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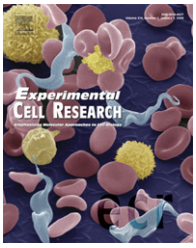

Research Article

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.

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

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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% CO_2. 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% CO_2.

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