FEBS Letters 581 (2007) 5185–5190

Cysteine cathepsins are not involved in Fas/CD95 signalling in primary skin fibroblasts

Lea Bojič[a], Ana Petelin[a], Veronika Stoka[a], Thomas Reinheckel[b], Christoph Peters[b], Vito Turk[a], Boris Turk[a,*]

[a] *Department of Biochemistry, Molecular and Structural Biology, J. Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia*
[b] *Institut für Molekulare Medizin und Zellforschung, Albert-Ludwigs-Universität, Breisacherstr. 66, D-79106 Freiburg, Germany*

Received 14 September 2007; revised 2 October 2007; accepted 2 October 2007

Available online 12 October 2007

Edited by Veli-Pekka Lehto

Abstract The potential role of cysteine cathepsins, especially cathepsin B, in Fas/CD95-induced apoptosis was investigated using wild-type and cathepsin B-deficient primary skin fibroblasts. Apoptosis was induced with an anti-Fas/CD95 antibody in the presence of cycloheximide and no difference was observed between the two genotypes. First cells with damaged mitochondria were observed ~3 h post apoptosis induction and their number was significantly increased after 11 h. In contrast, cells with damaged lysosomes were only seen after 15 h with no difference between the two genotypes. Moreover, Bid cleavage was found to be diminished in cathepsin B-deficient cells. These results suggest that cysteine cathepsins have no active role in Fas/CD95 apoptosis.

Keywords: Fas/CD95; Apoptosis; Caspases; Cathepsin; Cell death; Cathepsin B

1. Introduction

Fas/CD95 (APO-1) is one of the best characterized receptors from the tumor necrosis factor (TNF) receptor family with a major role in induction of apoptosis [1]. It has been found to be implicated in T- and B-cell apoptosis, in liver homeostasis and in a number of malignancies [2]. The mechanism of cell death triggered by Fas/CD95 is intimately linked with the formation of the death inducing signalling complex (DISC), which in addition to the receptor and its ligand involves the adaptor protein Fas-associated death domain containing protein (FADD) and the apoptosis-initiating protease caspase-8, which binds to FADD through DED and gets activated in the complex through proximity-induced dimerization [3]. The pathways downstream of caspase-8 activation differ based on the quantity of active caspase-8 formed. In type I cells, such as thymocytes, high amounts of caspase-8 are sufficient to directly activate the executioner caspases, in particular caspase-3, whereas in type II cells, such as hepatocytes, the level of caspase-8 formed is insufficient for direct caspase-3 activation and requires engagement of the mitochondrial system through the cleavage of proapoptotic Bcl-2 homologue Bid and subsequent release of proapoptotic factors from mitochondria [1,4–6].

In addition to caspases, the cathepsins [7], which have traditionally been viewed as lysosomal mediators of protein turnover, have been found to be implicated in apoptosis [8–10]. Lysosome-mediated apoptosis results from a destabilization of the lysosomal membrane followed by the release of cathepsins into the cytosol. Although lysosomes were found to be particularly sensitive towards oxidative stress [11,12], lysosomal destabilization was observed during apoptosis induced by different stimuli, including death ligands from the TNF family [10,13,14]. Although there is a clear picture that lysosomal cysteine cathepsins, in particular cathepsin B, are involved in TNF-α and TRAIL-induced apoptosis [15–17], the involvement of lysosomal proteases in Fas/CD95-induced apoptosis is still debated. While some authors suggest that activation of CD95 does not involve lysosomes [15,18], others suggest the opposite [19–21].

In order to address this question, Fas/CD95-induced apoptosis was investigated in wild-type and cathepsin B-deficient primary murine skin fibroblasts. We found that cysteine cathepsins including cathepsin B are not involved in apoptosis progression, although the lysosomes were found to be destabilized at a later time point and the amount of Bid cleaved was considerably diminished in cathepsin B-deficient cells as compared to the wild-type, suggesting that lysosomal destabilization and cathepsin-mediated Bid cleavage are probably not of physiological importance.

