

Conversion of CD95 (Fas) Type II into Type I signaling by sub-lethal doses of cycloheximide

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ABSTRACT

CD95 (Fas/Apo-1)-mediated apoptosis was shown to occur through two distinct pathways. One involves a direct activation of caspase-3 by large amounts of caspase-8 generated at the DISC (Type I cells). The other is related to the cleavage of Bid by low concentration of caspase-8, leading to the release of cytochrome c from mitochondria and the activation of caspase-3 by the cytochrome c/APAF-1/caspase-9 apoptosome (Type II cells). It is also known that the protein synthesis inhibitor cycloheximide (CHX) sensitizes Type I cells to CD95-mediated apoptosis, but it remains contradictory whether this effect also occurs in Type II cells. Here, we show that sub-lethal doses of CHX render both Type I and Type II cells sensitive to the apoptogenic effect of anti-CD95 antibodies but not to chemotherapeutic drugs. Moreover, Bcl-2-positive Type II cells become strongly sensitive to CD95-mediated apoptosis by the addition of CHX to the cell culture. This is not the result of a restraint of the anti-apoptotic effect of Bcl-2 at the mitochondrial level since CHX-treated Type II cells still retain their resistance to chemotherapeutic drugs. Therefore, CHX treatment is granting the CD95-mediated pathway the ability to bypass the mitochondria requirement to apoptosis, much alike to what is observed in Type I cells.

Keywords:

Apoptosis

Type I

Type II

CD95

Fas

Bid

FLIP

Bcl-2

Cycloheximide

Introduction

Exposure of many cell types to “stress signals” leads to the activation of a program of cell death called apoptosis, which is regulated at the mitochondrial level by molecules that belonged to the Bcl-2 family and is executed by certain members of the

caspase family of proteases [1]. At the mitochondrial level, one of the major apoptogenic events is the translocation of cytochrome c from the mitochondrial intermembrane space to the cytosol. There, cytochrome c, along with ATP, mediates APAF-1 oligomerization and activation of caspase-9, which sequentially activates caspase-3 [2]. Other molecules are released

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Abbreviations: CHX, cycloheximide; FADD, Fas-associated death domain protein; FLIP, FLICE/caspase-8 inhibitory protein; mAb, monoclonal antibody; pAb, polyclonal antibody; STS, staurosporine; VCS, vincristine sulfate; VP-16, etoposide

from the mitochondria, including AIF (“apoptosis-inducing factor”) [3], which seems to be involved in caspase-independent nuclear changes; EndoG, a nuclease implicated in cell death and proliferation [4,5]; and the endogenous inhibitors of the IAP (“inhibitor of apoptosis protein”) family, SMAC/DIABLO [6,7] and HtrA2/Omi [8].

CD95 (Fas/Apo-1) is a member of the death receptor family, composed of cell surface receptors able to initiate a cascade of pro-apoptotic signals [9]. Upon engagement, CD95 assembles the so-called DISC (“death-inducing signaling complex”), which consists of large CD95, FADD and caspase-8 molecules [10]. Two CD95 signaling pathways were identified [11]. In Type I cells, CD95-mediated apoptosis is initiated by large amounts of caspase-8 activation at the DISC complex. Caspase-8, in turn, is directly responsible for the processing of caspase-3 and initiation of the apoptotic demolition of the cell. In Type II cells, on the other hand, only a limited amount of caspase-8 is activated at the DISC complex and, consequently, the low level of enzyme activity is insufficient to induce the direct cleavage of caspase-3. As a result, these cells require an amplification step initiated by the cleavage of Bid by the small number of activated caspase-8 molecules, followed by translocation of the truncated Bid to the mitochondria where it is responsible for triggering the release of cytochrome c [12,13]. Since Type II cells employ a mitochondria-dependent pathway to ensure the full installation of the apoptotic program after CD95 engagement, overexpression of Bcl-2 or Bcl-x_L blocks CD95-mediated apoptosis in these cells. In contrast, neither Bcl-2 nor Bcl-x_L is able to prevent apoptosis triggered by CD95 in Type I cells.

It was recently proposed that protein synthesis inhibitors such as CHX sensitize cells to CD95-mediated apoptosis by down-regulating the expression of FLIP [14], a close homolog and inhibitor of caspase-8. In this study the authors suggested that only Type I cells are susceptible to the sensitizing effect of CHX since the down-regulation of FLIP contributes to increase caspase-8 activation only in cells that form a significant amount of the DISC. In fact, the authors supported their claim by showing that the inhibition of FLIP expression by specific antisense oligonucleotides could not sensitize Type II cells to CD95-mediated apoptosis [14]. However, a different group provided evidences that Type II cells may as well be sensitized by CHX, suggesting that additional biochemical pathways participating in the regulation of CD95-mediated apoptosis are affected by this metabolic inhibitor [15].

In the present work we further investigated the sensitivity of Type I and Type II cells to the combination of sub-lethal doses of CHX and anti-CD95 antibodies and compared the effect of CHX on a cell death pathway initiated by chemotherapeutic drugs. CHX sensitized both SKW6.4 (Type I) and CEM (Type II) cells to CD95-mediated apoptosis. Furthermore, CHX rendered CEM.Bcl-2 cells as sensitive to CD95-mediated apoptosis as the wild-type CEM, without affecting the resistance of these cells to etoposide, staurosporine or vincristine-induced apoptosis, which proceeds via the intrinsic mitochondrial pathway. Similar results were obtained with HL-60 (another Type II cell line) and HL-60.Bcl-2 cells. In contrast to previous work, sensitization of Type II cells by low doses of CHX was not associated with a down-regulation of protein levels of FLIP, but it was connected with a significant decrease of Bid. Taken together, our results indicate that CHX modifies the CD95

signaling pathway towards a mitochondria-independent apoptosis, thereby converting Type II into Type I cells, where Bcl-2 no longer protects from CD95-mediated apoptosis.

