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Sensitization of stefin B-deficient thymocytes towards staurosporin-induced apoptosis is independent of cysteine cathepsins

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Abstract **Stefin B (cystatin B) is an inhibitor of lysosomal cysteine cathepsins and does not inhibit cathepsin D, E (aspartic) or cathepsin G (serine) proteinases. In this study, we have investigated apoptosis triggered by camptothecin, staurosporin (STS), and anti-CD95 monoclonal antibody in the thymocytes from the stefin B-deficient mice and wild-type mice. We have observed increased sensibility to STS-induced apoptosis in the thymocytes of stefin B-deficient mice. Pretreatment of cells with pan-caspase inhibitor z-Val-Ala-Asp(OMe)-fluoromethylketone completely inhibited phosphatidylserine externalization and caspase activation, while treatment with inhibitor of calpains- and papain-like cathepsins (2*S*,3*S*)-*trans*-epoxysuccinyl-leucylamido-3-methylbutane ethyl ester did not prevent caspase activation nor phosphatidylserine exposure. We conclude that sensitization to apoptosis induced by STS in thymocytes of stefin B-deficient and wild-type mice is not dependent on cathepsin inhibition by stefin B.**

Keywords: Apoptosis; Cathepsin; Caspase; Inhibitor; Progressive myoclonus epilepsy; Thymocyte

1. Introduction

Programmed cell death (apoptosis) is essential to many biological processes in multicellular organisms, including embryonic development, immune responses, tissue homeostasis and normal cell turnover [1]. Proteolytic activity plays an important role in apoptosis and caspases appear to be essential for the execution of apoptotic process [2]. However, other cysteine proteases distinct from caspases have been suggested to be involved in apoptosis [3,4]. Cathepsins have traditionally been viewed as lysosomal mediators of protein turnover, however, recent findings have extended their role in other physiological processes including apoptosis [5,6]. The cysteine cathepsins B and L and the aspartyl cathepsin D, participate in both caspase-dependent and caspase-independent apoptosis induced by several stimuli, including death receptors of the TNFR family, B cell receptors, camptothecin (CPT), and bile salt oxidants [7–12].

Stefin B (also called cystatin B) belongs to cystatin family of endogenous cysteine protease inhibitors [13]. Mutations in the gene encoding stefin B are responsible for the primary defect in Unverricht–Lundborg disease (EPM1) [14–16]. Stefin B-deficient mice, produced by targeted disruption of the mouse stefin B gene, display a phenotype similar to the human disease with progressive ataxia and myoclonic seizures [17]. The mice exhibit apoptosis of cerebellar granule cells and have increased expression of apoptosis and glial activation genes [18]. Although, stefin B has long been known to inhibit in vitro papain-like cathepsins by tight and reversible binding, the physiological function of stefin B in the molecular pathogenesis of the disease remains unknown. In vitro stefin B binds tightly to cathepsins H, L, and S, and less tightly to cathepsin B [13]. Houseweart et al. [19] showed that the removal of cathepsin B from cystatin B-deficient mice greatly reduced the neuronal apoptosis, but did not rescue the ataxia and seizure phenotypes and concluded that besides cathepsin B there are other factors involved. Recent studies show that cathepsins cleave the pro-apoptotic Bcl-2 family member Bid, thereby activating it and allowing it to induce the mitochondrial release of cytochrome *c* and subsequent apoptosis [20,21]. At least in stefin B-deficient mice Bid signaling is not essential for apoptosis [22]. In contrast to T cell lines, PS exposure in primary T cells undergoing apoptosis by staurosporin (STS), etoposide, or interleukin 2 withdrawal is caspase-independent, while anti-CD95 triggered apoptosis is caspase-dependent [23].

We wanted to investigate if cell death and PS exposure in thymocytes of stefin B-deficient mice is cathepsin- or caspase-dependent. Therefore, stefin B-deficient thymocytes and thymocytes from wild-type mice were treated with CPT, STS, and anti-CD95 antibody. Thymocytes from stefin B-deficient mice exerted a markedly increased response, when they were exposed to STS, compared to thymocytes from wild-type mice. Preincubation of cells with (2*S*,3*S*)-*trans*-epoxysuccinyl-leucylamido-3-methyl-butane ethyl ester (E-64d) did not prevent apoptosis, while caspase inhibitor z-VAD-fmk completely prevented apoptosis in all cases tested. In thymocytes isolated from wild-type mice and of stefin B-deficient mice, apoptosis is cathepsin-independent and caspase-dependent.

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Abbreviations: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-AMC; AFC, 7-amino-4-trifluoromethyl coumarin; AMC, 7-amino-4-methyl coumarin; CPT, camptothecin; DMSO, dimethylsulfoxide; PS, phosphatidylserine; STS, staurosporin; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fmk; fmk, fluoromethyl ketone; E-64d, (2*S*,3*S*)-*trans*-epoxysuccinyl-leucylamido-3-methyl-butane ethyl ester

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2. Materials and methods

2.1. Materials

Horseradish peroxidase-conjugated goat-anti-rabbit IgG and goat-anti-mouse antibodies, fetal calf serum (FCS), were obtained from Sigma (USA). The caspase substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluorometyl coumarin (Ac-DEVD-AFC) was purchased from Bachem (Bachem AG, Switzerland). All other chemicals were of analytical grade.

2.2. Mice

Stefin B (cystatin B)-deficient mice mice were created as described previously [17]. Stefin B-deficient mice bred on a C57BL/6 background were kindly provided by Dr. R.M. Myers, Stanford University (USA) and bred in our local colony, where background was changed on Balb/c. All mice were genotyped by PCR as previously described [17].

