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Ethanol acts as a potent agent sensitizing colon cancer cells to the TRAIL-induced apoptosis

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Abstract Identification of mechanisms of modulation of the TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis is important for its potential use in anticancer therapy. Ethanol can induce cell death in vitro and in vivo by different signalling pathways. Its effect in combination with death ligands is unknown. We investigated how ethanol modulates the effects of TRAIL in colon cancer cells. After combined TRAIL and ethanol treatment, a potentiation of caspase-8, -9, -3 activation, a proapoptotic Bid protein cleavage, a decrease of mitochondrial membrane potential, a complete poly(ADP)ribose polymerase cleavage, and disappearance of antiapoptotic Mcl-1 protein were demonstrated. Ethanol acts as a potent agent sensitizing colon cancer cells to TRAIL-induced apoptosis.

Keywords: TNF-related apoptosis-inducing ligand; Ethanol; Apoptosis; Colon; Cancer

1. Introduction

Ethanol (C_2H_5OH, ethyl alcohol) has been frequently used and generally accepted as a solubilizing agent for a number of different reagents used in laboratory experiments with cell cultures. However, previous reports have demonstrated a role of ethanol in the apoptotic death of many cell types [1–4]. Apoptosis has also been shown to occur in both experimental and clinical alcoholic liver disease, but the signalling pathways remain not fully understood [5]. In addition to the effects of ethanol itself, it is also necessary to consider its interference with other factors, such as endogenous apoptotic regulators. TNF-related apoptosis-inducing ligand (TRAIL), a member of the tumour necrosis factor (TNF) family, is particularly interesting for its unique ability to induce cancer cell death while sparing most normal cells, which implies its use as a potent anti-cancer agent [6]. Cross-linking of TRAIL death receptors DR4 (TRAIL-R1, APO-2) and DR5 (TRAIL-R2, TRICK, Killer) results in activation of caspase-8 at the level of the death-inducing signalling complex (DISC). Activated caspase-8 then initiates the apoptosis executing caspase cascade [7,8].

However, in many cancer cell types, resistance to TRAIL was developed. We investigated the modulation of HT-29 human colon adenocarcinoma cell sensitivity to TRAIL by ethanol. Our results demonstrated a strong potentiation of TRAIL-induced apoptosis in the presence of ethanol and suggested some of the possible mechanisms involved in the interference of these two agents.

2. Materials and methods

2.1. Culture conditions

Human colon adenocarcinoma HT-29 cells (ATCC, Rockville, MD, USA) were cultured in Mc Coy's 5A medium (Sigma, Germany) with gentamycin (50 mg/l; Sigma) and 10% foetal calf serum (FCS; PAN Systems, Germany) at 37 °C in 5% CO_2 and 95% humidity. The attached cells (24 h after seeding) were treated with TRAIL (human Killer TRAIL, 100 ng/ml) and ethanol (4%) alone or in combination for 4 or 24 h in the medium with 5% of FCS. In the experiments using ethanol (0.1–6%) alone, the cells were treated for 48 h.

2.2. Stable transfections

SSFV.neo plasmids (LTR promoter, Neo resistance, *Eco*RI cloning site) [9] with or without (controls) Bcl-2 (kindly provided by Stanley Korsmeyer) were stably introduced into HT-29 cells by transfection using Tfx™ reagent (Promega, Czech Republic) according to the manufacturer's instructions. The clones were then selected with G418 (0.25 mg/ml, Genetica, Czech Republic). Two stable clones and one control clone with an empty vector were used in the experiments and treated as described above. After 4 h, poly(ADP)ribose polymerase (PARP) and pro-caspase-8 cleavage as well as Bcl-2 protein level were examined using immunoblotting.

2.3. Cell viability assay

Cell viability was determined microscopically by eosin (0.15%) dye exclusion assay from a total number of 100 cells.

