TNF-α treatment, based on a flow-cytometry analysis. In both cell lines Z-VAD-FMK completely preserved the integrity of the mitochondria, whereas E64d had no effect, consistent with the idea of the caspases, and not the cathepsins, being the major players in this model.

As a considerable number of reports suggested the release of cathepsins into the cytosol following the TNF-α treatment, we next examined the integrity of the lysosomes in both cell lines. As can be seen in Fig. 3B, the integrity of the lysosomes was compromised in a considerable number of cells in both cell lines, although this number was substantially lower in the T98G cells (15% vs. 40%).

3.3. Cathepsin B is differentially expressed in the T98G and U937 cell lines

Having found that a number of cells exhibited compromised lysosomal integrity, we next examined whether this loss of lysosomal integrity resulted in a release of the cysteine cathepsins into the cytosol. The major focus was on cathepsin B, which was most often found to be associated with TNF-α-induced cell death [6] and which is often used as a marker for lysosomes. As can be seen in Fig. 4A, a considerable amount of cathepsin B was observed in the cytosol of the U937 cells following the TNF-α treatment, confirming the lysosomal damage. Consistent with other experiments (see above), blocking the caspases with Z-VAD-FMK completely prevented cathepsin B translocation, whereas E-64d had no significant effect (Fig. 4A). In contrast, no cathepsin B was detected in the cytosol of T98G cells (not shown), suggesting that this cell line has a very low level of endogenous cathepsin B. In order to confirm that cathepsins released into the cytosol were indeed active, cytosolic extracts of TNF-α treated and untreated cells were examined using Z-Phe-Arg-AMC, which is a characteristic substrate for cysteine cathepsins including cathepsin B. A 2.2-fold increase of Z-Phe-Arg-AMC activity was observed upon TNF-α treatment of U937 cells (not shown), consistent with the

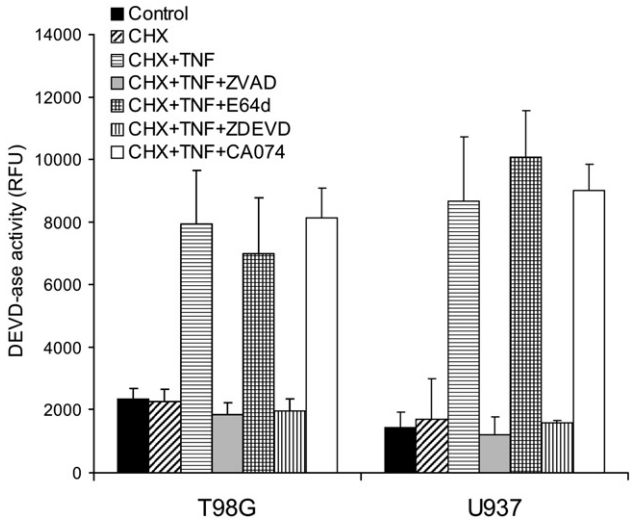

Fig. 2. Caspase activity after TNF-α-induced apoptosis. The U937 cells were treated with 10 ng/ml TNF-α and 0.2 μg/ml CHX for 3 h. The inhibitors Z-VAD-FMK (25 μM) and E64d (10 μM) were added 1 h before the treatment. The T98G cells were treated with 100 ng/ml TNF-α and 1 μg/ml CHX for 16 h. Before the treatment the inhibitors Z-VAD-FMK (15 μM), E64d (15 μM), CA-074Me (15 μM) and Z-DEVD-FMK (25 μM) were added. The caspase activity (DEVD-ase) is expressed in relative fluorescence units (RFU). The results are means ± S.D. of three independent experiments. The *P* value was below 0.0001.