2. Materials and methods

2.1. Materials

DMEM medium was purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum, horseradish peroxidase-conjugated rabbit-anti-goat IgG, cycloheximide (CHX) and protein inhibitor cocktail were obtained from Sigma (USA). The polyclonal antibodies against Bid were from R&D Systems (USA), the antibodies against CD95 mAb Jo-2 were from Pharmingen BD (USA), while the enhanced chemiluminiscence Western blotting reagents were obtained from Amersham Pharmacia Biotech (Stockholm, Sweden). The caspase substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-tri-fluoromethyl coumarin (Ac-DEVD-AFC) and the pancaspase inhibitor z-VAD-fmk were purchased from Bachem (Switzerland). The cysteine protease inhibitor E-64d was from Peptide Inc. (Japan). Fluorescent organelle-specific probe Mitotracker Red CMX-Ros was from Molecular Probes (Eugene, OR, USA) and acridine orange (AO) was from Sigma (St Louis, MO, USA). Annexin V-PE and 7-AAD for flow cytometry were purchased from BD Biosciences (USA) and the Bradford reagent was from Bio-Rad (Germany).

*Corresponding author. Fax: +386 1 477 3984.
E-mail address: boris.turk@ijs.si (B. Turk).

0014-5793/$32.00
doi:10.1016/j.febslet.2007.10.005

2.2. Cell cultures

Wild-type (WT) and cathepsin B knock out (BKO) primary murine skin fibroblasts were prepared from 3-day-old wild-type or cathepsin B-deficient mice [22] as described previously [23]. Cells were grown in DMEM medium supplemented with 10% FCS, 2 mM glutamine, and 1000 U/ml of penicillin-streptomycin at 37 °C in a humidified air atmosphere with 5% CO_2. Cells were maintained in plastic plates and subcultivated at confluence twice a week. For all experiments cells were used at passages 2–6.

2.3. Induction of apoptosis, cell lysis and Western blotting

Cells were cultured at 1×10^6 cells/ml in 6-well plates at least 16 h before the induction of apoptosis. The cell permeable inhibitors E-64d or z-VAD-fmk were added at the concentration of 15 μM in dimethylsulfoxide (DMSO) 2 h prior to induction of apoptosis with anti-CD95 mAb Jo-2 (0.2 μg/ml) in the presence of CHX (1 μg/ml) for 15 h, as used by others. Following apoptosis induction, cells were collected, pelleted by centrifugation at 1300 rpm for 5 min and washed twice with PBS. Whole-cell extracts were prepared in RIPA buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholic acid, 1 mM EDTA) containing protease inhibitor cocktail. Following 10 min incubation on ice, insoluble material was removed by centrifugation at 14000 rpm for 10 min. Total protein concentration was determined using the Bradford assay. Equal amounts (50 μg) of protein were resolved in 15% SDS–PAGE gels and electro-transferred to nitrocellulose membranes. Blots were probed with Bid specific goat IgG antibodies at 1:500 dilution. Anti-goat horseradish peroxidase-conjugated secondary antibodies (at 1:3000 dilution) were used, followed by visualization with ECL according to the manufacturer's instructions.

2.4. Flow cytometry

Phosphatidylserine exposure and the loss of membrane integrity were measured by labeling cells with Annexin V-PE and 7-amino-actinomycin D (7-AAD) according to the manufacturer's instructions. Cells were then subjected to FACS analysis using FACScalibur flow cytometer (Becton Dickinson, USA) and CellQuest software.

2.5. Measurement of DEVD-ase activity

For measurement of DEVD-ase activity, cellular proteins were prepared as described above for Western blotting assay. Fifty micrograms of protein were used to determine DEVD-ase activity by measuring the proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AFC (Bachem) by caspases, as described previously [24].

2.6. Lysosomal and mitochondrial stability assessment

2.6.1. Mitochondria membrane stability assay. Change in mitochondrial membrane potential ($\Delta\Psi m$) was assessed by use of MitoTracker Red CMX-Ros. MitoTracker Red CMXRos is a red-fluorescent dye that stains mitochondria in live cells and its accumulation is dependent upon membrane potential. Cells with an increased number of damaged mitochondria had decreased red fluorescence. To determine how treatment with CD95 mAb Jo-2 and CHX affected mitochondrial membrane stability in the primary wild-type (WT) and cathepsin B knock out (BKO) murine skin fibroblasts, cells were incubated with DMEM medium supplemented with 30 nM MitoTracker Red CMX-Ros after appropriate time post apoptosis induction. Following 10 min incubation at 37 °C, red fluorescence, corresponding to the $\Delta\Psi m$ of mitochondria, was determined by flow cytometry using the FL-3 channel and 5000 cells per sample.

