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Research paper

The combination of TRAIL and MG-132 induces apoptosis in both TRAIL-sensitive and TRAIL-resistant human follicular lymphoma cells

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ABSTRACT

We have previously shown that the human follicular lymphoma cell line, HF28GFP, is sensitive to TRAIL-mediated apoptosis. Nevertheless, when the same cells overexpress anti-apoptotic Bcl-2 family protein, Bcl-xL (HF28Bcl-xL), they become resistant to TRAIL. Thus, these cell lines help us to investigate the action of novel apoptosis inducing candidate drugs. In the present study, we examined the effects of MG-132 (a proteasome inhibitor), LiCl (a glycogen synthase kinase-3 inhibitor) and/or TRAIL on pro-apoptotic Bcl-2 family proteins such as Bim and Bid. Here we demonstrate that the combination of MG-132 and TRAIL induced significant apoptotic cell death in both cell lines, HF28GFP and HF28BclxL. Apoptosis correlated with a decrease of phospho-ERK1/2, the accumulation of Bim and translocation of truncated Bid (tBid) and jBid. In addition, the combination of MG-132 and TRAIL seemed to target other apoptotic factors, which led to the accumulation of active capsase-3. Furthermore, co-stimulation of LiCl and TRAIL induced apoptosis in HF28GFP cells. However, HF28Bcl-xL cells were far less sensitive to the combinatorial effects of LiCl and TRAIL. Interestingly, we observed that LiCl did not target Bim and Bid proteins. In conclusion, these data show that targeting of pro-apoptotic Bcl-2 family proteins simultaneously through a selective proteasome inhibition might help to overcome TRAIL resistance caused by overexpression of anti-apoptotic Bcl-2 family proteins. Moreover, the data may provide new strategies to develop targeted therapies against lymphomas.

1. Introduction

Apoptosis is a type of programmed cell death that plays an essential role in the development and homeostasis of normal tissues. The two main apoptotic pathways are the death receptor pathway (extrinsic pathway) and the mitochondrial pathway (intrinsic pathway). These pathways are executed by caspases which eventually lead to the demise of a cell [1].

The death receptor signaling pathway is initiated by cytotoxic ligands such TRAIL (tumor necrosis factor-related apoptosis inducing ligand) and Fas. The activation of transmembrane TRAIL death receptors DR4 or DR5 by TRAIL leads to the formation of death-inducing signaling complex (DISC), where caspase-8 is activated. The active caspase-8 then cleaves and activates caspase-3, which results in apoptosis. Moreover, TRAIL selectively initiates apoptosis in a variety of tumor cells but not in normal cells. This special feature of TRAIL makes it a promising candidate for treatment of cancers [2,3].

Mitochondrial signaling pathway, however, is activated by a variety of non-receptor-mediated apoptotic stimuli such as cytotoxic drugs. This pathway is mainly regulated by Bcl-2 family proteins, a group of structurally related proteins. The Bcl-2 family proteins can be classified into three groups; 1) anti-apoptotic proteins such as Bcl-xL, Bcl-2, and Mcl-1, 2) multi-BH pro-apoptotic effector proteins, Bax and Bak, and 3) BH3-only proteins (pro-apoptotic sensitizers/activators) such as Bim and Bid [4].

The intrinsic apoptotic stimuli lead to the activation of pro-apoptotic Bcl-2 family proteins and/or down-regulation of anti-apoptotic proteins. For instance, up-regulation of Bim results in the activation of pore forming Bcl-2 family proteins, Bax/Bak either by 1) direct activation – the binding of Bim with Bax/Bak, or 2) indirect activation – freeing Bax/Bak from anti-apoptotic Bcl-2 family proteins. This phenomenon triggers mitochondrial outer membrane permeabilization (MOMP), thereby apoptotic proteins such as cytochrome c, and smac are released from the mitochondrial interspace into the cytosol. The

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formation of cytochrome c, Apaf-1 and ATP complex leads to the activation of caspase-9. Moreover, the released smac neutralizes the anti-apoptotic effect of XIAP (X-linked inhibitor of apoptosis protein). Consecutively, the activation of caspase-3 by caspase-9 leads to apoptosis [5,6].

As Bim is an important protein for the induction of apoptosis, resting cells regulate the turnover of this protein through phosphorylation and degradation by the survival kinase, ERK1/2. Inhibition of Bim degradation, therefore, can lead to cell death [7–9].

The cells which do not require the involvement of mitochondria in Fas/TRAIL-mediated apoptosis are called type I, whereas the cells that depend on the engagement of mitochondria are known as type II [10,11]. Bid has an important role in both type I and type II cells because caspase-8 mediated cleavage of Bid, truncated Bid (tBid – 15 kDa) connects the death receptors-induced signaling to mitochondria. tBid translocation into the mitochondria, thus, leads to the releases apoptotic factors. Moreover, it is interesting that JNK might induce caspase-8 independent cleavage of Bid at a distinct site to generate an intermediate product jBid (21 kDa) and the translocation of jBid to mitochondria preferentially releases smac to the cytosol [12].

XIAP protein suppresses apoptotic pathways. It binds and inactivates both caspase-9 and caspase-3. This special feature of XIAP enables it to prevent death receptor- and mitochondrial-mediated apoptosis. Furthermore, it has been shown that inhibition of XIAP switches type II signaling to type I signaling [13].

The proteasome is a large catalytic complex that is responsible for most non-lysosomal intracellular protein degradation of misfolded/damaged proteins. It also regulates protein turnover [14]. The proteasome, thus, regulates cellular homeostasis by maintaining the normal functions of cellular proteins. The proteasome, however, is also play an important role in the progression of cancer because it regulates critical proteins such as transcription factors. Therefore, proteasome inhibition is an attractive target for the development of anti-cancer therapies [15].