2.3. Cell preparations and cell culture

Freshly prepared thymocytes from thymus glands of 6- to 7-week-old mice were plated in DMEM, 10% FCS, and 2 mM L-glutamine in 24-well culture plates at 5×10^6 cells/well. The cell-permeable inhibitors E-64d (Peptide Institute, Osaka, Japan), or z-VAD-fmk (Bachem AG, Switzerland) were added in adequate concentrations in dimethylsulfoxide (DMSO) 1 h prior to induction of apoptosis. The corresponding volume of DMSO was added to the control cultures. To induce apoptosis, cells were treated with CPT (Sigma, USA) (1 μM), STS (Sigma) (1 μM) or with anti-CD95 mAb Jo-2 (Pharmingen, USA) (500 ng/ml) for 4, 8, 12 and 18 h.

Human Jurkat T cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, and 1000 U/ml of penicillin–streptomycin. Cells were treated with CPT (1 μM), STS (1 μM) or with anti-CD95 mAb CH11 (Pharmingen) (1 μg/ml).

Preparation of cell lysates and immunoprecipitates. For protein analysis, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, and 5 mM EDTA). Cell lysates were clarified by centrifugation at 12 000 rpm for 10 min at 4 °C. Protein concentration of the supernatants were determined by Bradford assay, and equivalent amounts of protein from cell lysates were used for immunoprecipitation or analyzed directly by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blotting. For immunoprecipitations RACK-1 mAb (Pharmingen) was added to lysates and allowed to rock at 4 °C for 2 h. Then, 5 μg of anti-mouse IgM Ab and 50 μl of protein A–Sepharose beads (Amersham Pharmacia Biotech, Sweden) were added to the lysates, which were allowed to rock for another 2 h at 4 °C. Immunoprecipitates were washed twice in fresh cold RIPA buffer. Washed immunoprecipitates and total cell lysates were denatured by boiling after the addition of one-sixth of the volume of 6× reducing SDS–PAGE sample buffer (360 mM Tris, pH 6.6, 12% SDS, 600 mM DTT, 60% glycerol, and 0.6% bromophenol blue) and analyzed by immunoblotting.

Western blot. Proteins were separated on 15% SDS–PAGE and electrophoretically transferred to nitrocellulose filters in 192 mM glycine, 25 mM Tris, and 20% (v/v) methanol. The membrane was blocked by incubating in phosphate buffered saline pH 7.2 containing 0.5% (v/v) Tween-20 (PBST) containing 5% (wt/vol) nonfat dry milk and sequentially incubated with primary Ab: rabbit anti-human caspase-3 Ab or mouse anti-human caspase-7 mAb F5/1 (Gregorc, U., Doberšek, A., Salvesen, G., Turk V., Turk, B., Kopitar-Jerala, N. accepted to Immunology Lett.) and with anti-β-actin antibody (Sigma–Adrich, USA) in PBST for 1–2 h with shaking. Anti-RACK-1 immunoprecipitates were developed with anti-cystatin B (stefin B) Ab (Biogenesis, Poole, UK). The filters were incubated with horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit Ab (depending on the primary Ab). Membranes were then washed with PBST and developed by the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham Pharmacia Biotech).

2.4. Measurement of DEVD-ase activity

For measurement of caspase activities, treated cells were washed in PBS and pellets were lysed for 15 min at 4 °C in caspase lysis buffer (100 mM HEPES, 200 mM NaCl, 0.2% (w/v) CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 20% (w/v) sucrose, 2 mM EDTA, and 20 mM DTT, pH 7.0). After 15 min centrifugation at 12,000 × *g* and 4 °C, supernatant were collected and assayed for protein concentration by Bradford method (Bio-Rad Laboratories, USA). Caspase-3 and -7 activities were estimated on 100 μg of proteins by adding caspase buffer to the final volume of 90 μl into the 96-well plate. Following 10 min incubation at 37 °C, Ac-DEVD-AFC (Ac-Asp-Glu-Val-Asp-7-amido-4-methyl coumarin) was added to a final concentration of 100 μM and DEVD-ase activity was measured continuously in an LS50B fluorimeter with plate reader attachment (Perkin–Elmer, USA) at excitation and emission wavelengths of 400 and 505 nm, respectively.

2.5. Flow cytometry

Phosphatidylserine exposure was measured by labelling thymocytes with annexin V-PE and 7-amino-actinomycin D (7-AAD) (Becton Dickinson, USA) according to the manufacturer's instructions. Cells were then subjected to FACS analysis using a FACScalibur flow cytometer (Becton Dickinson) and CellQuest software.

3. Results

3.1. Stefin B-deficient thymocytes are more sensitive to protein kinase inhibitor staurosporin-induced apoptosis

As a first approach to assess the role of stefin B in extrinsic or intrinsic apoptotic signalling pathway the kinetics of caspase activation and PS externalization were compared in thymocytes from wild-type mice with thymocytes from stefin B-deficient mice treated with anti-CD95 mAb, STS, and CPT. Caspase activation was first studied by fluorimetric assay using the caspase-3 and -7 specific substrate Ac-DEVD-afc. The dose-dependent effect of STS on DEVD-ase activity is shown in Fig. 1. In thymocytes of stefin B-deficient mice as well as wild-type thymocytes the time course of cell death induced by anti-CD95 mAb was more rapid (4–8 h) than with CPT or STS (Fig. 2A–C). Examination of the time-dependent activation of DEVD-ase induced by STS revealed that DEVD-ase activity began to increase approximately 4 h after STS treatment and reached maximum after 8–12 h. Addition of STS caused a significantly faster caspase activation in the thymocytes of stefin B-deficient mice in comparison to wild-type mice (Fig. 2B). In the case of anti-CD95 mAb antibody and CPT the differences between DEVD-ase activities in stefin

