

Inhibition of caspase-1 induces cell death in pancreatic carcinoma cells and potentially modulates expression levels of bcl-2 family proteins

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Abstract Caspase-1 (interleukin-1 β -converting enzyme) is reported to play an important role in the regulation of apoptosis. We investigated the inhibition of caspase-1 by the cell permeable caspase-1 inhibitor Ac-AAVALLPAVLLALLAP-YVAD.CHO in pancreatic carcinoma cells. Inhibition of caspase-1 induced a non-apoptotic/necrotic-like cell death in AsPC-1, BxPC-3, MiaPaCa-2 and Panc-1 cells. Expression levels of bcl-2 and bax were up-regulated in caspase-1 inhibitor-treated cells while that of bcl-x_L remained unaltered. Our observations support our previous findings that caspase-1 is potentially involved in anti-apoptotic processes in pancreatic carcinoma. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Caspase-1; Epidermal growth factor; Bcl-2; Apoptosis; Necrosis; Pancreatic carcinoma

1. Introduction

Caspase-1 was the first identified member of a large family of cysteine proteases whose members play distinct roles in inflammation and apoptosis [1]. Caspases were implicated in apoptosis with the discovery that CED-3, the product of a gene required for cell death in the nematode *Caenorhabditis elegans*, is related to mammalian interleukin-1 β (IL-1 β)-converting enzyme (ICE, caspase-1) [2].

However, in recent studies we could demonstrate that ICE is overexpressed in the adenocarcinomas of the pancreas and correlated significantly with the overexpression of epidermal growth factor (EGF), EGF receptor and cyclin D1, pointing to possible aspects of ICE in proliferative processes in human pancreatic carcinoma cells [3]. This suggestion was also supported by experiments in EGF-stimulated pancreatic AsPC-1 cells demonstrating the reduction of cell proliferation by caspase-1 inhibition [4].

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Abbreviations: ICE, interleukin-1 β -converting enzyme (caspase-1); IL-1 β , interleukin-1 β ; EGF, epidermal growth factor; p-YVAD.CHO (caspase-1 inhibitor I), Ac-AAVALLPAVLLALLAP-YVAD.CHO (acetyl-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Tyr-Val-Ala-Asp-aldehyde); p-LEVD.CHO (caspase-4 inhibitor I), Ac-AAVALLPAVLLALLAP-LEVD.CHO (acetyl-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Leu-Glu-Val-Asp-aldehyde)

Peptide inhibitors designed to mimic known sequences of caspase substrates have been shown to suppress or alter effectively caspase-dependent effects in a number of cells [5]. In this study we used the polypeptide Ac-AAVALLPAVLLALLAPYVAD-CHO (p-YVAD.CHO) which is reported to be a specific cell permeable inhibitor of caspase-1 [6,7]. The C-terminal YVAD.CHO region is a highly specific, potent and reversible inhibitor of caspase-1. The long hydrophobic N-terminal sequence (residues 1–16) corresponds to the hydrophobic region of the signal peptide of the Kaposi fibroblast growth factor and confers cell permeability to the peptide without cytotoxic effects [8].

Members of the bcl-2 family proteins are key regulators of apoptosis and some of them are involved in necrosis as well [9,10]. Some of these proteins undergo post-translational modification, such as phosphorylation or proteolysis, that serves to alter their functions in some fashion [11–13]. Pro- and anti-apoptotic members can also heterodimerize and seemingly titrate one another's function, suggesting that their relative concentration may act as a rheostat for the suicide program [14].

In this study we investigated the influence of caspase-1 inhibition on the cell cycle of the pancreatic carcinoma cells AsPC-1, BxPC-3, MiaPaCa-2 and Panc-1 and the expression pattern of bcl-2 family proteins in these cells with and without caspase-1 inhibition.

