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Sensitization of mesothelioma to TRAIL apoptosis by inhibition of histone deacetylase: role of Bcl-x_L down-regulation

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Abstract

The TNF-related apoptosis-inducing ligand (TRAIL) is an immunological inducer of apoptosis selectively killing many, but not all, cancer cells. Malignant mesothelioma (MM) is fatal neoplasia with no current treatment, most likely due to high resistance of MM cells towards inducers of apoptosis, including TRAIL. We studied whether inhibition of histone deacetylase (HDAC), recently shown to sensitize malignant cells to a variety of apoptogenic substances, renders MM cells susceptible to TRAIL. Indeed, sub-apoptotic doses of the HDAC inhibitor suberohydroxamic acid (SBHA) sensitized MM cells to TRAIL apoptosis. Of the apoptotic mediators tested, the anti-apoptotic protein Bcl-x_L was strongly down-regulated by combined treatment of the cells with SBHA and TRAIL but not by the HDAC inhibitor alone, while little or no change in the expression of other Bcl-2 family members highly expressed in MM cells, including Mcl-1 and Bax, was observed. Our data suggest a cross-talk between HDAC inhibition and TRAIL that results in modulation of expression of specific apoptotic mediators, and point to the potential of their combinatorial use in treatment of TRAIL-resistant neoplastic disease.

Keywords: TRAIL; Apoptosis; Sensitization; Histone deacetylase inhibitor

Malignant mesothelioma (MM) is a fatal neoplastic disease with no current cure and poor prognosis [1,2]. It is typically associated with occupational asbestos exposure [1]. A complicating factor in MM is the frequent occurrence of SV40 genes in the cells that override the natural defense mechanisms, including execution of the cell cycle arrest and induction of the apoptosis program [3]. For these reasons, MM cells are highly malignant and, generally, resistant to apoptosis. Moreover, normal mesothelial cells have been reported to be more susceptible to inducers of apoptosis compared to their malignant counterparts [4,5].

MM cells are also resistant to the immunological apoptogen of the TNF family, the TNF-related apoptosis-inducing ligand (TRAIL) [6]. It is thus important to understand the mode of resistance of MM cells to TRAIL and find ways in which this can be bypassed, such as by combinatorial treatment with apoptogens that may use different modes of action and/or overcome the block in TRAIL-specific signaling. TRAIL triggers apoptosis by interacting with two cognate death receptors, DR4 and DR5 that, in turn, recruit pro-caspase-8 to the associated intracellular death domains. The activated caspase-8 then relays the signal down-stream, either directly to the effector caspases and/or indirectly via mitochondria [7]. The indirect pathway requires formation of the death-inducing signaling complex associated with the DR4/DR5 death domain, followed by Bid cleavage with ensuing activation of mitochondrial pro-apoptotic events [8].

Resistance to apoptosis of MM cells is most likely governed by multiple mechanisms. It can be expected that both the proximal (receptor-dependent) and distal (mitochondrial) pathways are impaired in MM cells. This includes low expression of death receptors and/or high expression of decoy receptors [9] and over-expression of the anti-apoptotic mitochondrial proteins, including Bcl-x_L or Mlc-1 [10]. High expression of the caspase agonists, members of the inhibitor of apoptosis

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protein family, may inhibit down-stream signaling following activation of both the proximal and distal pathways [11].

A number of chemical agents have been shown to sensitize cancer cells to TRAIL-induced apoptosis, although only scarce data are available in case of MM cells [12]. Recent focus has been given to inhibitors of histone deacetylase (HDAC). These agents block the DNA methylation–demethylation cycle and, therefore, efficiently arrest the cell cycle progression [13]. Their action impairs expression of a number of proteins, some of which are implicated in regulation of apoptosis [14]. Here we tested whether inhibition of HDAC might sensitize MM cells to apoptosis induction by TRAIL. We report that sub-apoptotic levels of an HDAC inhibitor, suberohydroxamic acid (SBHA), rendered MM cells susceptible to apoptosis when the cells were simultaneously treated with SBHA and TRAIL, a process likely involving a cross-talk between the proximal and distal pathways.

Materials and methods

Cell culture and treatment. The MM-BI (sarcomatose) and Ist-Mes2 (epithelioid) human MM cell lines were used [15]. The cells were cultured in the DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. Cells were treated with human recombinant TNF-related apoptosis-inducing ligand (hrTRAIL) prepared as described elsewhere [16,17] at 30 ng/ml and the histone deacetylase (HDAC) inhibitor suberohydroxamic acid (SBHA; Aldrich) at 10–100 μM. SBHA was dissolved in DMSO and added to cells at $\leqslant$0.1% (v/v) of DMSO.