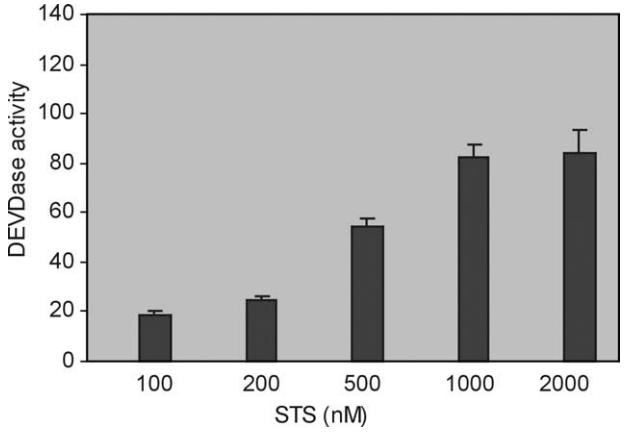

Fig. 1. Dose-dependent effect of STS on DEVD-ase activity. Thymocytes from Balb/c mice were treated with increasing concentrations of STS for 8 h. DEVD–AFC cleavage activity was determined as described in Section 2.

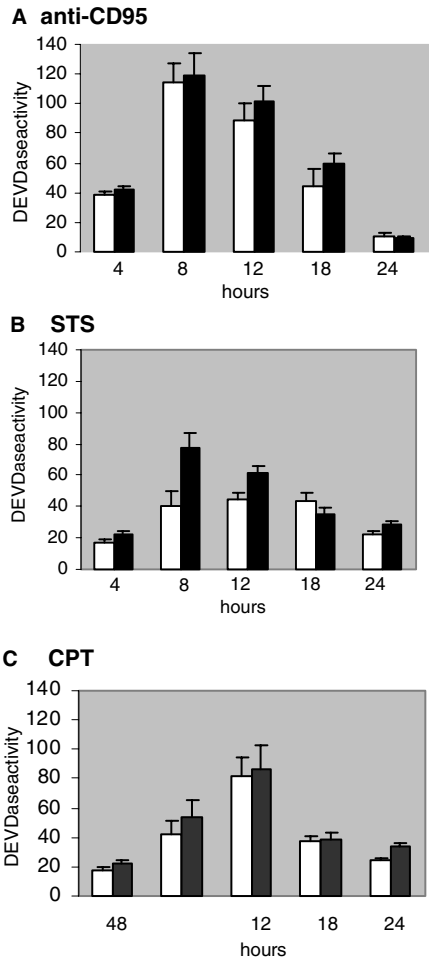

Fig. 2. Kinetic analysis of caspase activation associated with apoptosis triggered by different stimuli in thymocytes from wild-type mice and stefin B-deficient mice. Thymocytes from wild-type mice (□) and stefin B-deficient mice (■) were treated with (A) anti-CD95 mAb (0.5 μg/ml); (B) STS (1 μM); (C) or CPT (1 μM). At the indicated time, DEVD–AFC cleavage activity was determined as described in Section 2. The results are means ± S.D. of at least three independent experiments performed in duplicate.

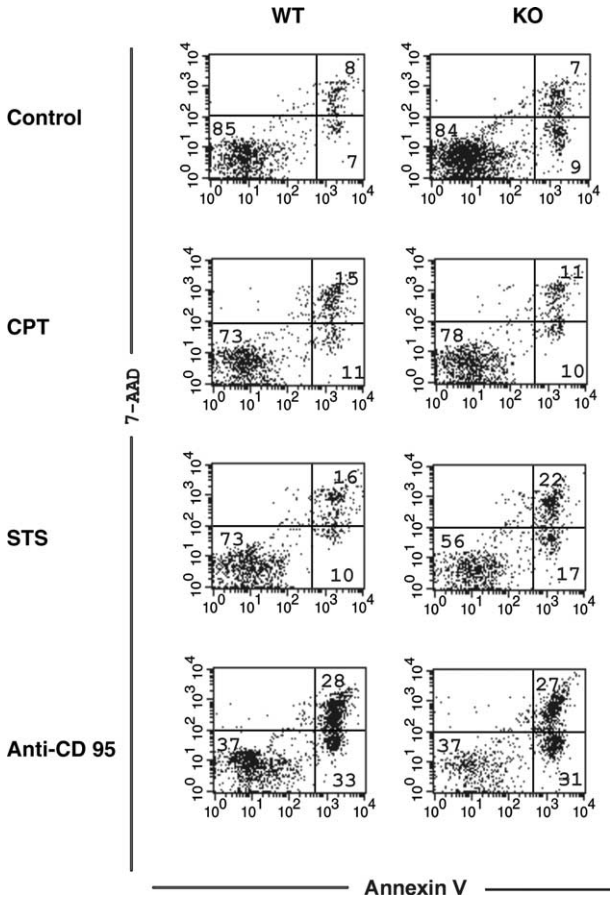

Fig. 3. PS surface exposure in thymocytes isolated from wild-type (WT) and stefin B-deficient (KO) mice undergoing apoptosis by different stimuli. Thymocytes were treated with CPT (1 μM), STS (1 μM), or anti-CD95 mAb (0.5 μg/ml) for 6 h. At this time cells were stained with annexin V-FITC and 7AAD. The percentages of annexin $V^-/7AAD^-$, annexin $V^+/7AAD^-$, and annexin $V^+/7AAD^+$ are indicated on each dot plot. Results are from a typical experiment of three performed that showed similar percentages.