2. Materials and methods

2.1. Cell culture

AsPC-1 cells were grown in monolayers at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin and glutamine (Biochrom, Berlin, Germany). Cells were seeded at a density of about 3×10^5 cells in 50 mm (\varnothing) plates, starved for 24–30 h and then incubated with EGF (25 ng/ml in FCS-free DMEM). To investigate functional properties of caspase-1, the following cell permeable caspase inhibitors were used: caspase-1 inhibitor I (p-YVAD.CHO), caspase-1 inhibitor II (Ac-YVAD.cmk), caspase inhibitor III (Boc-D(OMe)-fmk), caspase-1 inhibitor IV (Ac-YVAD.aom), caspase-1 inhibitor VI (Z-YVAD(OMe)-fmk) and caspase-4 inhibitor I (Ac-AAVALLPAVLLALLAP-LEVD.CHO, p-LEVD.CHO) (Calbiochem). Cells were incubated with EGF (25 ng/ml) and a caspase inhibitor (dissolved in dimethyl sulfoxide, DMSO) or DMSO alone in DMEM. The inhibitors p-YVAD.CHO and p-LEVD.CHO were used in a concentration of 25 μ M, the others in a concentration between 100 and 200 μ M. After incubation, cells were collected by mechanical scraping and used for Western blot or cell cycle analyses. All of the results were validated in at least three independent experiments.

2.2. Reverse transcription (RT) and polymerase chain reaction (PCR)

mRNA was isolated using a guanidinium thiocyanate method and oligo(dT)-cellulose column chromatography (QuickPrep, Micro mRNA Purification Kit; Pharmacia Biotech). The mRNA was dissolved in the elution buffer (30 μ l) provided in the kit. cDNA was prepared by RT of mRNA using SuperScript RT RNase-H-reverse transcriptase (Life Technologies). A 399 bp ICE fragment was amplified using the following primers: 5'-GGAAATTACCTTAATATG-CAAGAC-3' (sense) and 5'-CATGAACACCAGGA-ACGTGCT-GTC-3' (antisense). A 5 μ l aliquot of cDNA was amplified under the following cycling conditions: 94°C for 60 s, 55°C for 30 s and 72°C for 120 s for 40 cycles. The PCR products were electrophoresed through a 1% agarose gel containing ethidium bromide and visualized by UV light. β -Actin was used as an internal standard to confirm equal loading in each experiment.

2.3. Western blot analysis

Cells were lysed in 10 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1 μ g (each) of aprotinin, leupeptin and pepstatin/ml. 10 μ g protein of each sample were loaded on a denaturing 15% polyacrylamide gel. After electrophoresis the resolved proteins were transferred on a nitrocellulose membrane (Schleicher and Schuell) using a semi-dry transblot apparatus (Phase; Lübeck, Germany). Non-specific protein interactions were blocked by pre-incubation of the membranes with 5% skim milk in phosphate-buffered saline (PBS) overnight at 4°C. After incubation of the membranes with antibodies, specific binding was detected using the enhanced chemiluminescence system (Amersham). The monocytic cell line BJAB served as a positive control for bcl-2 and bcl-x_L. Blots were re-probed with an antibody against actin (Ser-Gly-Pro-Ser-Ile-Val-His-Arg-Lys-Cys-Phe, Sigma) to control for equal protein loading of the gel. The rabbit polyclonal serum of anti-bcl-x_L (H-5), were purchased from Santa Cruz Biotechnology (CA, USA), mouse monoclonal antibodies against bcl-2 (Ab-1) and bax (Ab-5) from Calbiochem.

2.4. Immunostaining

Cells were fixed with 70% ethanol for 20 min at -20°C, washed twice with PBS and incubated with anti-bcl-2 (Ab-1, Calbiochem), anti-bcl-x_L (H-5), and anti-bax (B-9) (Santa Cruz Biotechnology, CA, USA) at 37°C followed by incubation with rhodamine or DTAF-conjugated secondary antibody for 1 h at 37°C. Stained cells were visualized under a fluorescence microscope.

2.5. Cell cycle analysis

For cell cycle analysis, cell nuclei were stained with propidium iodide using Cell Cycle Plus (Becton and Dickinson). Analysis was performed by flow cytometry carried out on a FACScan using the Cell Quest program (Becton and Dickinson). For determination of cell size, trypsinized unstained cells were used. Cell cycle experiments were performed in a total of five independent experiments.