2.4. Fluorescence microscopy

The cells treated as described above were stained with 4,6-diamidino-2-phenyl-indole (DAPI, Fluka, Buche, Switzerland) solution (1 μg DAPI / ml ethanol) at room temperature in the dark for 30 min. They were then mounted in Mowiol 4-88 (Calbiochem, San Diego, CA, USA) and the percentage of apoptotic cells (with chromatin condensation and fragmentation) was determined using a fluorescence microscope (Olympus 1X70, Prague, Czech Republic).

2.5. Detection of mitochondrial membrane potential

The cells were incubated (20 min) in Hanks' balanced salt solution (HBSS) with 100 nM of tetramethylrhodamine ethyl ester perchlorate

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Abbreviations: DR, death receptor; FLIP, FLICE inhibitory protein; MMP, mitochondrial membrane potential; PARP, poly(ADP)ribose polymerase; ROS, reactive oxygen species; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand

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(TMRE, 49/574 nm, abs/em, Molecular Probes, Eugene, OR, USA), then washed twice with HBSS, and analysed (2×10^4 cells per sample) by a flow cytometer (585/42 band pass filter, FACSCalibur, Becton–Dickinson, argon ion laser, 488 nm for excitation, San Jose, CA, USA). Forward and side scatters were used to gate the viable cell population. The data were evaluated (Cell Quest software, Becton–Dickinson) as a percentage of the cells with decreased mitochondrial membrane potential (MMP).

2.6. Production of reactive oxygen species

The intracellular production of reactive oxygen species (ROS) was detected by flow cytometric analysis using dihydrorhodamine-123 (DHR-123, Fluka, Switzerland) as described previously [10].

2.7. Caspase activity assay

The cells were lysed in lysis buffer (250 mM HEPES, 25 mM CHAPS, 25 mM DTT, and 40 μM protease inhibitor cocktail, Sigma–Aldrich Corp., St. Louis, MO, USA) on ice for 20 min and then centrifuged ($15\,000 \times g$, 15 min, 4°C). The acquired proteins (equal concentrations) were incubated with caspase-3 (Ac-DMQD-AMC, 50 μM, Alexis), caspase-8 (Ac-IETD-AMC, 50 μM, Sigma) or caspase-9 (Ac-LEHD-AMC, 50 μM, Alexis) substrates overnight in assay buffer (40 mM HEPES, 20% glycerol, and 4 mM DTT). Fluorescence was measured (355/460 nm) using a Fluostar Galaxy fluorometer (BMG Labtechnologies GmbH, Offenburg, Germany).

2.8. Immunoblotting

The cells were lysed in a sample buffer (100 mM Tris, pH 7.4; 1% sodium dodecyl sulfate; and 10% glycerol), diluted to an equal concentration, mixed with bromphenol blue(0.01%) and mercaptoethanol (1%), and subjected (20 μg) to SDS–PAGE. The polyacrylamide gels were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA) electrophoretically in a buffer containing 192 mM glycine, 25 mM Tris, and 15% methanol. The membranes were blocked overnight in 5% nonfat milk in wash buffer (0.05% Tween 20 in 20 mM Tris; pH 7.6; and 100 mM NaCl) and then probed with rabbit anti-PARP (1:500, SC-7150, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-caspase-8 (1:500, #9746, Cell Signalling Technology, Inc., Beverly, MA, USA), rabbit anti-caspase-9 (1:500, RB-1617-100, Neomarkers, Fremont, CA, USA), mouse anti-caspase-3 (1:500, SC-7272, Santa Cruz Biotechnology), rabbit anti-Bid (1:500, #550365, Pharmingen, BD Biosciences, San Jose, CA, USA), rabbit anti-FLICE inhibitory protein (anti-FLIP) (1:1000, AAP-440, Stress Gen Biotechnologies Corp., Victoria, BC, Canada), mouse anti-Bax (1:500, B73520, Transduction Laboratories, BD Biosciences, San Jose, CA, USA), rabbit anti-Bak (1:4000, #66026E, Pharmingen), rabbit anti-Mcl-1 (1:4000, M8434, Sigma–Aldrich, Prague, Czech Republic), mouse anti-Bcl-2 (1:1000, SC-509, Santa Cruz Biotechnology), anti-calpain I (1:500,3189-100, Biovision Corp., CA, USA), or mouse anti-β-actin (1:5000, A5441, Sigma) antibodies. The proteins recognized were detected using horseradish peroxidase-labelled secondary antibodies: mouse anti-IgG (1:2000 to 1:3000, #NA931, Amersham Biosciences, Bucks., UK), rabbit anti-IgG (1:6000, #NA934, Amersham Biosciences), and enhanced chemiluminescence kit (ECL, Amersham Biosciences). An equal loading was verified using β-actin quantification as well as non-specific amidoblack staining of proteins after immunoblotting.