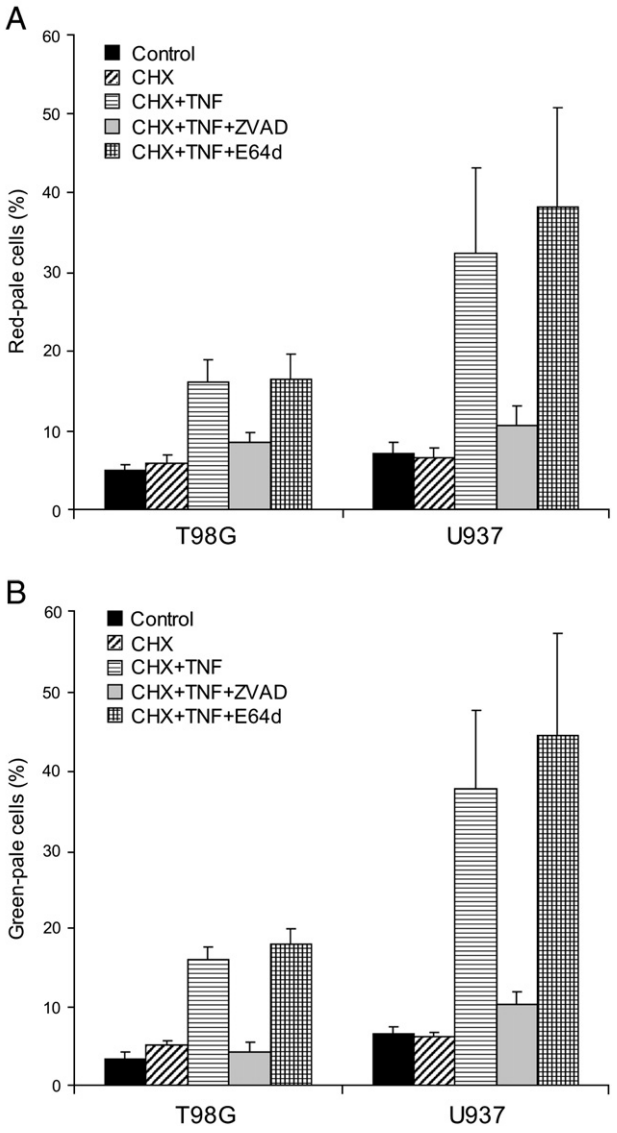

Fig. 3. Assessment of mitochondrial and lysosomal integrity in TNF-α-induced apoptosis. The T98G and U937 cells were treated with TNF-α and CHX for 3 h and 16 h respectively. Mitotracker Red CMXRos- (A) and Lysotracker Green- (B) uptakes were determined by flow cytometry, as described in Materials and methods (section 2.6.). Each histogram indicates the percentage of cells with decreased fluorescence. The results are means ± S.D. of three independent experiments. For U937 cells, the *P* value was 0.0006 whereas for T98G cells the *P* value was below 0.0001.

release of cathepsins in the cytosol. In contrast, only marginal differences were observed in T98G cells, in agreement with very low levels of cathepsin B in this cell line.

Since it is known that different cells or tissues express different levels of cathepsins [23], we wanted to verify the above findings and therefore investigated the levels of endogenous cathepsin B in four different cell lines in total cell extracts. In addition to the U937 and T98G cells, THP-1, another immune-type cell line, and HaCaT, as a keratinocyte type cell line, were included in this experiment. Both immune-type cell lines, THP-1 and U937, expressed very high levels of single-chain mature cathepsin B (~35 kDa), HaCaT cells expressed only moderate levels of the enzyme, whereas no cathepsin B could be detected in the T98G cells, consistent with the above findings (Fig. 4B). Since the recombinant single-chain cathepsin B used as a control was nonglycosylated due to its expression in *Escherichia coli*, it exhibited a slightly lower molecular weight than mammalian cathepsin B from different cell lines (D. Caglič, U. Repnik. and B. Turk, unpublished). The levels of cathepsin L, which were also examined in the four cell lines, differed to a lesser extent (data not shown).

4. Discussion

Death-receptor-mediated cell death, as one of the major mechanisms for the removal of cancer cells, has been extensively studied for the past 10–15 years with a major focus on the TNF pathway [24–26]. Here we report that TNF-α-induced apoptosis is largely independent of the cysteine cathepsins, although lysosomes were found to be damaged in a number of cells and significant levels of cathepsin B were observed in the cytosol as a result of this damage. Our results thus clearly support the majority of studies suggesting that caspases are the major executioners of cell death in the death-receptor pathway [1,2,4,5]. However, this outcome poses an obvious question about the role of lysosomal cysteine cathepsins in this pathway. The lysosomal damage observed linked with increased level of cathepsins in the cytosol are also in agreement with other reports suggesting that TNF-α and TRAIL-induced apoptosis in various cancer cells and hepatocytes involve lysosomal cysteine cathepsins, in particular cathepsin B [8–12]. The simplest explanation is that the involvement of cathepsins in an apoptotic pathway is dependent on the cell and/or tissue type. However, the situation is probably more complex and cathepsins have been suggested to trigger caspase-independent cell death [7], as well as to act in concert with caspases, with the major function being to assist the latter by helping to activate them [27–29]. The major problem with the idea of caspase-independent cell death is that a single intracellular cathepsin substrate has not been identified, a