2.6. Caspase-1 activity assay

To confirm selectivity of the inhibitor p-YVAD.CHO for caspase-1 under our experimental conditions, i.e. in EGF-treated AsPC-1 cells, we performed the caspase-1 colorimetric assay (R&D Systems, Germany) according to the manufacturer's protocol. Briefly, 24 h after incubation of EGF-stimulated AsPC-1 cells with 25 μ M p-YVAD.CHO, cells were lysed and then incubated with a caspase-1-specific substrate, the peptide WEHD-p-nitroanilide, for 1–2 h at 37°C. Cleavage of the peptide by active caspase-1 releases the chromophore p-nitroanilide, which can be quantitated spectrophotometrically at 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the color reaction.

2.7. Apoptosis assay

To detect morphological changes in the nuclear chromatin of cells undergoing apoptosis, cells were stained with the DNA-binding fluorochrome bis-benzimide (Hoechst 33258; Sigma). Cells were fixed with 3.7% paraformaldehyde in PBS for 15 min and washed twice with PBS. Staining of the nuclei was performed by incubation of the cells with bis-benzimide (15 μ g/ml) for 15 min followed by washing with PBS. A 5 μ l aliquot of cells was placed on a glass slide and the average number of nuclei per field was scored for the incidence of condensed chromatin fragments in apoptotic cells under a fluorescence microscope.

2.8. DNA fragmentation assay

DNA was extracted from 4×10^5 cells using the apoptotic DNA ladder Kit (Roche, Switzerland). Isolated DNA was electrophoresed through a 1% agarose gel containing ethidium bromide and visualized by UV light. β -Actin was used as an internal standard to confirm equal loading in each experiment.

2.9. Statistical analysis

For statistical analysis the paired Student's *t*-test was used. Significance was defined as $P < 0.05$.

3. Results

3.1. p-YVAD.CHO induces cell death in AsPC-1 cells

As we have previously shown, EGF-stimulated AsPC-1 cells express caspase-1 in its active form as demonstrated by the detection of the proteolytically active subunits p20 and p10, the caspase-1-dependent fragmentation of lamin A/C and the detection of active IL-1 α and IL-1 β without leading to cell death [4]. In order to induce expression of active caspase-1, AsPC-1 cells were stimulated with 25 ng/ml EGF and incubated with the caspase-1 inhibitor p-YVAD.CHO (25 μ M) or DMSO alone for 0, 6, 12, 18, 24 and 48 h. Approximately 6 h after incubation inhibitor-treated cells began to detach from the matrix surface displaying typical morphological evidence for cell death. Detached cells were stained with 0.2% trypan blue and counted in a hemocytometer. The observed cell death occurred highly significant after 18 h ($15.8 \pm 3\%$, $P < 0.02$), 24 h ($24.5 \pm 10.3\%$, $P < 0.01$) and 48 h ($25.4 \pm 3\%$, $P < 0.005$) of inhibitor incubation. The ratio between detached (dead) cells and total cell amount treated with p-YVAD.CHO is compared to that of the DMSO-treated cells in Fig. 1. We also tested other caspase-1 inhibitors: Ac-YVAD.cmk (200 μ M), Ac-YVAD.aom (100 μ M) and Z-YVAD(OMe)-fmk (100 μ M) and the pan-caspase inhibitor Boc-D(OMe)-fmk (100 μ M). Only p-YVAD.CHO had a significant cell-death effect on pancreatic AsPC-1 cells. As we demonstrated in previous work, Ac-YVAD.cmk induces a sig-