Materials and methods

Cell culture and reagents

The T cell lines CEM.neo and CEM.Bcl-2 and the B lymphoblastoid cell lines SKW6.4.neo and SKW6.4.Bcl-2 were kindly provided by Dr. Henning Walczak (German Cancer Research Center, Heidelberg, Germany). Human acute myeloid leukemia HL-60 cells ectopically over-expressing Bcl-2 or not were previously described [16]. T cells and myeloid HL-60 were maintained in RPMI 1640–10% FCS, supplemented with 10 mM HEPES, 2 mM L-glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37 °C in 5% CO₂. SKW6.4 and SKW6.4.Bcl-2 were cultured in DMEM 10% FCS, supplemented with 10 mM HEPES, 2 mM L-glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37 °C in 5% CO₂.

Cycloheximide (CHX), etoposide (VP-16), staurosporine (STS) and vincristine sulfate (VCS), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). CHX was prepared as 100 mM stock solution in ethanol whereas VP-16 was made up as 100 mM in DMSO. z-DEVD-fmk were purchased from Enzyme Systems (Livermore, USA). Anti-CD95 mAb (CH11, MBL, Nagoya, Japan) was prepared as 0.5 mg/ml stock solution in PBS. Anti-Bcl-2 mAb (clone 100, Santa Cruz, Carpinteria, CA, USA), anti-Bid mAb (clone 7, Pharmingen, San Diego, USA), anti-caspase-3 mAb (clone 19, Pharmingen), anti-caspase-3 pAb (Calbiochem, CA, USA), anti-cytochrome-c mAb (clone 6H2-B4, Pharmingen), anti-c-IAP-1 mAb (clone B75-1, Pharmingen), anti-c-IAP-2 pAb (R&D systems, MN, USA), anti-XIAP (clone 48, Transd. Laboratories, CA, USA), anti-actin mAb (clone C4, ICN Biomedicals Inc, Warrenale, PA, USA), anti-caspase-8 pAb (Pharmingen), anti-cFLIP pAb (Upstate, Lake Placid, NY, USA) and anti-SMAC pAb (kindly provided by Dr. Seamus Martin, Ireland) were used for immunoblot detection of the specific proteins. Anti-mouse Ig and anti-rabbit Ig conjugated with horseradish peroxidase (HRP) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Immunostaining

Surface expression of CD95 was determined by cell staining with anti-CD95-PE-conjugated mAb clone DX2 (Pharmingen) or isotype control. Two hundred and fifty thousand cells were washed in PBS and blocked for 30 min with PBS containing 2% bovine serum albumin (BSA). Cells were then incubated for 1 h on ice with anti-CD95 antibody in PBS buffer. Then, cells were washed in PBS/2% BSA and resuspended in 300 µl of same solution for flow cytometry. Staining was analyzed by flow cytometry using the Cell Quest software (BD Bioscience, San Jose, CA). Results were expressed as percentage of stained cells.

Measurement of DNA content by flow cytometry

DNA fragmentation as an indicative of apoptosis was evaluated according to Nicoletti et al. [17]. Briefly, after culture

with or without the specific drugs, cells were harvested, washed in PBS and resuspended in 0.3 ml of a hypotonic fluorochrome solution (HFS, 50 µg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100). The fluorescence of individual nuclei was measured using a FACScalibur flow cytometer (Becton-Dickinson, Mountain View, CA, USA). The percentage of hypodiploid nuclei correlates with the extent of apoptosis in the samples. The results represent the average \pm SD in triplicate samples. Every experiment was repeated at least three times.

Determination of the loss of mitochondrial transmembrane potential

The collapse of the inner mitochondrial transmembrane potential ($\Delta\psi_m$) was measured using DiOC₆ or CMXRos dye (Molecular Probes) as described [18]. Briefly, cells were incubated in 200 µl of RPMI-FCS containing 50 nM of the dye at 37 °C for 45 min before analyzed by flow cytometry.

Western-blot analysis

Western-blot of total cell lysates were performed as previously described [19]. For preparation of cytosolic fractions, cells were washed once with ice-cold PBS and permeabilized for 5 min on ice at a density of 3×10^7 /ml in cytosolic extraction buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.2, 100 µM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin, containing 250 µg/ml digitonin). Samples were then centrifuged at 1000 g for 5 min at 4 °C, the supernatants were collected and appropriately diluted with 5× SDS-PAGE sample buffer. A total of 20 to 30 µg of protein was loaded per lane and western-blot reactions on PVDF membranes were detected using enhanced chemiluminescence (ECL, Amersham, Arlington, IL, USA).

Caspase activity fluorimetric assay

Cell-free extracts were generated from CEM.Bcl-2 untreated cells or treated with CHX (0.2 µM), CH11 (0.25 µg/ml) or CHX plus CH11 for 6 h. Four million cells were washed twice with PBS (pH 7.2) and lysed in 50 µl of ice-cold lysis buffer (150 mM NaCl, 20 mM Tris pH 7.5, 1% Triton X-100, 10 µg/ml leupeptin, 5 µg/ml aprotinin and 100 µM PMSF). Cell lysates were incubated on dry ice for 1 min followed by 10 min incubation at 4 °C, and supernatants were obtained by centrifugation at 15,000 g for 15 min at 4 °C. Fifty microliters of lysates were then transferred to a 96-well flat bottom microplate containing 100 µl of caspase-3 (DEVD-AFC, Calbiochem) or caspase-8 (IETD-AFC, Calbiochem) fluorogenic substrates at 50 µM final concentration. Samples were read on a spectrofluorometer (SpectroMax, GEMINI XS, Molecular Device, CA, USA) at 400 nm excitation and 505 nm emitted light. Results represent relative fluorescence units (RFU) at different time points.