Apoptosis detection. Apoptosis was quantified using the annexin V–FITC method, which detects phosphatidylserine externalized in the early phases of apoptosis [18]. Briefly, cells were plated at 10^5 per well in 24-well plates, and after an overnight incubation, treated with hrTRAIL or SBHA alone or in combination. Floating and attached cells were combined, washed twice with PBS, resuspended in 0.1 ml binding buffer (10 mM Hepes, 140 mM NaCl, and 5 mM $CaCl_2$, pH 7.4), incubated for 20 min at room temperature with 2 μl annexin V–FITC (Pharmingen), supplemented with 10 μl propidium iodide (PI) (10 μg/ml), and analyzed by flow cytometry (FACScalibur, Becton–Dickinson), using channel 1 for annexin V–FITC binding and channel 2 for PI staining. Cell death was quantified as the percentage of cells with high annexin V binding.

Assessment of protein expression. Cells were treated as indicated and harvested by trypsinization. Following fixation with 2% formalin in PBS and permeabilization with 0.2% saponin in PBS containing 2% FBS (both at room temperature for 1 h), the cells were reacted with the primary antibody against Bcl-2, Bcl-x_L, Mcl-1, Bax, Bak, Bad, and Bik, followed by incubation with a secondary antibody conjugated to FITC (both at room temperature, 1 h). The level of protein expression was estimated by analyzing the cells in a flow cytometer and expressed as mean fluorescence intensity (MFI) following subtraction of non-specific background fluorescence estimated in cells reacted with irrelevant antibody in place of the primary antibody.

Caspase-3 activation assay. Activation of caspase-3 was assessed using a specific antibody recognizing the activated, cleaved form of the protease. In brief, cells were harvested, fixed, and permeabilized (see above), after which they were incubated with a specific anti-caspase-3 antibody (PharMingen), followed by treatment with a secondary, FITC-conjugated antibody. The population of cells with activated caspase-3 was determined on the basis of high green fluorescence, as estimated using a flow cytometer.

Transfections. The plasmids used for transfections harbored wild-type Bcl-x_L fused to EGFP and its EGFP-fused mutant lacking the mitochondria-docking C-terminus (ΔBcl-x_L) [19]. Briefly, cells were seeded in 6-well plates and left to reach 50–60% confluency. After this, the cells were washed and incubated with 1 μg plasmid DNA per well pre-mixed with Lipofectamine-2000 (Invitrogen) and combined with 0.5 ml OptiMEM (Invitrogen) per well. After 2–3 h, cells were washed and incubated in complete DMEM for 24 h, after which they were incubated in a selection medium, i.e., complete DMEM supplemented with 1 μg/ml G418 (Sigma). After five passages, the cells were considered stably transfected. The efficacy of over-expression of the Bcl-x_L and Δbcl-x_L genes was assessed by inspecting the cells in a fluorescence microscope and scoring them for green fluorescence. Typically, almost 100% of the cells revealed green fluorescence, which showed punctate (mitochondrial) pattern in case of Bcl-x_L and diffuse (cytosolic) staining in case of Δbcl-x_L.

siRNA treatment. RNA oligonucleotides with 3′-dTdT overhangs specific for caspase-8 [20] and caspase-9 [21] were synthesized by Proligo. A control, scrambled siRNA was used as a non-specific control. Transfections with individual siRNAs were performed using Oligofectamine (Invitrogen) and OptiMEM as suggested by the producer. In brief, cells were seeded in 12-well plates in complete DMEM and allowed to reach 50–60% confluency, after which they were transfected with 60 pmol siRNA per well. Fourty-eight hours later, the cells were assessed by flow cytometry for the level of expression of (pro)-caspase-8 and -9 using antibodies anti-caspase-8 and anti-caspase-9 IgG (Santa Cruz), and used in experiments.

Fluorescence microscopy. MM–BI cells stably transfected with Bcl-x_L-EGFP or ΔBcl-x_L-EGFP were grown on cover slips to near confluence. The cells were mounted in Vectashield containing DAPI (Vector Laboratories) and inspected in the Leica DMIR fluorescence microscope.

Statistical analysis. All experiments were conducted at least three times and data are shown as means ± SD. Statistical significance was calculated using Student's t test and data were considered significantly different when $p < 0.05$.

Results and discussion

Malignant mesothelioma (MM) cells are generally resistant to inducers of apoptosis because of as yet not well-understood mechanism(s). This includes low susceptibility to the immunological inducer TRAIL that, due to its selectivity for cancer cells, holds substantial promise as a potential clinically relevant anti-cancer agent. Therefore, sensitization of MM cells to TRAIL by other agents is of great interest. Thus far, MM cells have been reported to show synergism between TRAIL and certain chemical inducers of apoptosis that activate the distal (mitochondrial) apoptotic pathways and/or upregulate the cognate death receptors.