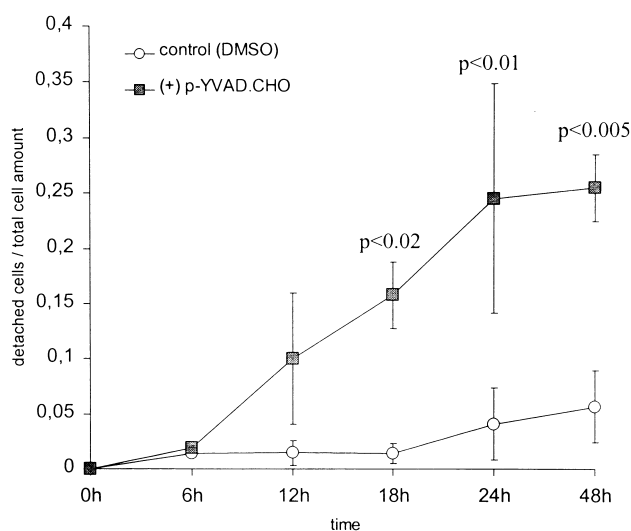


Fig. 1. Incubation with p-YVAD.CHO induces cell death in EGF-stimulated AsPC-1 cells. Ratio of detached (dead) AsPC-1 cells to total cell amount after incubation with EGF (25 ng/ml) and p-YVAD.CHO (25 μ M) or DMSO alone for 0, 6, 12, 18, 24 and 48 h. The data represent the mean \pm S.D. of at least four independent experiments (P -values as indicated).

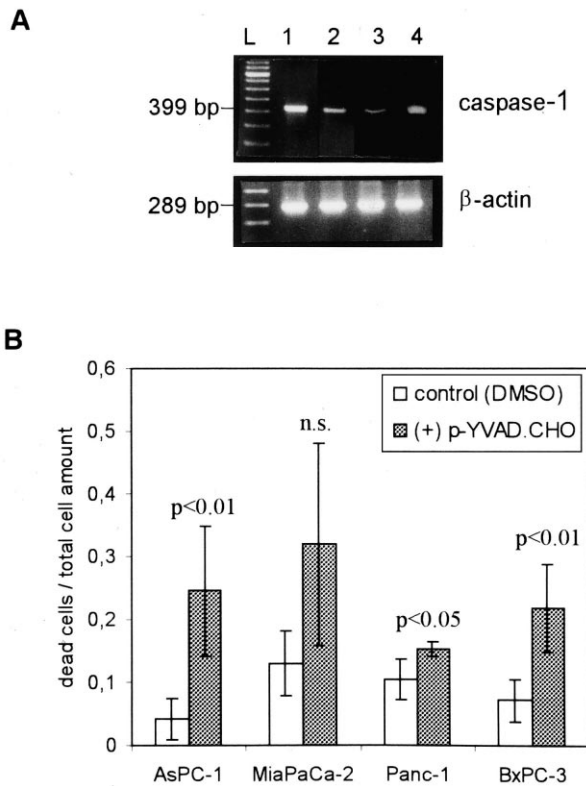


Fig. 2. Induction of cell death in EGF-stimulated AsPC-1, BxPC-3, MiaPaCa-2 and Panc-1 cells 24 h after incubation with p-YVAD.CHO. A: Expression of caspase-1 mRNA in AsPC-1 (lane 1), MiaPaCa-2 (lane 2), Panc-1 (lane 3) and BxPC-3 (lane 4) after incubation with EGF (25 ng/ml) and p-YVAD.CHO (25 μ M) for 24 h. Equal loading was confirmed by β -actin mRNA analysis. L, 100 bp DNA ladder. B: Cell death in AsPC-1, BxPC-3, MiaPaCa-2 and Panc-1 determined as the ratio of detached (dead) cells to total cell amount in the absence or the presence of 25 μ M p-YVAD.CHO after 24 h (S.D. and *P*-values as indicated; n.s.: not significant).

nificant reduction of cell proliferation on EGF-stimulated AsPC-1 cells and a slight increase of the apoptotic fraction although it did not reach statistical significance [4]. Inhibitors Ac-YVAD.aom (100 μ M), Z-YVAD(Ome)-fmk (100 μ M) and Boc-D(OMe)-fmk (100 μ M) did not display any cell-death effect on AsPC-1 cells.

In order to confirm the selectivity of p-YVAD.CHO for caspase-1 and its inhibition under our experimental conditions, i.e. in EGF-stimulated AsPC-1 cells, we measured caspase-1 activity by a colorimetric assay described in Section 2. Absorption at 405 nm indicating the caspase-1-dependent release of the chromophore *p*-nitroanilide which is directly proportional to caspase-1 activity is significantly reduced ($P<0.05$) in EGF-stimulated AsPC-1 cells 24 h after incubation with 25 μ M p-YVAD.CHO as compared to EGF-stimulated AsPC-1 cells treated with DMSO alone.