Evaluation of mRNA expression by quantitative PCR (qPCR)

Total RNA was extracted from CEM.neo and CEM.Bcl-2 after 4 and 8 h of treatment with sub-lethal dose of CHX (0.2 µM) using

Trizol (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were determined spectrophotometrically by measuring fluorescence at 260 nm and 280 nm. One microgram of RNA was reverse transcribed into cDNA in a total volume of 20 µl using Superscript III (Invitrogen) transcription reagents according to the manufacturer's instructions. After obtaining the cDNA, genes expression was quantified by qPCR methodology using Quantitect SYBRGreen Kit (Qiagen, USA) in ABI Prism 7300 sequence detector equipment (Applied Biosystem, CA).

qPCR conditions were as follows: enzyme activation at 94 °C for 15 min, initial denaturation at 94 °C for 15 s, annealing temperature specific for each gene during 25 s and extension at 72 °C for 30 s (50 cycles). Primer sequences and their respective annealing temperature are listed in Table 1. Results were given as relative expression, *amplicon* ratio: investigated gene/*gapdh* housekeeping gene.

Results and discussion

Sensitivity of cell lines to CD95- or drug-induced apoptosis

In Type I cells, apoptosis occurs by a direct activation of caspase-3 by caspase-8 whereas in Type II cells a mitochondria-dependent pathway is required for apoptosis to take place after CD95 engagement [11]. In addition, in both Type I and Type II cells, apoptosis initiated by chemotherapeutic drugs also depend at a large extent on the mitochondrial pathway [20,21]. Therefore, overexpression of Bcl-2 or Bcl-x_L should block CD95-mediated apoptosis only in Type II cells. In comparison, expression of anti-apoptotic members of the Bcl-2 family should prevent drug-induced apoptosis in both Type I and Type II cells. Taking all into account, we first compared the four cell lines SKW6.4, SKW6.4.Bcl-2, CEM, and CEM.Bcl-2 in respect to their resistance to CD95- and to staurosporine-mediated apoptosis. Our results show that SKW6.4 cells are only slightly more sensitive to CD95-mediated apoptosis than

Table 1 – Sequences of primers and annealing temperatures

Genes	Sequences (5' → 3')	Annealing temperature (°C)
Beta-actin	F: GCC CTG AGG CAC TCT TCC A R: CCA GGG CAG TGA TCT CCT TCT	58
Bid	F: GCT TCC AGT GTA GAC GGA GC R: GTG CAG ATT CAT GTG TGG ATG	63
c-IAP-1	F: AGT CTT GCT CGT GCT GGT TT R: ATG GAC AGT TGG GAA AAT GC	59
c-IAP-2	F: AGT CTT GCT CGT GCT GGT TT R: TGC TTT TGC CAG ATC TGT TG	58
Fas	F: CAA GGG ATT GGA ATT GAG GA R: TGG AAG AAA AAT GGG CTT TG	55
FasL	F: AGG AAA GTG GCC CAT TTA AC R: CAA GAT TGA CCC CGG AAG TA	55
cFLIP _L	F: GCC GAG GCA AGA TAA GCA R: GCC CAG GGA AGT GAA GGT	54
gapdh	F: GGA GAA GGC TGG GGC TCA T R: GTC CTT CCA CGA TAC CAA AGT T	60

CEM cells (Fig. 1A). In agreement with the reports in the literature, overexpression of Bcl-2 protected the Type II CEM cells but not the Type I SKW6.4 cells from CD95-mediated apoptosis (Fig. 1A). In comparison, both CEM.Bcl-2 and SKW6.4.Bcl-2 cells were resistant to apoptosis induced by staurosporine (Fig. 1B) and other chemotherapeutic drugs (Fig. 3B and data not shown). The expression of Bcl-2 was monitored by western-blot of total cell lysates confirming the nature of Bcl-2-overexpressed cells (Fig. 1C). Since differences in the degree of CD95 stimulation could potentially affect the cell sensitivity to CD95-mediated apoptosis, we monitored the cell surface expression of CD95 in all cell lines by flow cytometry. Our results showed that similar levels of CD95 were expressed in all cell lines tested (Fig. 1D and data not shown).

Sub-lethal dose of CHX overcomes Bcl-2 resistance to CD95-mediated apoptosis in Type II cells

Although CHX was shown to sensitize cells to CD95-mediated apoptosis, controversy still remains as whether this effect is restricted to Type I cells or it may also occur in Type II cells. Moreover, the role of the mitochondrial pathway, or more specifically the role of Bcl-2, in this process remains uncertain. In order to approach this question and to define the proper sub-lethal dose of CHX for each cell line, we initially treated SKW6.4, SKW6.4.Bcl-2, CEM, and CEM.Bcl-2 cells with different concentrations of CHX and established a dose-response curve of induction of apoptosis. Based on our results (Fig. 1E), we defined the sub-lethal dose of 0.2 μ M as the appropriate concentration of CHX to be used in our experiments. Pre-