Recently, focus has been given to inhibitors of HDAC, since these agents are potent inducers of apoptosis of transformed cells via their effects on the DNA methylation–demethylation cycle [13,22]. A recent communication reported on a cooperative pro-apoptotic activity between HDAC inhibitors and TRAIL, although the mechanism has not been elucidated [23].

We therefore asked whether HDAC inhibitors might also synergize with TRAIL in killing of MM cells and, if so, what the molecular basis of this process might be.

Fig. 1 shows a cooperative effect between the HDAC inhibitor SBAH and TRAIL in apoptosis induction in two phenotypically different MM cells. While TRAIL at a pharmacologically relevant dose of 30 ng/ml caused only ~15% apoptosis, its combination with sub-apoptotic levels of SBHA significantly increased the extent of cell death. Thus, for both cell lines, apoptosis extent was significantly higher compared to TRAIL only when TRAIL was combined with 20 μM SBHA (itself causing only 10–15% apoptosis), resulting in 45–50% cell death.

Previous reports suggest that TRAIL induces apoptosis in MM cells via the mitochondrial pathways, suggesting that MM cells belong to the 'type II' cells [24]. Therefore, high level of expression of the apoptosis-modulatory mitochondrial proteins may be responsible, at least partially, for the resistance of the MM cells to TRAIL. We report here that both MM–BI and Ist-Mes2 cells expressed high levels of the mitochondrial anti-apoptotic proteins Bcl-x_L (mean fluorescence intensity, MFI, 152 ± 25 and 106 ± 21, respectively) and Mcl-2 (325 ± 68 and 341 ± 52, respectively), and of the pro-apoptotic protein Bax (595 ± 101 and 504 ± 62, respectively). Expression of several other Bcl-2 family proteins, including that of Bcl-2, Bak, Bad, and Bik was relatively low, i.e., with MFI insignificantly different from non-specific MFI (data not shown).

It has been shown that cells can be sensitized to TRAIL by down-regulation of the anti-apoptotic and/or up-regulation of the pro-apoptotic Bcl-2 family proteins. We therefore studied the effect of SBHA on the candidates of modulation of susceptibility of MM cells to TRAIL, i.e., Bcl-x_L, Mcl-1, and Bax. Fig. 2 reveals that SBHA alone, up to 50 μM, had little effect on the expression of the proteins, perhaps with some effect, albeit mild, on Bax. However, combination of SBHA with TRAIL showed an early and significant down-regulation

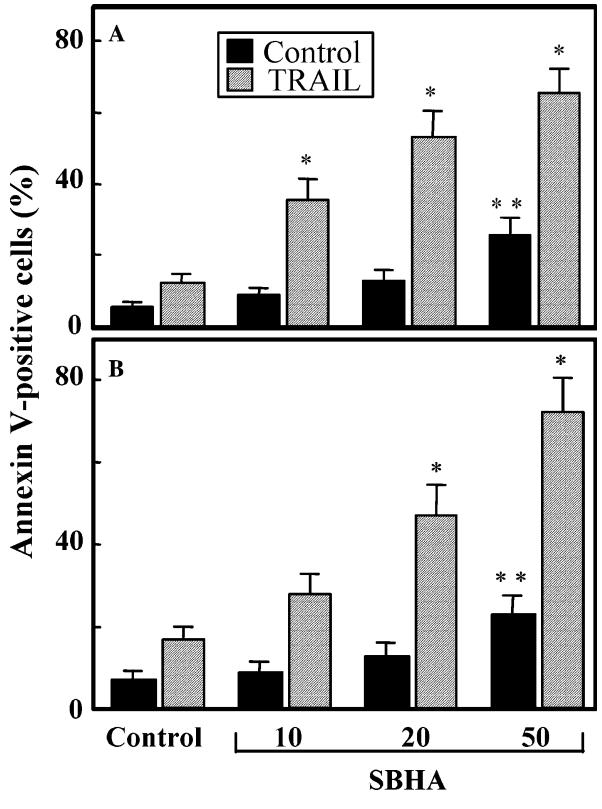

Fig. 1. SBHA sensitizes MM cells to TRAIL-induced apoptosis. MM–BI (A) and Ist-Mes2 cells (B) were plated at 60–70% confluency and treated with hrTRAIL (30 ng/ml) and/or SBHA at 10, 20 or 50 μM. After 24 h, the cells were harvested and apoptosis was evaluated using the annexin V-binding method. The symbol "*" denotes significant difference of cells treated with TRAIL in combination with SBHA and those treated with TRAIL alone, while the symbol "**" denotes significant difference for cells treated with SBHA only and the control cells.