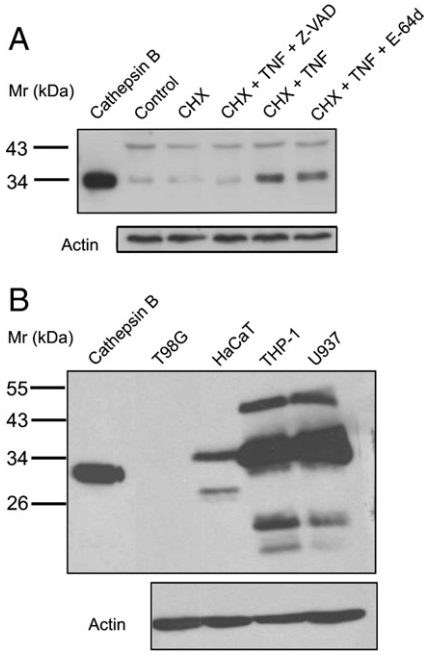

Fig. 4. (A) Release of cathepsin B in the cytosol of the U937 cells during TNF-α-induced apoptosis. Equal amount of proteins were loaded and separated on 15% SDS-PAGE, followed by immunoblotting with anti-cathepsin B antibodies. Actin was used as a loading control. Upper band in all lanes corresponds to procathepsin B. (B) Expression of cathepsin B in different tumour-cell lines. Whole-cell extracts of different cell lines were immunoblotted against cathepsin B. Equal amount of proteins were loaded and separated on 15% SDS-PAGE, followed by immunoblotting with the anti-cathepsin antibodies. The different bands observed in various cell lines correspond to procathepsin B (~46 kDa), single-chain mature cathepsin B (~35 kDa) and differentially processed heavy chain of mature cathepsin B (23–28 kDa) (D. Caglič, U. Repnik. and B. Turk, unpublished).

discovery that would help unravel this pathway. The only cysteine cathepsin substrates identified so far are Bid, the anti-apoptotic Bcl-2 family members Bcl-2, Bcl-Xl, Mcl-1 and, to a minor extent, the caspase inhibitor XIAP, which largely points to the activation of the mitochondrial pathway and not toward caspase-independent cell death [6,18,20,30–32]. However, this might not be important in the death-receptor pathway, as lysosomal destabilization linked with the appearance of cathepsins in the cytosol was observed in Fas apoptosis in type-II cells, although apoptosis was not dependent on the cathepsins [33,34]. The latter studies suggested that lysosome breakdown is a late event in death-receptor apoptosis and therefore not critical for apoptosis, which could also explain our results. Furthermore, this is also consistent with the results of Vasiljeva et al. [14], who found that cathepsin B was dispensable for TNF-induced apoptosis in primary mouse-cancer cells, whereas Leu-LeuOMe-induced apoptosis, which clearly involves lysosomal cathepsins, was attenuated in cathepsin B knockout cells.

Another unclear issue in the pathway is the lysosomal permeabilization. There have been several ideas suggesting how this may happen, but they all contain some flaws. The initial study using mouse hepatocytes suggested that caspase-8 may directly destabilize lysosomes [11], which is highly unlikely as a protease, in general, cannot degrade membranes consisting of non-protein components. A few studies suggested Bax-induced lysosomal permeabilization, which in principle is possible [35,36]. However, Bax also extremely efficiently permeabilizes mitochondria [37–39], which would lead to a mitochondrial apoptotic pathway anyway, without the need for any lysosome involvement, making this hypothesis less likely. Although our results neither provide direct evidence for the mechanism of lysosome permeabilization, one possibility is that the higher sensitivity of the U937 cells to TNF-α could be linked to the higher expression level of cathepsins B and L in this cell line, compared to the T98G cell line. However, different levels of TNF receptors and caspase-8 in the two cell lines could equally well explain these results.

5. Conclusion

We have shown that TNF-α-induced apoptosis is largely independent of cysteine cathepsins, although lysosomes were found to be damaged in a number of cells and significant levels of cathepsin B were observed in the cytosol as a result of this damage. Moreover, these results support the studies suggesting that caspases are the major executioners of cell death in the death-receptor pathway. However, controversies are likely due to the fact that this pathway is largely cell-dependent and that lysosomal cathepsins have differential roles, depending on the cell type used and on the time of their release into the cytosol. Therefore, the possibility that lysosomes may serve as a downstream event to amplify the signal through the engagement of mitochondria cannot be excluded.

Acknowledgements

We are grateful to Dr. Urška Repnik and Dr. Nataša Kopitar-Jerala for providing us with the cathepsins B and L antibodies. In addition, we acknowledge Dr. Urška Repnik's advice on flow cytometry and Dr. Paul McGuiness for critical reading of the manuscript. This work was supported by grants from the Slovenian Research Agency to V.T. (P1-0140), B.T. (J1-0711) and V.S. (J1-9520).

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