2.9. Statistical evaluation

The results of at least three independent experiments were expressed as means ± S.E.M. Statistical significance ($P < 0.05$) was determined by one-way ANOVA followed by Tukey test.

3. Results

3.1. Cell death

We demonstrated only a limited cytotoxicity of TRAIL (100 ng/ml) in HT-29 cells. After 24-h-treatment, the cell viability was 82%. However, when TRAIL was combined with ethanol, only 40% of cells remained viable (Table 1). This effect was preceded by a significantly increased percentage of cells with characteristic apoptotic nuclear morphology (Table 1) after 4 h compared to the control as well as to the agents used alone.

3.2. Mitochondrial membrane potential and reactive oxygen species production

As shown in Table 1, no significant changes in ethanol-treated cells and about 2-fold enhancement of the number of cells with decreased MMP after TRAIL treatment (4 h) compared to control were detected. This effect was further significantly potentiated (3.2-fold compared to control) after the combination of TRAIL and ethanol.

No significant changes were observed in ROS production (DHR-123) after any type of treatment compared to control cells.

3.3. Proteins involved in regulation of apoptosis

Changes in pro-caspase levels. A significant decrease in pro-caspase-8 (55/57 kDa) immunoreactivity accompanied by an increase of its 43/41 kDa cleavage products, and a decrease of pro-caspase-3 and -9 expression were demonstrated in HT-29 cells after 4 h of TRAIL treatment. While ethanol alone had no significant effect, it significantly potentiated these effects of TRAIL (Fig. 1(a)).

Caspase activities. The effects on pro-caspase expressions correlated well with the corresponding caspase activities. TRAIL alone induced a significant increase in caspase-8, -9, and -3 activities in HT-29 cells and these effects were significantly potentiated after 4 h of combined TRAIL and ethanol treatment (Fig. 2).

Cleavage of cellular Bid protein. TRAIL alone induced cleavage of Bid protein. However, in combination with ethanol, a further significant decrease of the full-length 24 kDa Bid protein level was detected (Fig. 1(b)).

Mcl-1 protein expression. TRAIL alone induced a marked increase in Mcl-1 protein level compared to the control. On the

Table 1
ROS production (DHR-123, flow cytometer), cells with decreased MMP (TMRE, flow cytometer), apoptotic cells (DAPI, fluorescence microscope), and HT-29 cell viability (eosin, microscope) after 4- or 24-h-treatment with TRAIL (100 ng/ml), ethanol (4%) or their combination

Treatment	ROS production (median of fluorescence)	Cells with decreased MMP (% of control)	Apoptotic cells (%)	Cell viability (%)	
Time (h)	4	4	4	4	24
Control	138.1 ± 26.3	100.0 ± 10.0	2.3 ± 0.4	92.3 ± 0.8	89.3 ± 6.6
TRAIL	142.6 ± 29.5	209.6 ± 14.6*	15.8 ± 1.2*	89.3 ± 3.6	81.7 ± 2.2
Ethanol	142.3 ± 28.5	95.7 ± 20.0	2.5 ± 0.4	88.0 ± 3.1	87.0 ± 2.8
TRAIL + ethanol	111.0 ± 27.5	329.4 ± 25.8*+×	43.9 ± 2.8*+×	87.0 ± 1.2	40.0 ± 4.9*+×

Values are means ± S.E.M., $P < 0.05$, (*) vs. control, (+) vs. TRAIL, (×) vs. ethanol.