B-deficient and wild-type thymocytes were not as pronounced as in the case of STS (Fig. 2A and C).

The kinetics of PS externalization was observed by cytofluorometric analysis using annexin V-7AAD, respectively. Upon induction of apoptosis with STS, thymocytes from stefin B-deficient mice showed increased PS exposure in comparison to thymoctes from wild-type mice (Fig. 3).

Together these results show a concomitant caspase activation and PS exposure after STS, CPT, anti-CD95 mAb-induced apoptosis. Such kinetics suggest that in thymocytes PS exposure is dependent on caspase activation after STS, CPT, or anti-CD95 mAb-induced apoptosis. Extracts of apoptotic cells were tested with Western blots for the caspase-3 and -7 processing. In the extracts from the thymocytes of stefin B-deficient and wild-type mice both caspase-3 and -7 were present in proteolytically processed activated form (Fig. 4). Processing of caspase-3 in untreated thymocytes is due to the spontaneous apoptosis in thymocytes in primary culture, after 8 h.

3.2. Stefin B and RACK-1 do not co immunoprecipitate in the thymocytes of Balb/c mice

We tested if the increased sesnsitivity of stefin B-deficient thymocytes is to STS-induced apoptosis is due to interactions of stefin B with RACK-1, since co-immunoprecipitation of the two proteins was described in rat cerebellar cell extracts [35].

With specific antibodies we were able to detect RACK-1 as well as stefin B in thymocyte lysates of Balb/c mice using Western blot, but we could not detect the interaction of the two proteins with co-immunoprecipitation (data not shown).

3.3. Cysteine proteinase inhibitor E-64d does not prevent apoptosis in stefin B-deficient nor in wild-type thymocytes

To determine whether cysteine endopeptidases (lysosomal papain-like cathepsins or calpains) have an active role within apoptotic pathways in thymocytes we used the previously characterized cell-permeable inhibitor E-64d. Our previous results on Jurkat T cells show that preincubation with E-64d even in concentrations as low as 5 μM was sufficient to completely block any cathepsin activity [24]. Here we present evidence that inhibition of cathepsin activity did not protect

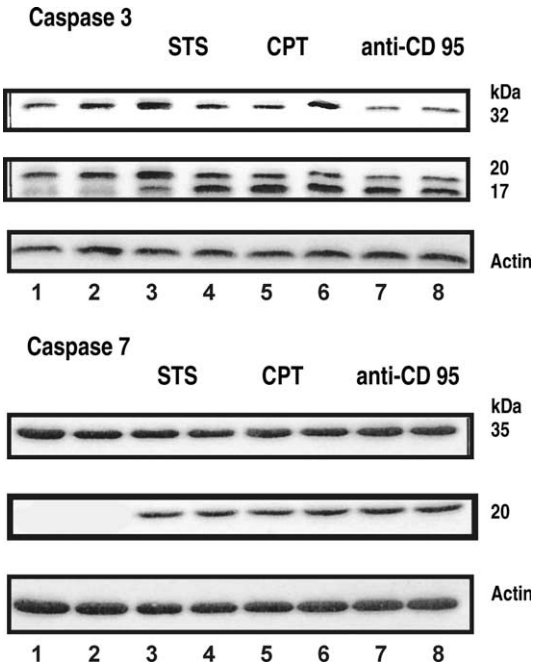

Fig. 4. Caspase-7 and -3 activation during CD95-, STS-, and CPT-induced apoptosis in thymocytes. Cells from wild-type (lines 1, 3, 5, 7) and stefin B-deficient mice (2, 4, 6, 8) were left untreated (1, 2) treated with STS (3, 4), CPT (5, 6), or anti-CD95 mAb (7, 8) for 8 h. After washing with PBS, cells were lysed as described in Section 2. An equal amount of proteins was loaded and separated on 15% SDS–PAGE, followed by Western blotting with the anti-caspase-3 rabbit polyclonal and sera anti-caspase-7 mAb F5/1. Membranes were stripped and redeveloped with anti-β-actin Ab. Results are from a typical experiment of two performed that showed similar results.