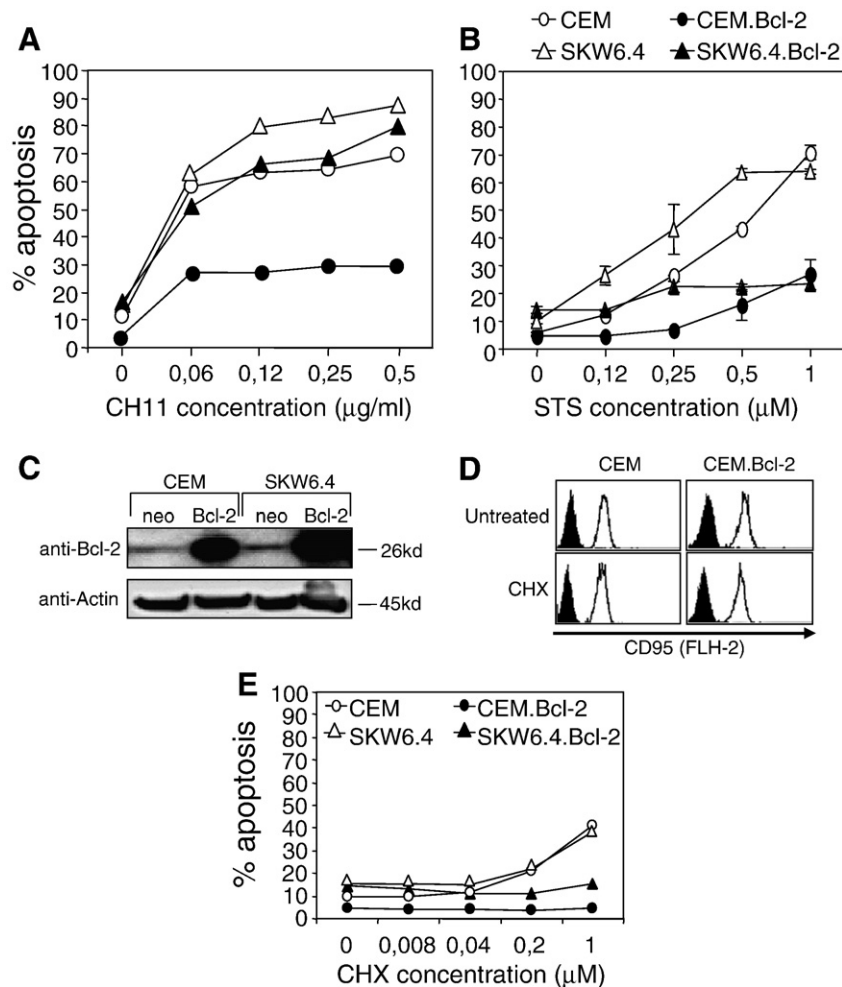


Fig. 1 – Characterization of Type I and Type II cells. Cells were incubated for 16 h with different concentrations of CH11 antibody (A) or STS (B). Apoptosis was estimated by cell cycle analysis. Numbers represent the average percentage \pm SD of cells with sub-diploid DNA content. Figure shows representative data of three independent experiments. (C) Western-blot analysis of Bcl-2 expression in CEM.neo, CEM.Bcl-2, SKW6.4.neo and SKW6.4.Bcl-2 cells. (D) Cell surface expression of CD95 was determined in CEM and CEM.Bcl-2. Cells were incubated or not with 0.2 μ M of CHX for 8 h followed by immunostaining with CH11 antibody or isotype control and PE-conjugated secondary antibody. Fluorescence intensity was analyzed and quantified by Cell Quest software. Data represents the average obtained in triplicate samples. Assay was repeated at least three times. (E) Dose-response curve of CHX in CEM.neo, CEM.Bcl-2, SKW6.4.neo and SKW6.4.Bcl-2 cells. Cells were incubated or not with different concentrations of CHX for 12 h. Apoptosis was estimated by cell cycle analysis of DNA content.

incubation with 0.2 μ M of CHX sensitized both SKW6.4 and CEM cells to apoptosis induced by different concentrations of CH11 antibodies (data not shown).

We then sought to investigate whether this effect could be blocked by overexpression of Bcl-2. Treatment with a combination of CHX and an optimal dose of CH11 antibodies did not substantially affect the fate of SKW6.4.Bcl-2 cells as they are already as sensitive to CD95-mediated apoptosis as the wild-type SKW6.4 (Fig. 2A). However, when we treated the CD95-

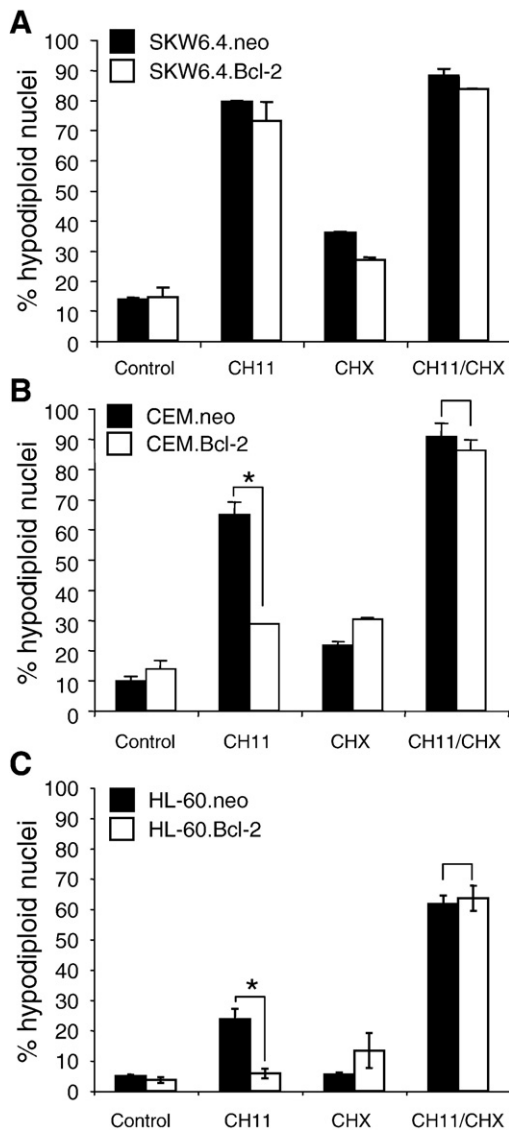


Fig. 2 – CHX overcomes Bcl-2 resistance to CD95-mediated apoptosis in Type II cells. (A) SKW6.4 and SKW6.4.Bcl-2 and (B) CEM and CEM.Bcl-2 were pre-treated or not with 0.2 μ M CHX for 1 h and then incubated with or without 0.25 μ g/ml of CH11 antibody for 18 h. (C) HL-60 and HL-60.Bcl-2 were pre-treated or not with 0.2 μ M CHX for 1 h and then incubated with or without 1 μ g/ml of CH11 antibody for 18 h. Apoptosis was estimated by cell cycle analysis of DNA content. Numbers represent the average percentage \pm SD of cells with sub-diploid DNA content. Figure shows representative data of three independent experiments.