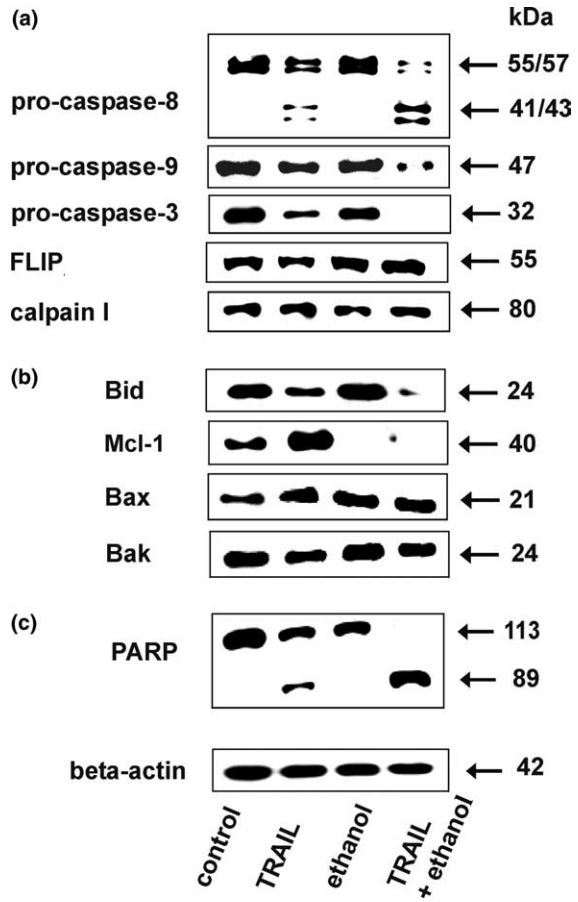

Fig. 1. Expression of (a) pro-caspases-8, -9, -3, cFLIP$_L$, and calpain I, (b) selected Bcl-2 family members, and (c) cleavage of PARP after 4-h-treatment of HT-29 cells with TRAIL (100 ng/ml), ethanol (4%) or there combination detected by Western blotting. The results are representatives of at least three independent experiments.

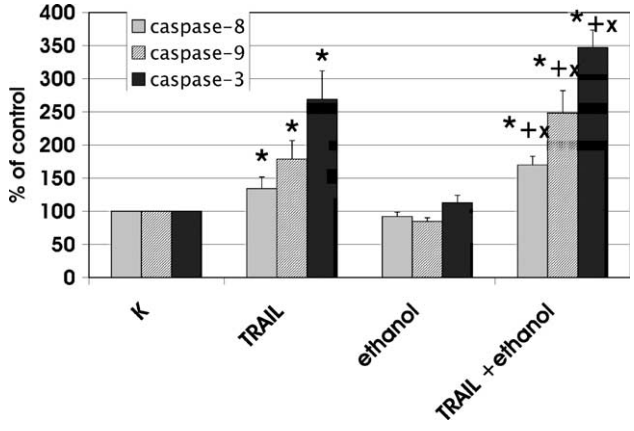

Fig. 2. Caspase-8, -9, and -3 activities (% of control) after 4-h-treatment with TRAIL (100 ng/ml), ethanol (4%) or their combination. The values are means ± S.E.M., $P < 0.05$, vs. (*) untreated control, (+) TRAIL, (×) ethanol.

other hand, this protein completely disappeared after treatment with ethanol or its combination with TRAIL (Fig. 1(b)).

FLIPL, calpain I, Bax, and Bak protein expression. Compared to the control cells, no significant effects on the level of these proteins were observed after any type of treatment (Fig. 1(a) and (b)).

Degradation of PARP. No apoptosis-associated cleavage of PARP was detected in control or ethanol-treated cells. TRAIL alone induced cleavage of PARP (113 kDa) to its 89 kDa fragment. This effect was significantly potentiated after combined TRAIL and ethanol treatment, when the full-length PARP completely disappeared (Fig. 1(c)).

3.4. The effects of Bcl-2 overexpression

Stable HT-29-Bcl-2 transfectants were prepared and treated with TRAIL, ethanol, and their combination. After combined TRAIL and ethanol treatment of HT-29-Bcl-2 transfectants, a significant potentiation of the pro-caspase-8 and PARP cleavage was demonstrated compared to the agents used alone (Fig. 3). These results were similar to those obtained in non-transfected HT-29 cells.