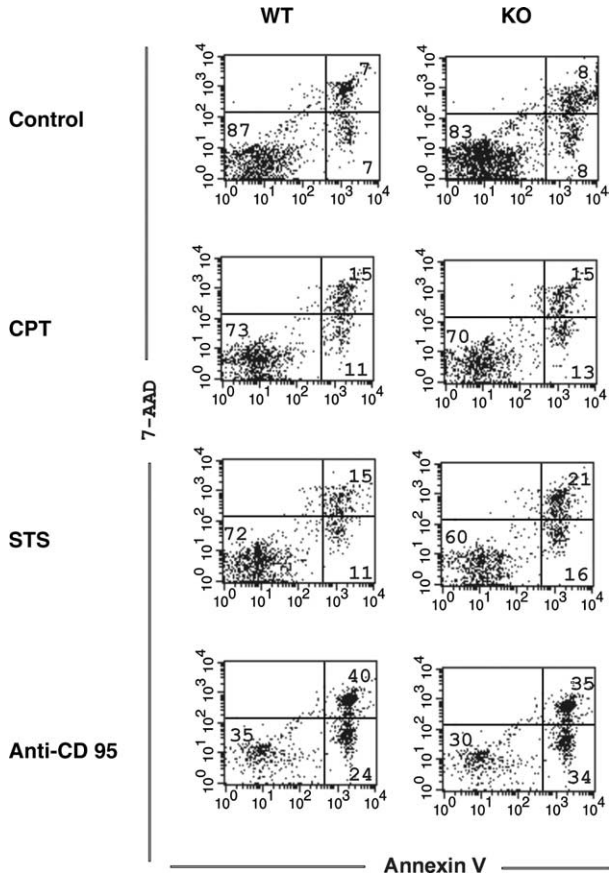

Fig. 5. Effects of cathepsin inhibitor E-64d on PS exposure in thymocytes isolated from from wild-type (WT) and stefin B-deficient (KO) mice undergoing apoptosis by different stimuli. Thymocytes were incubated in the presence of E-64d (25 μM) for 1 h before treatment with CPT (1 μM), STS (1 μM), or anti-CD95 mAb (0.5 μg/ml) for 6 h. At this time cells were stained with annexin V-PE and 7AAD. The percentages of annexin $V^-/7AAD^-$, annexin $V^+/7AAD^-$, and annexin $V^+/7AAD^+$ are indicated on each dot plot. Results are from a typical experiment of three performed that showed similar percentages.

primary culture of thymocytes from apoptosis. The effect of E-64d on the anti-CD95 mAb, STS and CPT-induced apoptosis was examined by flow cytometry. Pretreatment of thymocytes with up to 25 μM of E-64d for 1 h prior to induction of apoptosis had no effect on apoptosis determined by flow cytometry (Fig. 5). DEVD-ase activity after induction of apoptosis in cells pretreated with E-64d and untreated cells was also comparable (data not shown).

3.4. Apoptosis is prevented by caspase inhibitor z-VAD-fmk in both stefin B-deficient and wild-type thymocytes

To assess the role of caspases in apoptosis induced in thymocytes isolated from stefin B-deficient and wild-type mice we examined the effect of the pan-caspase inhibitor Z-VAD-fmk on PS exposure. z-VAD-fmk was found to inhibit also cathepsins in intact cells and in our experiments we used z-VAD-fmk at concentrations that do not significantly inhibit the cathepsins (25 μM) [24]. Cells preincubated with z-VAD-fmk inhibitor for 1 h and treated with anti-CD95 mAb, STS, and CPT were harvested for analysis of DEVD-ase activity and PS exposure. Preincubation of thymocytes with 25 μM z-VAD-fmk prevented not only DEVD-ase activity (data not shown) in thymocytes isolated from stefin B-deficient and wild-type mice, but also PS exposure (Fig. 6), indicating that caspase activation is required for apoptosis progression in this model. DEVD-ase activity and PS exposure was low also in negative controls indicating that also spontaneous apoptosis in thymocytes in primary culture was prevented with z-VAD-fmk. Jurkat T cells were treated with the same apoptosis-inducing stimuli, anti-CD95 mAb, STS, or CPT in the presence or the absence of the pan-caspase inhibitor z-VAD-fmk or E-64d. In Jurkat z-VAD-fmk inhibited PS exposure induced by anti-CD95 mAb, as well as with CPT and STS while E-64d did not inhibit caspase activation nor PS exposure induced by anti-CD95 mAb (data not shown). Our results on induction of apoptosis in Jurkat cell line are in accordance with previously published results [23].

4. Discussion

In this study, we investigated the effect of the lack of stefin B in apoptotic pathways in thymocytes. In T cells and thymocytes, caspases are not the only proteases that are able to induce apoptosis, cathepsins and calpains could also participate [25–27]. In thymocytes of stefin B deficient mice as well as in thymocytes isolated from wild-type mice the time course of cell death induced by anti-CD95 mAb was more

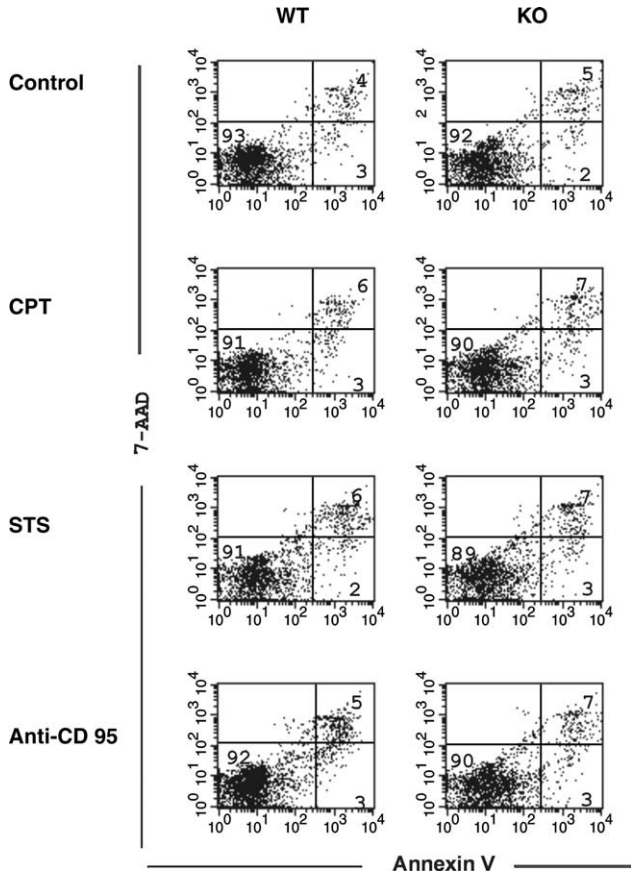

Fig. 6. Effects of caspase inhibitor z-VAD-fmk on PS exposure in thymocytes from wild-type (WT) and stefin B-deficient (KO) mice undergoing apoptosis by different stimuli. Thymocytes were incubated in the presence of z-VAD-fmk (25 μM) for 1 h before treatment with CPT (1 μM), STS (1 μM), or anti-CD95 mAb (0.5 μg/ml) for 6 h. At this time cells were stained with annexin V-PE and 7AAD. The percentages of annexin $V^-/7AAD^-$, annexin $V^+/7AAD^-$, and annexin $V^+/7AAD^+$ are indicated on each dot plot. Results are from a typical experiment of three performed that showed similar percentages.