2.6.2. Lysosome membrane stability assay. Cells were assessed for lysosomal stability using acridine orange (AO) uptake and relocation methods. AO is a lysosomotropic, weak base ($pKa = 10.3$), which is retained in its charged form (AOH^+) inside the acidic vacuolar compartment by proton trapping. Briefly, cells treated with anti-CD95 mAb Jo-2 and CHX were incubated with DMEM medium supplemented with AO (5 μg/ml for 15 min at 37 °C) after 3, 7, 11 and 15 h post apoptosis induction. Cells displaying a reduced number of intact AO-accumulating lysosomes, the so-called 'pale cells', were analyzed as having decreased red fluorescence (the AO uptake method) [25]. Red fluorescence (FL-3 channel) of 5000 cells per sample was measured by flow cytometry. Although LysoTracker Green DND-26 probe worked well in fluorescence microscopy, the probe could not have been used in flow cytometry measurements in fibroblasts, in agreement with manufacturer's instructions (http://probes.invitrogen.com/handbook/print/1203.html).

3. Results

3.1. Treatment with anti-CD95 mAb and CHX induced cathepsin-independent and caspase-dependent cell death in primary murine skin fibroblasts

In order to address the role of cathepsin B and other cysteine cathepsins in Fas/CD95 signalling pathway, the kinetics of DEVD-ase activity, characteristic of caspase activation, and phosphatidylserine externalization were analyzed in primary murine skin fibroblasts from wild-type and cathepsin B-deficient mice, treated with anti-CD95 mAb Jo-2. Stimulation of cells with 0.2 μg/ml anti-CD95 mAb Jo-2 alone did not induce increase in caspase activity or percentage in phosphatidylserine-positive cells in any of the two genotypes of fibroblasts. However, 1 μg/ml CHX sensitized the fibroblasts to anti-CD95 mAb-induced cell death, as judged on the basis of morphology changes (not shown), increased DEVD-ase activity and increased number of apoptotic cells (Fig. 1). This is in agreement with results on human dermal fibroblasts where Fas activation resulted in caspase-8 activation and apoptosis only in the presence of CHX [26], and consistent with type II cells' behavior. However, no difference was observed between the wild-type (WT) and cathepsin B-deficient (BKO) cells (Fig. 1), suggesting that cathepsin B is not important for caspase activation and apoptosis progression in this type of cell death. Serial passaging of fibroblast cultures did not significantly modify the apoptotic susceptibility of both cell lines to anti-CD95 mAb Jo-2 and CHX between passages 2 and 6 (data not shown), implying that the passage number has no effect on apoptosis progression.

As in addition to cathepsin B some other cysteine cathepsin could contribute to Fas/CD95 apoptosis in murine fibroblasts, we next used the broad-spectrum cell-permeable cysteine cathepsin inhibitor E-64d. As can be seen in Fig. 1, pretreatment of fibroblasts with 15 μM E-64d for 2 h prior to induction of apoptosis, which is known to completely block cysteine cathepsin activity in cells [27], had no effect on apoptosis progression as determined by flow cytometry (Fig. 1B), suggesting that none of the cysteine cathepsins is implicated in Fas/CD95 apoptosis in primary skin fibroblasts. In a control experiment, preincubation of cells with 15 μM pan-caspase inhibitor z-VAD-fmk completely abolished all signs of apoptosis in both genotypes, in agreement with the essential role of caspases in this cell death model (Fig. 1). Very similar results were obtained 18 h post apoptosis induction with slightly increased number of apoptotic cells. The percentage of annexin V/7-AAD double positive cells, indicative of late apoptotic or necrotic cells was very low at both 15 and 18 h (~5–11%; Fig. 1B).

3.2. Fas-induced apoptosis induced mitochondrial destabilization and is accompanied by disruption of lysosomes

Although cysteine cathepsins including cathepsin B were found to be dispensable for Fas/CD95-induced apoptosis in murine skin fibroblasts, an earlier report suggesting that Fas signalling induced early lysosomal destabilization [19], prompted us to explore this possibility. In a preliminary experiment, wt and cathepsin B-deficient fibroblasts were incubated