To examine whether caspase-4, which shares similar substrate specificities with caspase-1 [6,7], is also involved in the induction of the observed cell death, AsPC-1 cells were incubated with caspase-4 inhibitor p-LEVD.CHO, a polypeptide with the same hydrophobic N-amino-terminal tail (residues 1–16) as the caspase-1 inhibitor p-YVAD.CHO. The amount of detached (dead) cells after incubation with 25 μ M p-LEVD.CHO is similar to that of DMSO-treated cells in EGF-stimulated AsPC-1 cells. Hence, involvement of caspase-4 in the p-YVAD.CHO-induced cell death is very unlikely. This finding excludes also the cytotoxicity of the hydrophobic tail of the polypeptide itself.

Incubation with p-YVAD.CHO (25 μ M) also induced significant cell death in EGF-stimulated (caspase-1-expressing) pancreatic BxPC-3 ($P<0.01$) and Panc-1 ($P<0.05$) cells whereas in MiaPaCa-2 cells the observed cell death did not reach statistical significance ($P=0.06$) (Fig. 2A,B). The activity of caspase-1 and its inhibition in these cells after 24 h was confirmed by the colorimetric caspase-1 activity assay described above. In the presence of p-YVAD.CHO, caspase-1 activity was significantly reduced ($P<0.05$).

3.2. Evidence of a non-apoptotic/necrotic cell death

In order to characterize the mode of cell death induced by

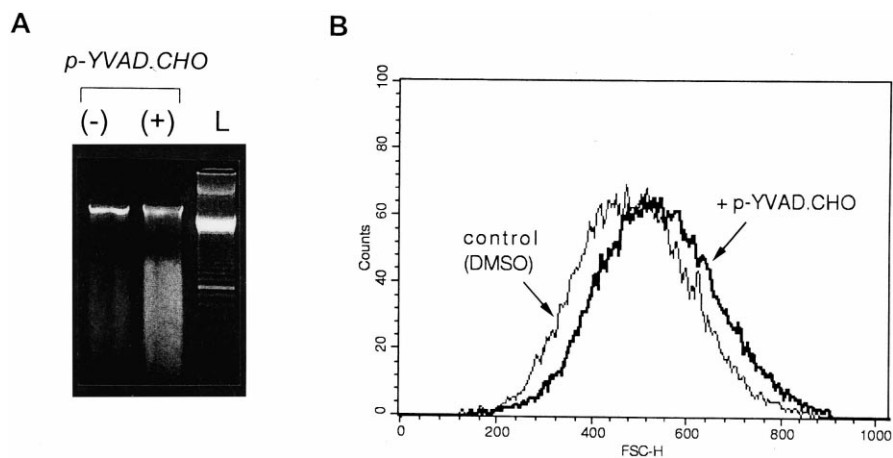


Fig. 3. Induction of a necrotic-like cell death in EGF-stimulated AsPC-1 after treatment with the caspase-1 inhibitor p-YVAD.CHO. A: Agarose gel electrophoresis of total DNA from EGF-stimulated (25 ng/ml) AsPC-1 cells cultured for 48 h in the presence and the absence of 25 μ M p-YVAD.CHO. L, 100 bp DNA ladder. B: Determination of AsPC-1 cell size after incubation with p-YVAD.CHO (25 μ M) or DMSO: histogram of FSC light intensity of analyzed EGF-stimulated (25 ng/ml) AsPC-1 cells in the presence and the absence of 25 μ M p-YVAD.CHO for 24 h. FSC light intensity is related to cell size. Incubation with p-YVAD.CHO leads to a significant increase in cell size ($9.4\pm1.4\%$; $P<0.01$, *t*-test).

p-YVAD.CHO we performed an apoptosis assay using bis-benzimide for the detection of condensed nuclear chromatin indicating apoptosis. We observed no difference between inhibitor-treated and -untreated EGF-stimulated AsPC-1 cells. In both cases the amount of condensed (apoptotic) nuclei was lower than 6%. This finding was confirmed by cell cycle analysis where negligible sub-G1 fractions could be observed (data not shown).