rapid (4–8 h) than with CPT or STS (Fig. 2). We observed comparable increase in caspase activity and cells surface PS exposure in stefin B-deficient and wild-type mice in apoptosis induced by anti-CD95 mAb and concluded that stefin B is not involved in extrinsic apoptotic pathway (Fig. 2A and Fig. 3). We tested two reagents that act through intrinsic pathway: in the apoptosis induced by CPT, topoisomerase inhibitor which is also known to cause activation of NF-κB, there was not a major difference between stefin B-deficient and wild-type thymocytes (Fig. 2C) NF-κB suppresses apoptosis through induction of multiple target genes coding for inhibitors of the extrinsic or intrinsic signalling pathways that regulate apoptosis [28]. Recently, it has been shown that NF-κB protects cells from TNF-α-induced apoptosis by the up regulation of serine protease inhibitor 2A (Spi2A), also an inhibitor of cathepsin B [29]. The most pronounced difference between time course of apoptosis in thymocytes of stefin B-deficient and wild-type mice we found with STS, a broad-specificity protein kinase inhibitor. Although STS inhibits several protein kinases, it shows the highest affinity to the protein kinase C (PKC) isoenzymes [30]. Despite the common use of STS as an inducer of apoptosis, the mechanism by which STS initiates apoptosis still not clear. It is apparent that STS induces apoptosis through the mitochondrial pathway [31]. Cells from mice lacking both BAX and BAK are completely resistant to STS as well as several other apoptotic stimuli that act through disruption of mitochondrial function [32]. STS induces mitochondrial outer membrane permeabilization, which is followed by the release of cytochrome *c* and the downstream activation of caspase-9 and -3 [31,33]. In STS induced apoptosis caspase-3 to feeds back on permeabilized mitochondria and cleave the 75-kDa subunit (NDUFS1) of respiratory complex I, which leads to a disruption of electron transport, mitochondrial transmembrane potential ($\Delta\psi_m$) and the production of reactive oxygen species (ROS) [34]. The increased sensitivity of stefin B-deficient thymocytes towards STS-induced apoptosis could be at least partially due to the ROS.

Di Giaimo et al. [35] using yeast two hybrid system, identified five proteins interacting with stefin B in rat cerebella, none of which was cathepsin. We find particularly interesting interaction of stefin B with protein kinase C receptor (RACK-1) which was also confirmed by co-immunoprecipitation of two proteins in rat cerebellar cell extracts, and in immunofluorescence analysis of differentiated cultured primary cerebellar granule cells. RACK-1 was originally identified and cloned from a rat brain cDNA expression library as a protein that specifically bound activated PKCβ.

We tested the possibility that stefin B interacts with RACK-1 and in this way interferes with PKC signalling in the cells. With specific antibodies we were able to detect RACK-1 as well as stefin B in thymocyte lysates of Balb/c mice using Western blot, but we could not confirm the interaction of the two proteins with co-immunoprecipitation. The interaction of stefin B with RACK-1 is probably tissue specific, characteristic only to cerebellum. Why stefin B-deficient thymocytes are more sensitive to STS-induced apoptosis is still not clear, but at least in murine thymocytes, it is not due to stefin B–RACK-1 interactions. Apoptotic pathways consist of a cascade of signalling proteins before the final decision is made. We cannot exclude the possibility that the increased sensitivity of stefin B-deficient thymocytes towards STS-induced apoptosis is due to STS inhibition of PKC and lack of phosphorylation of signalling proteins.

Two independent studies identified cathepsin B as a key player in microglial neuronal cell death [36,37]. In WEHI-S fibro sarcoma cells, TNF-α induced an increase in cytosolic cathepsin B activity followed by death with apoptotic features. Apoptosis was enhanced by low concentrations of pan-caspase inhibitors z-VAD-fmk. Contrary to caspase inhibitors, a panel of pharmacological cathepsin B inhibitors, the endogenous cathepsin inhibitor stefin A (cystatin A) as well as antisense-mediated depletion of cathepsin B rescued WEHI-S cells from apoptosis [7]. Since stefin B is inhibitor of cysteine proteinases and does not inhibit cathepsin D, E (aspartic) or cathepsin G (serine) proteinases we examined the effect of E-64d on apoptosis in stefin B deficient thymocytes. In our experiments inhibition of cathepsin activity with E-64d did not rescue thymocytes isolated from stefin B-deficient mice nor wild-type mice from apoptosis with any apoptotic inducers tested. Calpains, calcium-dependent cysteine proteases, which are also inhibited by E-64d, are required for polymorphonuclear neutrophil apoptosis, as well as for glucocorticoid-induced apoptosis thymocytes [4,38]. But the lack of inhibition of apoptosis with E-64d, suggested that in our test system calpains were not involved. It can be therefore suggested that

the sensitization of thymocytes to STS-induced apoptosis is not related to lack of inhibition of cathepsins by stefin B.