resistant cell line CEM.Bcl-2 they became as susceptible as the wild-type CEM cells (Fig. 2B). Similar results were obtained using HL-60 and HL-60.Bcl-2 cells, another Type II cell line (Fig. 2C). Our results suggest that CHX is not only able to sensitize both Type I and Type II cells to CD95-induced apoptosis, but it can also surmount the anti-apoptotic effect that Bcl-2 grants to Type II cells. This effect was not due to a possible down-regulation of Bcl-2 by CHX, as the levels of Bcl-2 in both CEM and CEM.Bcl-2 cells remained comparable in the presence or absence of CHX (Fig. 3A). Similar results were obtained with HL-60 and HL-60.Bcl-2 cells (data not shown).

Sensitization of Type II cells by CHX is restricted to CD95-mediated apoptosis

At this point, our data suggest that CHX should be either opposing the anti-apoptotic effect of Bcl-2 at the mitochondrial level, despite the similar expression of Bcl-2 in all experimental samples, or bypassing the mitochondria pathway much alike to what is observed in Type I cells. To confirm that Bcl-2 levels were still sufficiently high, we investigated whether remaining levels were sufficient to protect cells from apoptosis induction by triggers of the intrinsic pathway, i.e. chemotherapeutic drugs. CEM and CEM.Bcl-2 cells were thus pre-treated with 0.2 μ M CHX for 1 h and then incubated overnight with VP-16, VCS and STS.

Our results revealed that CHX is unable to sensitize CEM.Bcl-2 cells to apoptosis induced by these chemotherapeutic drugs (Fig. 3B). On the contrary, it seems that CHX protected, at least in part, CEM and CEM.Bcl-2 cells from these apoptogenic drugs, demonstrating that the gain in sensitivity after CHX incubation is restricted to the CD95 pro-apoptotic signaling. Taken together, our results clearly indicate that the presence of sub-lethal concentration of CHX is neither interfering with Bcl-2 expression (Fig. 3A) nor neutralizing its anti-apoptotic effect for triggers of the mitochondrial pathway (Fig. 3B). Instead, it seems to alter the signaling pathway initiated by the engagement of CD95.

Mitochondrial changes in CEM and CEM.Bcl-2 cells after CHX plus anti-CD95 treatment

Although we have already established that sub-lethal doses of CHX do not interfere with the anti-apoptotic activity of Bcl-2, we aimed to investigate whether this treatment may promote any mitochondrial changes in CEM.Bcl-2 cells when associated with CD95 stimulation. First, we analyzed the loss of mitochondrial transmembrane potential ($\Delta\psi_m$) in CEM and CEM.Bcl-2 cells incubated with CH11 antibodies in the presence or absence of CHX. Similarly to what we reported above, incubation with CH11 antibodies alone induced loss of $\Delta\psi_m$ only in CEM cells. However, the combination of CHX and CH11 antibodies induced a substantial decrease of $\Delta\psi_m$ in both CEM and CEM.Bcl-2 (Fig. 4A).

Second, we investigated the release of cytochrome c and SMAC at the same experimental conditions. In agreement with our results obtained by analyzing $\Delta\psi_m$, CD95 stimulation alone induced a significant release of cytochrome c and SMAC only in CEM cells (Fig. 4B). Moreover, in this respect, CEM cells respond similarly to CH11 antibodies or the combination of

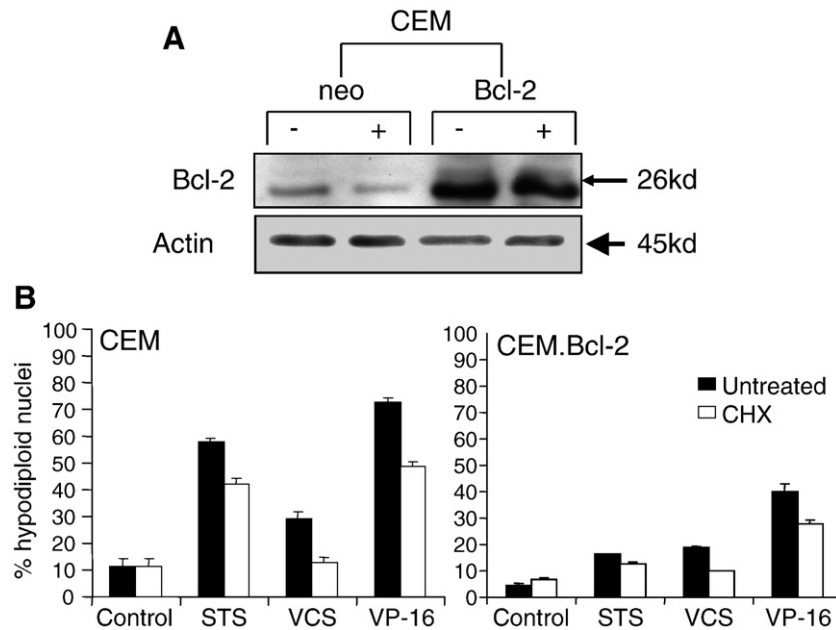


Fig. 3 – CHX does not interfere with Bcl-2 expression or resistance to mitochondrial cell death in Type II cells. (A) CEM and CEM.Bcl-2 were treated with or without 0.2 μ M of CHX for 8 h and Bcl-2 protein expression determined by western-blot analysis. (B) CEM and CEM.Bcl-2 cells were pre-treated or not with 0.2 μ M CHX for 1 h and incubated for 18 h with or without STS (1 μ M), VCS (10 μ M), or VP-16 (10 μ M). Apoptosis was estimated by cell cycle analysis of DNA content. Numbers represent the average percentage \pm SD of cells with sub-diploid DNA content. Figure shows representative data of three independent experiments.