4. Discussion

Recent data obtained in our laboratory indicated a significant role of ethanol in the sensitization of colon cancer cells to the TRAIL-induced apoptosis. The intracellular events of ethanol-induced cell death are still only partially understood. It was reported that upon ethanol treatment, caspase activation, modulation of extracellular signal-regulated kinase 1/2 (ERK1/2) activity, changes in Bcl-2 family protein expression, increase in intracellular calcium ion concentration and DNA fragmentation occur [2–4,11]. Ethanol was also described to promote apoptosis through activation of the intrinsic or mitochondrial pathway as documented by an increased mitochondrial permeability transition, translocation of cytochrome *c*, and caspase-9 activation [1,4]. We demonstrated that human colon cancer HT-29 cells are relatively resistant to ethanol effects. After 48 h of treatment with ethanol (0.1–6%), no statistically significant changes in cell viability were apparent compared to the controls (not shown).

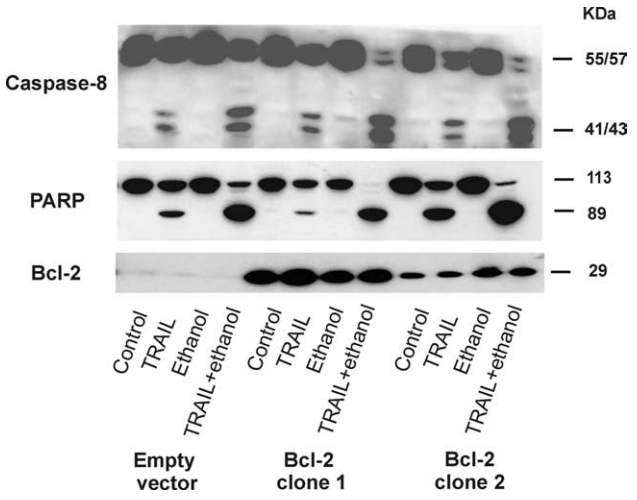

Fig. 3. Cleavage of pro-caspase-8 and PARP and Bcl-2 expression in stable Bcl-2 transfectants (clones 1, 2) and controls after 4-h-treatment with TRAIL (100 ng/ml), ethanol (4%) and their combination, detected by Western blotting. The results are representatives of three independent experiments.

For the experiments studying the effects of ethanol and TRAIL (100 ng/ml) treatment after a short time interval of 4 and 24 h, the concentration of 4% ethanol was selected. Detecting several apoptotic parameters (nuclear morphology, caspase-3 activation and cleavage of its substrate PARP), we demonstrated a potentiation of TRAIL-induced apoptosis of HT-29 cells by ethanol. The mechanisms involved in this strong cooperative effect were therefore the subject of our study.

Cross-linking of DR4 and DR5 results in the recruitment of an adaptor Fas-associated death domain protein (FADD) and pro-caspase-8 to the receptor forming the DISC [7,8]. Activation of caspase-8 at the DISC can lead to two signals: (i) a mitochondria-independent signal that activates caspases downstream of caspase-8 and (ii) a mitochondria-dependent caspase activation following caspase-8 activation and Bid cleavage [12]. The truncated Bid was described to translocate to mitochondria [13] and trigger cytochrome *c* release, which can be accompanied by a loss of the MMP and activation of caspase-9 [14]. In our experiments, TRAIL alone induced only a partial caspase-8 and -9 activation, Bid cleavage and decrease in MMP in HT-29 cells. However, all these effects were significantly potentiated by its combination with ethanol.