The inhibition of caspase activity with general caspase inhibitor z-VAD-fmk completely prevented apoptosis in thymocytes isolated from stefin B-deficient mice as well as wild-type mice (Fig. 6). In our test system of apoptosis induced in murine thymocytes PS exposure is connected to caspase activation, while in primary T cells, PS exposure was not affected by caspase inhibitors [23].

As a conclusion, we can say that in thymocytes isolated from wild-type mice and stefin B-deficient mice, apoptosis is cathepsin-independent and caspase-dependent, while the stefin B deficiency affects predominantly apoptotic process which is triggered by STS by a currently unknown mechanism.

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References

[1] Wyllie, A.H., Kerr, J.F. and Currie, A.R. (1980) Cell death: the significance of apoptosis. Int. Rev. Cytol. 68, 251–306.
[2] Hengartner, M.O. (2000) The biochemistry of apoptosis. Nature 407, 770–776.
[3] Jaattela, M. and Tschopp, J. (2003) Caspase-independent cell death in T lymphocytes. Nat. Immunol. 4, 416–423.
[4] Squier, M.K., Sehnert, A.J., Sellins, K.S., Malkinson, A.M., Takano, E. and Cohen, J.J. (1999) Calpain and calpastatin regulate neutrophil apoptosis. J. Cell Physiol. 178, 311–319.
[5] Leist, M. and Jaattela, M. (2001) Four deaths and a funeral: from caspases to alternative mechanisms. Nat. Rev. Mol. Cell Biol. 2, 589–598.
[6] Turk, B., Stoka, V., Rozman-Pungercar, J., Cirman, T., Droga-Mazovec, G., Oresic, K. and Turk, V. (2002) Apoptotic pathways: involvement of lysosomal proteases. Biol. Chem. 383, 1035–1044.
[7] Foghsgaard, L., Wissing, D., Mauch, D., Lademann, U., Bastholm, L., Boes, M., Elling, F., Leist, M. and Jaattela, M. (2001) Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. J. Cell Biol. 153, 999–1010.
[8] Guicciardi, M.E., Deussing, J., Miyoshi, H., Bronk, S.F., Svingen, P.A., Peters, C., Kaufmann, S.H. and Gores, G.J. (2000) Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome *c*. J. Clin. Invest. 106, 1127–1137.
[9] Roberg, K., Johansson, U. and Ollinger, K. (1999) Lysosomal release of cathepsin D precedes relocation of cytochrome *c* and loss of mitochondrial transmembrane potential during apoptosis induced by oxidative stress. Free Rad. Biol. Med. 27, 1228–1237.
[10] Roberts, L.R., Kurosawa, H., Bronk, S.F., Fesmier, P.J., Agellon, L.B., Leung, W.Y., Mao, F. and Gores, G.J. (1997) Cathepsin B contributes to bile salt-induced apoptosis of rat hepatocytes. Gastroenterology 113, 1714–1726.
[11] Roberts, L.R., Adjei, P.N. and Gores, G.J. (1999) Cathepsins as effector proteases in hepatocyte apoptosis. Cell Biochem. Biophys. 30, 71–88.
[12] van Eijk, M. and de Groot, C. (1999) Germinal center B cell apoptosis requires both caspase and cathepsin activity. J. Immunol. 163, 2478–2482.
[13] Turk, B., Turk, V. and Turk, D. (1997) Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. Biol. Chem. 378, 141–150.
[14] Lalioti, M.D., Mirotsou, M., Buresi, C., Peitsch, M.C., Rossier, C., Ouazzani, R., Baldy-Moulinier, M., Bottani, A., Malafosse, A. and Antonarakis, S.E. (1997) Identification of mutations in cystatin B, the gene responsible for the Unverricht–Lundborg type of progressive myoclonus epilepsy (EPM1). Am. J. Hum. Genet. 60, 342–351.
[15] Lalioti, M.D., Scott, H.S., Buresi, C., Rossier, C., Bottani, A., Morris, M.A., Malafosse, A. and Antonarakis, S.E. (1997) Dodecamer repeat expansion in cystatin B gene in progressive myoclonus epilepsy. Nature 386, 847–851.
[16] Pennacchio, L.A., Lehesjoki, A.E., Stone, N.E., Willour, V.L., Virtaneva, K., Miao, J., D'Amato, E., Ramirez, L., Faham, M., Koskiniemi, M., Warrington, J.A., Norio, R., de la Chapelle, A., Cox, D.R. and Myers, R.M. (1996) Mutations in the gene encoding cystatin B in progressive myoclonus epilepsy (EPM1). Science 271, 1731–1734.
[17] Pennacchio, L.A., Bouley, D.M., Higgins, K.M., Scott, M.P., Noebels, J.L. and Myers, R.M. (1998) Progressive ataxia, myoclonic epilepsy and cerebellar apoptosis in cystatin B-deficient mice. Nat. Genet. 20, 251–258.
[18] Lieuallen, K., Pennacchio, L.A., Park, M., Myers, R.M. and Lennon, G.G. (2001) Cystatin B-deficient mice have increased expression of apoptosis and glial activation genes. Hum. Mol. Genet. 10, 1867–1871.
[19] Houseweart, M.K., Pennacchio, L.A., Vilaythong, A., Peters, C., Noebels, J.L. and Myers, R.M. (2003) Cathepsin B but not cathepsins L or S contributes to the pathogenesis of Unverricht–Lundborg progressive myoclonus epilepsy (EPM1). J. Neurobiol. 56, 315–327.
[20] Cirman, T., Oresic, K., Mazovec, G.D., Turk, V., Reed, J.C., Myers, R.M., Salvesen, G.S. and Turk, B. (2004) Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. J. Biol. Chem. 279, 3578–3587.
[21] Stoka, V., Turk, B., Schendel, S.L., Kim, T.H., Cirman, T., Snipas, S.J., Ellerby, L.M., Bredesen, D., Freeze, H., Abrahamson, M., Bromme, D., Krajewski, S., Reed, J.C., Yin, X.M., Turk, V. and Salvesen, G.S. (2001) Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. J. Biol. Chem. 276, 3149–3157.
[22] Houseweart, M.K., Vilaythong, A., Yin, X.M., Turk, B., Noebels, J.L. and Myers, R.M. (2003) Apoptosis caused by cathepsins does not require Bid signaling in an in vivo model of progressive myoclonus epilepsy (EPM1). Cell Death Differ. 10, 1329–1335.
[23] Ferraro-Peyret, C., Quemeneur, L., Flacher, M., Revillard, J.P. and Genestier, L. (2002) Caspase-independent phosphatidylserine exposure during apoptosis of primary T lymphocytes. J. Immunol. 169, 4805–4810.
[24] Rozman-Pungercar, J., Kopitar-Jerala, N., Bogyo, M., Turk, D., Vasiljeva, O., Stefe, I., Vandenabeele, P., Bromme, D., Puizdar, V., Fonovic, M., Trstenjak-Prebanda, M., Dolenc, I., Turk, V. and Turk, B. (2003) Inhibition of papain-like cysteine proteases and legumain by caspase-specific inhibitors: when reaction mechanism is more important than specificity. Cell Death Differ. 10, 881–888.
[25] Gil-Parrado, S., Fernandez-Montalvan, A., Assfalg-Machleidt, I., Popp, O., Bestvater, F., Holloschi, A., Knoch, T.A., Auerswald, E.A., Welsh, K., Reed, J.C., Fritz, H., Fuentes-Prior, P., Spiess, E., Salvesen, G.S. and Machleidt, W. (2002) Ionomycin-activated calpain triggers apoptosis. A probable role for Bcl-2 family members. J. Biol. Chem. 277, 27217–27226.
[26] Jung, K.C., Park, W.S., Kim, H.J., Choi, E.Y., Kook, M.C., Lee, H.W. and Bae, Y. (2004) TCR-independent and caspase-independent apoptosis of murine thymocytes by CD24 cross-linking. J. Immunol. 172, 795–802.
[27] Michallet, M.C., Saltel, F., Flacher, M., Revillard, J.P. and Genestier, L. (2004) Cathepsin-dependent apoptosis triggered by supraoptimal activation of T lymphocytes: a possible mechanism of high dose tolerance. J. Immunol. 172, 5405–5414.
[28] Karin, M. and Lin, A. (2002) NF-kappaB at the crossroads of life and death. Nat. Immunol. 3, 221–227.
[29] Liu, N., Raja, S.M., Zazzeroni, F., Metkar, S.S., Shah, R., Zhang, M., Wang, Y., Bromme, D., Russin, W.A., Lee, J.C., Peter, M.E., Froelich, C.J., Franzoso, G. and Ashton-Rickardt, P.G. (2003) NF-kappaB protects from the lysosomal pathway of cell death. EMBO J. 22, 5313–5322.
[30] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Staurosporine, a potent inhibitor of phospholipid/Ca++ dependent protein kinase. Biochem. Biophys. Res. Commun. 135, 397–402.