CH11 plus CHX. In contrast, the appearance of cytochrome c and SMAC in the cytosol of CEM.Bcl-2 cells could only be clearly detected when these cells were co-treated with CHX and CH11 antibodies (Fig. 4B). However, maximal translocation of cytochrome c and SMAC in CEM.Bcl-2 cells was clearly less than the translocation seen in CEM cells (Fig. 4B). Collectively, these results suggest that the combination of CHX and CD95 stimulation promotes changes in the mitochondria that cannot be blocked by Bcl-2.

Downstream caspases seem to be responsible for the mitochondrial modifications associated with CHX plus CD95 stimulation in CEM.Bcl-2 cells

Considering that, in the presence of low doses of CHX, i) Bcl-2 is still functionally capable of preventing apoptosis at the mitochondrial level and ii) apparently the mitochondrial pathway is not required anymore for CD95-induced apoptosis in CHX-treated cells; then, how mitochondrial changes were observed after CH11 plus CHX in CEM.Bcl-2 cells. We explored the possibility of a positive feedback mechanism mediated by downstream caspases that could account for these mitochondrial changes. In this regard, it has been shown that caspases-3, -6 and -7 can indeed induce loss of mitochondrial transmembrane potential and release of apoptogenic factors from the mitochondria to the cytosol in a Bcl-2-insensitive manner [22,23]. To test whether the above mentioned observations related to mitochondrial changes were due to early activation of caspase-3, we pre-treated or not CEM.Bcl-2 cells with 150 μ M of z-DEVD-fmk 1 h before incubation with CHX plus CD95 stimulation. In fact, z-DEVD-fmk prevented the activation of

caspase-3 (data not shown) and blocked the loss of $\Delta\psi_m$ in CEM.Bcl-2 cells treated with CH11 plus CHX (Fig. 4C). These results suggest that the combination of CH11 and CHX promotes early activation of caspase-3, which precedes and is necessary to promote subsequent mitochondrial changes associated with apoptosis. Indeed, a kinetic study of caspase activation showed by western-blot that the loss of the pro-form of caspase-3 and appearance of its active forms p20 and p17, an indicative of caspase-3 activation, occurs only in CEM.Bcl-2 cells treated with the association of CHX and CH11 antibodies but not after single treatment with CH11 (Fig. 4D). This result was confirmed by analyzing caspase-3 activity using the fluorimetric substrate DEVD-AFC (Fig. 4E). Interestingly, this early activation of caspase-3 is very much reminiscent to what has been observed in Type I cells after CD95 stimulation.

The ratio of caspase-8 activation vs protein levels of Bid is significantly modified by CHX plus CD95 stimulation

As we discussed before, by definition, Type I cells are characterized by high levels of caspase-8 activation at the DISC, whereas in Type II cells only a small amount of caspase-8 is activated after CD95 stimulation. If sub-lethal doses of CHX converts CD95 Type II cells to Type I cells, one would expect that the combination of CHX and CH11 antibodies would increase the amount of caspase-8 activation in CEM.Bcl-2 cells, compared to CH11 alone. Indeed, western-blot analysis of caspase-8 activation showed that more of the p43 and p41 active forms appeared after the combination of CHX plus CH11, compared with CH11 alone (Fig. 5A). Increased caspase-

8 activity by CHX/CH11 was further confirmed using the fluorimetric substrate IETD-AFC (Fig. 5B).

It is well known that the two major targets of caspase-8 in CD95-mediated cell death is the pro-apoptotic member of the Bcl-2 family, Bid, and the effector caspase, caspase-3 [24-27]. Apparently,

when the availability of active caspase-8 is low, Bid is the preferred target. In comparison, when a large amount of active caspase-8 is produced, caspase-3 is activated directly by caspase-8 [25].

On the other hand, we hypothesized that in the absence of Bid it is possible that even low/moderate amounts of active