Proteasome inhibition can lead to cellular cytotoxicity through several mechanisms including, Bim accumulation by decreasing the amount of phospho-ERK1/2 [16], inhibition of NF-kB activity, and stabilization of p53. MG-132 (a proteasome inhibitor) is commonly used to investigate the proteasome activity in a range of cell types [17]. Moreover, MG-132 enhances TRAIL-induced apoptosis through up-regulation of DR5 in cancer cells [18].

Glycogen synthase kinase-3β (GSK3β) involved in multiple cellular processes including proliferation, glucose regulation and apoptosis [19]. Inhibition of GSK-3 by LiCl is found to augment the apoptotic effect of TRAIL in human lung carcinoma cells through up-regulation of death receptors DR4 and DR5 [20].

Overexpression of anti-apoptotic Bcl-2 family proteins prevents mitochondria-mediated apoptosis by blocking the permeabilization of outer mitochondrial membrane. We have previously shown that TRAIL induces apoptosis in HF28GFP cells (Type II cell model) but the apoptosis is completely prevented by overexpression of Bcl-xL (HF28Bcl-xL cells) [10]. In the present study, we examined the effects of apoptosis inducing agents: MG-132, LiCl, and/or TRAIL on Bid, Bim, and XIAP using HF28GFP cells (vector control) and HF28Bcl-xL cells.

2. Materials and methods

2.1. Cell lines and culture conditions

The origin and characteristics of human follicular lymphoma cell lines, HF28GFP and HF28Bcl-xL have been previously described [21]. The cells were cultured in RPMI 1640 medium (Lonza, Belgium) supplemented with 5% heat inactivated fetal bovine serum (GIBCO, Invitrogen, USA). 2 mM L-glutamine (Lonza), 106 U/ml streptomycin, 106 U/ml penicillin (Lonza), 10 mM HEPS buffer (Lonza), 0.1 mM nonessential amino acids (Lonza), 1 mM Na-pyruvate (Lonza), 20 μM 2-mercaptoethanol (Fluka-Chemie, Buch, Switzerland) at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Cell treatments

HF28GFP and HF28Bcl-xL cells were seeded on 6 or 12-well flat bottom polystyrene cell culture plates (Corning Inc. NY, USA) and treated with 50 ng/ml His-tagged recombinant human soluble killer TRAIL™ (Enzo life sciences, USA), 50 mM LiCl, (Sigma-Aldrich, USA) 1 μM MG-132 (Calbiochem, USA), 50 μM caspase-8 specific inhibitor, Z-IETD-FMK (Calbiochem, USA), 20 μM JNK specific inhibitor, SP600125 (Sigma-Aldrich, USA).

2.3. Flow cytometric analysis of apoptotic cells

Apoptotic cells were determined by flow cytometric analysis after propidium iodide (PI) staining. Cells with sub-G1 DNA content/hypodiploid cells were considered as apoptotic. Fixation and staining of cells were performed according to a standard protocol. In brief, at the end of stimulation times, samples containing one million cells were collected, resuspended in ice-cold PBS and fixed with ice-cold 70% V/V ethanol. After overnight incubation at +4 °C, cells were centrifuged at 1500 RPM for 10 min, resuspended in PBS containing 150 μg/ml RNAase (Sigma, USA) and incubated for 1 h at +50 °C. PI (Molecular Probes, Sigma) was added to the final concentration of 8 μg/ml and incubation was further continued 2 h at +37 °C. FACSCanto II flow cytometer with FACSDiva version 6.1.2 software (Becton Dickinson, USA) and FlowJo v10 software were used for the analysis.

2.4. Detection of changes in mitochondrial membrane potential

Depolarization of mitochondrial membrane was detected by TMRM (methyl ester of tetramethylrhodamine) staining. After incubation of cells with stimuli, 5×10^5 cells were collected and stained with 100 nM TMRM (Molecular Probes) for 20 min at +37 °C in the dark. After staining, the cells were immediately analyzed using a FACSCanto II flow cytometer (Becton Dickinson). The fluorescence excitation and emission maxima are 548 nm and 574 nm, respectively. The forward and side scatters were used to gate living or early apoptotic cells.

2.5. Preparation of total cell lysates

At the end of stimulation times, 2×10^6 cells were collected, washed in phosphate buffered saline (PBS) and centrifuged (600 × *g*, 5 min). The cell pellet was resuspended with lysis buffer containing 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 3% NP-40, 100 mM NaCl, 50 mM NaF, 1 mM PMSF, 1 mM VO_4, 5 μg/ml aprotinin, and 5 μg/ml Leupeptin. After 1 h incubation on ice, samples were centrifuged (10 000 × *g*, 15 min, + 4 °C). The protein concentration of the lysates was measured, equalized with sodium dodecylsulfate- polyacrylamide gel electrophoresis (SDS-PAGE) buffer (0.125 M Tris-Hcl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, bromophenol blue) and boiled for 5 min.

2.6. Preparation of mitochondrial and cytosolic fractions

Mitochondrial and cytosolic fractions were separated by an Apoalert cell fractionation kit (Clontech Laboratories, Inc., USA) according to the manufacturer's protocol. In brief, 2×10^7 cells were collected and washed with wash buffer. The cell pellet was resuspended in 0.8 mL fractionation buffer containing protease inhibitors and dithiothreitol (DTT) and incubated on ice for 10 min. Subsequently, cells were homogenized by passing the cell suspension through a 27G syringe needle. The homogenate was centrifuged (700 × *g*, 10 min, + 4 °C). The remaining supernatant was centrifuged (10 000 × *g*, 25 min, + 4 °C). The supernatant (cytosolic fraction) was collected and the pellet (mitochondrial fraction) was resuspended in 0.1 mL fractionation buffer.