DNA fragmentation assays of p-YVAD.CHO-treated cells failed to display the enzymatic cleavage of DNA into multimers of 180 bp fragments ('DNA ladder') typical for apoptosis. In contrast DNA appears as a continuous spectrum of sizes resulting in a smear, pointing to DNA fragmentation during necrosis (Fig. 3A).

Another effect of p-YVAD.CHO was observed regarding cell size. The size of AsPC-1 cells in the presence and the absence of p-YVAD.CHO was determined in the flow cytometer. In EGF-stimulated AsPC-1 cells incubated with 25 μ M p-YVAD.CHO for 24 h, a significant increase of cell size could be observed ($9.4 \pm 1.4\%$; $P < 0.01$, t -test) detected as an increase in intensity of the FSC light measured in the flow cytometer which can be interpreted as cell swelling during necrosis [15] (Fig. 3B). The intensity of the SSC light which correlates to the granularity of the cells was unaltered as compared to the cells treated with DMSO alone indicating the lack of formation of apoptotic bodies (data not shown).

3.3. Expression of *bcl-2* and *bax* is up-regulated in caspase-1 inhibitor-treated cells

The expression pattern of *bcl-2* family proteins known to be involved in the regulation of cell-death processes was determined by Western blot analysis. Cells were stimulated with 25 ng/ml EGF and incubated with 25 μ M p-YVAD.CHO or DMSO alone for 0, 6, 12, 18 and 24 h. In caspase-1 inhibitor-treated cells the expression of *bcl-2* and *bax* protein was up-regulated as compared to EGF-stimulated cells incubated with DMSO alone whereas expression levels of *bcl-x_L* remained unaltered.

In order to confirm our observations from Western blot analysis immunofluorescence staining was also performed using specific antibodies for *bcl-2*, *bax* and *bcl-x_L*. EGF-stimulated AsPC-1 cells incubated with 25 μ M p-YVAD.CHO display a higher immunofluorescence reactivity for *bax* and *bcl-2* than untreated cells whereas that of *bcl-x_L* remained unchanged (data not shown).

Expression levels of *bcl-2* and *bax* protein were also up-regulated in EGF-stimulated BxPC-3 and MiaPaCa-2 cells, after incubation with p-YVAD.CHO. In Panc-1 cells no difference in *bax* expression could be observed between inhibitor-treated and -untreated cells whereas only very low levels of *bcl-2* could be detected. In all examined pancreatic cells *bcl-x_L* levels remain unchanged (data not shown).

4. Discussion

In recent studies we could demonstrate that stimulation with EGF induces a time-dependent expression of active caspase-1 in AsPC-1 cells without leading to cell death [4]. Here we investigated the influence of the cell permeable p-YVAD.CHO on the cell cycle of pancreatic carcinoma cell lines. We demonstrated that incubation with caspase-1 inhibitor, p-YVAD.CHO, significantly induces cell death in EGF-stimu-

lated caspase-1-expressing AsPC-1 cells. Cells incubated with the inhibitor are larger in size than untreated cells that can be interpreted as cell swelling during necrosis [15] (Fig. 2). The absence of a DNA 'ladder', chromatin condensation and sub-G1 events are also evident for a non-apoptotic/'necrotic-like' cell death after caspase-1 inhibition. These results seem to be in contradiction with the general assumption that inhibition of caspases would rescue cells from death. However, a similar observation was made in thymocytes where treatment with a broad spectrum caspase inhibitor leads also to a necrotic cell death [16]. In camptothecin-treated leukemia U-937 cells, the inhibition of caspase activities is coupled with a shift from apoptosis to transient G1 arrest followed by massive necrosis [18]. In B-lymphocytes, the inhibition of caspase activity also induces a switch from apoptotic death to a necrotic form suggesting that apoptosis and necrosis share common initiation pathways [17]. The necrotic effect of p-YVAD.CHO in pancreatic AsPC-1 cells as compared to other tested caspase-1 inhibitors may be partially due to its nuclear permeability provided through the hydrophobic polypeptide tail as demonstrated by Lin et al. [8]. As we know from our immunohistochemical studies in pancreatic carcinoma tissues the overexpression of caspase-1 is cytoplasmatic and nuclear as well in pancreatic tumor cells [3].