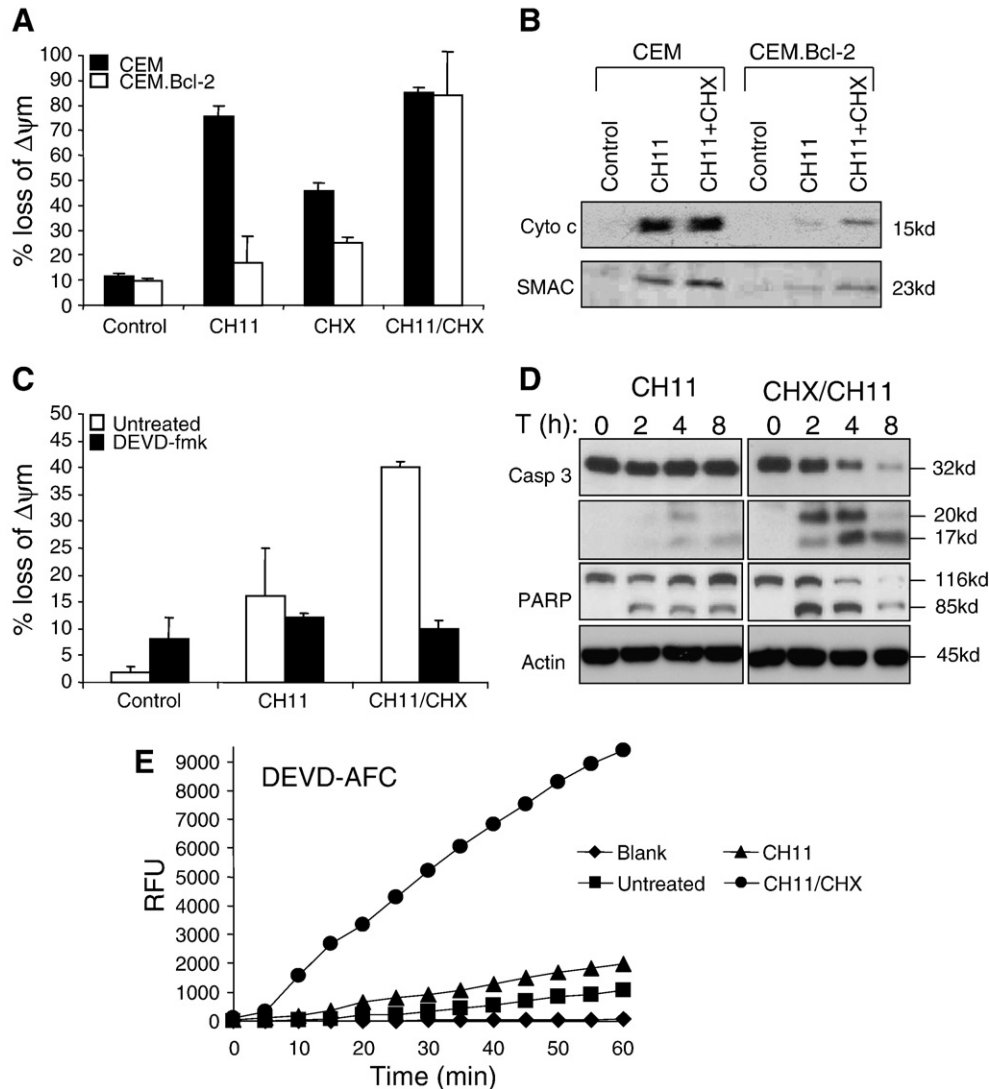


Fig. 4 – Cycloheximide induces loss of mitochondrial potential, release of cytochrome c and earlier caspase-3 activation in CD95-mediated apoptosis in CEM.Bcl-2 cells. (A) Change in $\Delta\psi_m$ was determined in CEM and CEM.Bcl-2 cell lines by the DiOC₆ assay. Cells were treated with or without 0.2 μ M of CHX for 1 h followed by incubation with or without 0.25 μ g/ml of CH11 antibody for 12 h. Cells were then stained with 50 nM DiOC₆ for 45 min and fluorescence intensity was analyzed and quantified by FACS. Numbers represent the average percentage of triplicates \pm SD of cells with loss of $\Delta\psi_m$. Figure shows representative data of two independent experiments. **(B)** Release of cytochrome c and SMAC from the mitochondria to the cytosol was determined in cells pre-treated or not for 1 h with 0.2 μ M CHX followed by incubation with or without 0.25 μ g/ml of CH11 antibody. **(C)** Mitochondrial depolarization is blocked by z-DEVD-fmk in CEM.Bcl-2 cells. Type II CEM.Bcl-2 cells were pre-treated or not for 1 h with 150 μ M z-DEVD-fmk followed by 1 h incubation with or without 0.2 μ M CHX and then treated with or without 0.25 μ g/ml of CH11 antibody for 12 h. Change in $\Delta\psi_m$ was determined by the CMXRos assay by flow cytometry. Numbers represent the average percentage \pm SD of cells with loss of $\Delta\psi_m$. Figure shows representative data of two independent experiments. **(D)** Kinetics of caspase-3 activation was monitored in CEM.Bcl-2 cells by western-blot analysis. Cells were pre-treated or not for 1 h with 0.2 μ M CHX and incubated with 0.25 μ g/ml of CH11 antibody for the depicted periods of time. Expression of caspase-3 pro and active forms were analyzed in total cell lysates by immunoblot. **(E)** Caspase-3 activity in cell-free extracts of CEM.Bcl-2, pre-treated or not for 1 h with 0.2 μ M CHX and incubated with 0.25 μ g/ml of CH11, was determined by the ability of hydrolysis of the fluorogenic caspase substrate peptide DEVD-AFC. Data are representative of two independent experiments.