Bid cooperates with other members of the Bcl-2 family [15]. We proved that HT-29 cells do not express any detectable level of Bcl-2 protein. No significant changes in Bax or Bak protein levels were apparent after any type of the treatment. Interestingly, a strong upregulation of an antiapoptotic Mcl-1 protein was demonstrated in TRAIL-treated HT-29 cells. It was reported that Mcl-1 can provide an initial viability – enhancing or death-delaying response and may allow time for the cells to make a decision either to undergo apoptosis or to prevent cell death [16]. Increased expression of Mcl-1 was also responsible for the blockage of TRAIL-induced apoptosis mediated by EGF/ErbB1 signalling pathway in epithelial cells [17]. Complete disappearance of Mcl-1 after ethanol and/or combined ethanol and TRAIL treatment suggests possible connection of this protein with the sensitivity of HT-29 cells to apoptosis.

Generation of free radicals was shown to be associated with the induction of apoptosis by both TRAIL [18] and ethanol [4] in several cell lines. No significant changes in ROS production by colon cancer cells in our experiments suggest that the ROS generation (at least hydrogen peroxide production) may not be directly involved in the effects observed.

The TRAIL signalling pathway can be regulated in a cell type-dependent manner at different levels. In our experiments, significant changes at the level of mitochondria were demonstrated concerning MMP, caspase-9 activation, and Bid cleavage. Using stable Bcl-2-HT-29 transfectants prepared in our laboratory, the effect of Bcl-2 protein, which is supposed to block the mitochondrial pathway [19], was investigated. As no significant differences in the response of Bcl-2-transfected and non-transfected cells with respect to the presented apoptotic parameters were demonstrated after TRAIL and ethanol treatment, we concluded that mitochondria do not play a crucial role in the potentiation of TRAIL-induced apoptosis by ethanol.

To examine the possibility that non-caspase proteases may be involved in the potentiation of TRAIL-induced apoptosis, the expression of calpain I in HT-29 cells was studied. Although calpain I-mediated Bid cleavage was demonstrated to promote cell death in various cell systems [20], no significant changes in 80 kDa calpain protein subunit level were observed after any type of the treatment in our experiments. Thus, we do not suppose the role of this non-caspase protease in our model.

As further mechanisms, we suggest that ethanol may interfere with some pro-survival signalling pathways that have been implicated in TRAIL resistance. In addition to the induction of apoptosis, TRAIL may activate other intracellular signalling pathways such as the transcription factor NF-κB [21] and members of the mitogen-activated protein kinase (MAPK) family [22]. NF-κB was shown to regulate the expression of several antiapoptotic proteins such as IAPs, Bcl-2 family members or FLIP [23,24]. The absence of modulation of the steady $cFLIP_L$ level by any type of treatment in HT-29 cells implies that the downregulation of this protein, which is responsible for caspase-8 inhibition directly at the receptor level [25], is not involved in ethanol-mediated sensitization of the cells to TRAIL-induced apoptosis.

As potentiation of caspase-8 activation was demonstrated after TRAIL and ethanol treatment, we suggest the existence of another ethanol-dependent mechanism responsible for regulation of this apical caspase activity. Among the MAPKs, ERK1/2 appears to be of particular importance in that it protects cells against apoptosis induced by a variety of stimuli including death ligands [26]. Inhibition of ERK1/2 was shown to enhance the sensitivity of HeLa [22] and Jurkat [27] cells to TRAIL-induced apoptosis by influencing events at or above the level of caspase-8, whereas in pancreatic cell carcinoma, downregulation of Bcl-2, Bel-XL, and Mcl-1 was observed [28]. Since inhibition of ERK1/2 increased the apoptotic effects of TRAIL in HT-29 cells (our unpublished results), a possible role of this pathway in our experiments is also suggested.

Taken together, ethanol was shown to significantly modulate the TRAIL effects on HT-29 human colon cancer cells. We demonstrated a significant potentiation of pro-apoptotic Bid protein cleavage, caspase-8, -9, and -3 activation, a decrease of MMP, a complete PARP cleavage and a disappearance of Mcl-1 protein after combined TRAIL and ethanol treatment. No significant changes of $cFLIP_L$, Bax or Bak level, and ROS production were detected. Mitochondria do not seem to play a crucial role in the potentiation of TRAIL-induced apoptosis by ethanol. To conclude, ethanol acts as a potent agent sensitizing colon cancer cells to death ligand-induced apoptosis.

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