[31] Duan, S., Hajek, P., Lin, C., Shin, S.K., Attardi, G. and Chomyn, A. (2003) Mitochondrial outer membrane permeability change and hypersensitivity to digitonin early in staurosporine-induced apoptosis. J. Biol. Chem. 278, 1346–1353.
[32] Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B. and Korsmeyer, S.J. (2001) Proapoptotic BAX and BAK, a requisite gateway to mitochondrial dysfunction and death. Science 292, 727–730.
[33] Arnoult, D., Gaume, B., Karbowski, M., Sharpe, J.C., Cecconi, F. and Youle, R.J. (2003) Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J. 22, 4385–4399.
[34] Ricci, J.E., Munoz-Pinedo, C., Fitzgerald, P., Bailly-Maitre, B., Perkins, G.A., Yadava, N., Scheffler, I.E., Ellisman, M.H. and Green, D.R. (2004) Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain. Cell 117, 773–786.
[35] Di Giaimo, R., Riccio, M., Santi, S., Galeotti, C., Ambrosetti, D.C. and Melli, M. (2002) New insights into the molecular basis of progressive myoclonus epilepsy: a multiprotein complex with cystatin B. Hum. Mol. Genet. 11, 2941–2950.
[36] Gan, L., Ye, S., Chu, A., Anton, K., Yi, S., Vincent, V.A., von Schack, D., Chin, D., Murray, J., Lohr, S., Patthy, L., Gonzalez-Zulueta, M., Nikolich, K. and Urfer, R. (2004) Identification of cathepsin B as a mediator of neuronal death induced by Abeta-activated microglial cells using a functional genomics approach. J. Biol. Chem. 279, 5565–5572.
[37] Kingham, P.J. and Pocock, J.M. (2001) Microglial secreted cathepsin B induces neuronal apoptosis. J. Neurochem. 76, 1475–1484.
[38] Squier, M.K. and Cohen, J.J. (1997) Calpain, an upstream regulator of thymocyte apoptosis. J. Immunol. 158, 3690–3697.