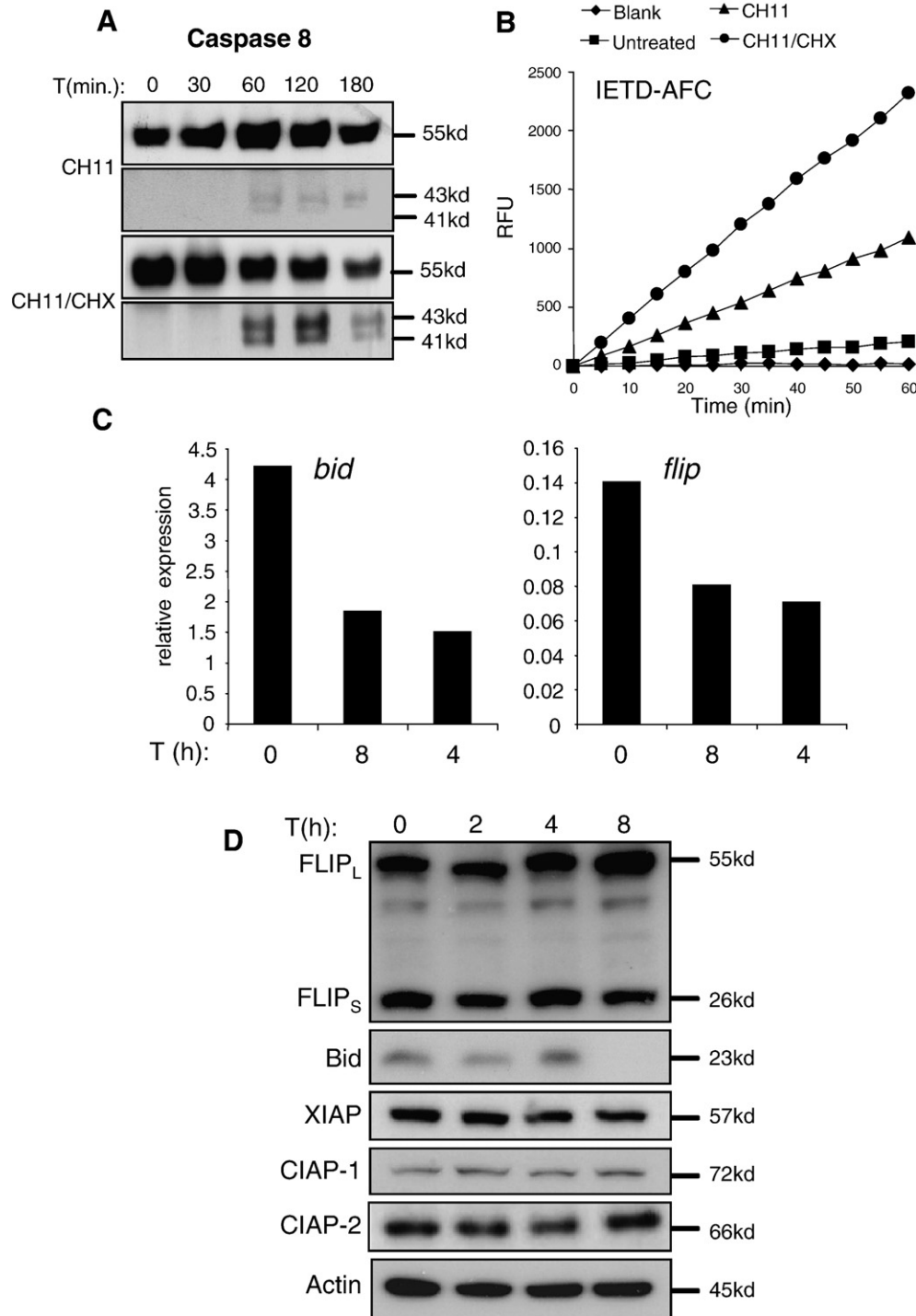


Fig. 5 – Caspase-8 activation and expression of Bid, FLIP and IAP members after pre-treatment of CEM.Bcl-2 cells with CHX. (A) Western-blot analysis of caspase-8 activation in CEM.Bcl-2 cells pre-treated or not for 1 h with 0.2 μ M CHX and incubated with 0.25 μ g/ml of CH11 antibody for the depicted periods of time. (B) Cell-free extracts from CEM.Bcl-2 cells, untreated or treated with CHX and CHX plus CH11 for 6 h, were assayed for the ability to cleave IETD-AFC. Data are representative of two independent experiments. (C) RNA expression of Bid and cFLIP_L in CEM.Bcl-2 cells after sub-lethal doses of CHX. Cells were treated with 0.2 μ M CHX for different time points and mRNA expression was evaluated by qPCR. Data representative of three independent experiments (D) cFLIP_L, cFLIP_S, Bid, c-IAP1, c-IAP2 and XIAP protein expression determined by western-blot in a time course of total lysate of CEM.Bcl-2 cells treated with 0.2 μ M CHX. Data are representative of three independent experiments.

caspase-8 can directly activate caspase-3. Therefore, we looked at the expression of Bid at both mRNA and protein levels and compared it to the expression of cFLIP, a previously described target of CHX [28].

As we predicted, sub-lethal doses of CHX induced an early and significant drop of the expression of Bid mRNA (Fig. 5C) that was accompanied by a complete disappearance of Bid protein (Fig. 5D). This observation strongly supports our hypothesis that CHX is converting Type II cells into Type I cells, since in the absence of Bid, the death signal initiated by CD95 stimulation could not be directed to the mitochondria.

Resistance to death receptor-induced apoptosis has been long associated to NF κ B activation and regulation of anti-apoptotic genes such as IAPs, A1, Bcl-x_L and cFLIP. Overexpression of cFLIP through NF κ B activation, provides cells with a death resistance phenotype that can be overcome by overexpression of NF κ B inhibitor I κ B or by treatment with CHX [29,30]. Thus, to evaluate the importance of cFLIP on the conversion of Type II to Type I cells, cFLIP mRNA and protein levels were analyzed in CEM.Bcl-2 cells after CHX treatment. Interestingly, although we observed a down-regulation of FLIP mRNA (Fig. 5C), we could not observe any difference at the protein level, under our experimental conditions (Fig. 5D). In addition, the protein levels of XIAP, c-IAP-1 and c-IAP-2 were not modified by CHX treatment (Fig. 5D).

Even if the inhibition of Bid expression fits with our aforementioned hypothesis, it is important to point out that the sole absence of Bid is unlikely to account for the increased sensitivity of CHX-treated CEM.Bcl-2 cells to anti-CD95 antibodies. Incidentally, at least hepatocytes (considered to be Type II cells) from Bid^{-/-} mice are more resistant to CD95-mediated apoptosis both *in vivo* and *in vitro* when compared to Bid^{+/+} hepatocytes [31]. Therefore, we believe that it is the combination of the increased amount of active caspase-8, the elimination of Bid, and possibly yet unidentified factors such as a different subcellular distribution of caspase-3 that accounts for the exquisite sensitivity of CHX-treated CEM.Bcl-2 cells to CD95 stimulation. In this regard, it has been proposed that recruitment of CD95 and molecules involved on DISC formation (FADD, caspase-8 and cFLIP), and perhaps caspase-3, to lipid rafts sensitizes cells to CD95-induced apoptosis [31–35]. Verifying the effects of CHX in lipid rafts formation might provide us new insights of how sub-lethal doses of this substance can have such a strong effect in death receptor-mediate apoptosis.

In conclusion, to our knowledge we show here for the first time that inhibition of protein synthesis can alter the expression of apoptosis signaling molecules and convert CD95 Type II cells into Type I cells. These findings may have likely implications in the development of novel therapeutic approaches targeting the apoptosis resistance and signaling of cancer cells.

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