Searching for factors potentially influencing the balance between cell survival and cell death we examined the expression pattern of *bcl-2* family proteins known to be involved in the regulation of cell death in its apoptotic and necrotic form as well [9,10]. Interestingly, incubation with the caspase-1 inhibitor is accompanied by an upregulation of the 'anti-apoptotic' *bcl-2* protein in AsPC-1 cells (Fig. 3). An increased *bcl-2* expression was also found in apoptotic and necrotic brain tissue after head injury [10], in macrophages after *Shigella*-induced apoptosis [19] as well as in necrotic muscle fibers in several human myopathies [20]. However, some reports speculate that the anti-cell-death function of *bcl-2* depends not on the expression level but on its phosphorylation state [11,12]. Other studies presented a model in which the ratio of *bcl-2* to its heterodimerization partner *bax* determines survival or death following a cell-death stimulus [14] whereas investigations in U251 glioblastoma cells demonstrated that the anti-cell-death function of *bcl-2* is impaired at a high level of expression [21].

Another interesting finding is that the inhibition of caspase-1 also coincides with an increase in the pro-apoptotic *bax*

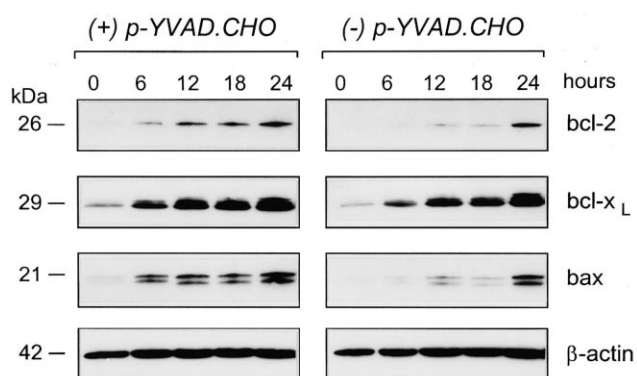


Fig. 4. Western blot analysis of the expression pattern of *bcl-2*, *bcl-x_L* and *bax* in EGF-stimulated (25 ng/ml) AsPC-1 cells treated with p-YVAD.CHO (25 μ M) or DMSO alone for different incubation times. Equal loading is confirmed by reprobing the gel with β -actin.

protein expression levels in our examined AsPC-1 cells whereas bcl-x_L expression levels remained unchanged. This may be consistent with the hypothesis that the ratio of bax/bcl-x_L expression levels determines the mode of cell death [22,23]. Shinoura et al. suggest that if a cancerous cell expresses a level of bcl-x_L which prevent bax-induced apoptosis, the overexpression of bax leads to necrotic cell death [22]. Wood et al. reported that cleavage of bax (21 kDa) into a 18 kDa protein fragment enhances its cell-death function [24]. The lower protein band in the Western blot analysis of bax (Fig. 4) may represent this 18 kDa protein fragment and support bax's function to shift the biochemical balance towards necrosis.

The observed upregulation of bcl-2 and bax expression levels by unchanged bcl-x_L levels in the pancreatic cell lines AsPC-1, BxPC-3 and MiaPaCa-2 may support the suggestion that the relative expression levels of bcl-2 family proteins to each other is a crucial factor in the regulation of cell death. A similar mechanism may also be assumed for the observed cell death in Panc-1 cells although the protein ratios in these cells cannot be determined because only very low levels of bcl-2 could be detected.

In conclusion, we have demonstrated the induction of a 'necrotic-like' cell death in EGF-stimulated, caspase-1-expressing, pancreatic cells by p-YVAD.CHO and a parallel upregulation of protein expression levels of bcl-2 and bax. Although the determination of the exact interrelations between the upregulation of bcl-2 and bax expression levels and the observed cell death warrants further investigation, a caspase-1-dependent modulation of the expression of these proteins cannot be excluded. However, our results support our previous suggestion that caspase-1 has an anti-cell-death function in pancreatic carcinoma and may provide new aspects in the role of caspase-1 in cell proliferation and death.

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