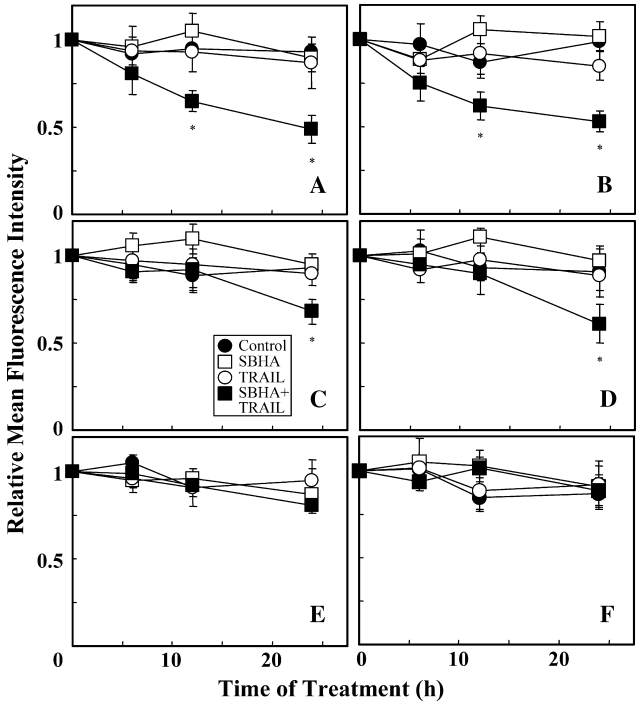

Fig. 2. Treatment of MM cells with TRAIL and SBHA leads to early decrease in Bcl-x_L. MM-BI (A,C,E) and Ist-Mes2 cells (B,D,F) were seeded and allowed to reach 60–70% confluency. The cells were then treated with 30 ng/ml hrTRAIL and/or SBHA at 20 μM for 6, 12, and 24 h. The cells were then harvested, fixed, and permeabilized, after which they were reacted with anti-Bcl-x_L IgG (A,B), anti-mcl-1 IgG (C,D), and anti-Bax IgG (E,F) as indicated, followed by a secondary antibody conjugated to FITC. Fluorescence was assessed by FACS analysis. The data are presented as relative mean fluorescence intensity after subtraction of MFI of the background fluorescence (non-specific fluorescence of cells reacted with isotype IgG instead of the specific primary IgG). The symbol "*" denotes significant difference of cells treated with TRAIL in combination with SBHA and the other treatments.

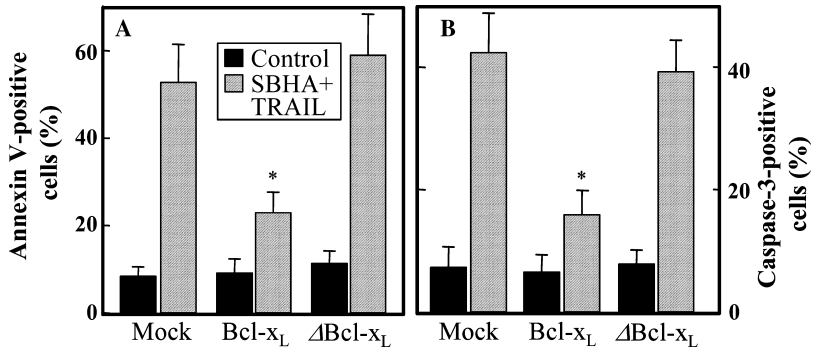

Fig. 3. Over-expression of Bcl-x_L confers resistance to TRAIL. Stable MM–BI cells selected for transfection with the empty plasmid (mock transfection), wild-type Bcl-x_L or ΔBcl-x_L were seeded and allowed to reach 60–70% confluency. The cells were then treated for 24 h with 30 ng/ml hrTRAIL in the presence of 20 μM SBHA. Apoptosis was assessed by the annexin V-binding method. Activation of caspase-3 was estimated by FACS analysis of cells reacted with antibody specifically recognizing the activated form of caspase-3. The symbol "*" denotes significant difference of cells overexpressing Bcl-x_L and mock-transfected cells, treated with TRAIL in combination with SBHA.

of Bcl-x_L expression, while the drop in the level of Mcl-1 in cells co-treated with the two agents was delayed. The extent of the Bcl-x_L protein level decrease was proportional to SBHA concentration. TRAIL alone exerted modest and non-significant effect on Bcl-x_L. Thus, SBHA at 20 μM together with TRAIL increased the ratio of relative protein expression of Bcl-x_L and Bax in early periods of treatment, while the change in the ratio of Mcl-1 and Bax increased later. These data suggest that down-regulation of Bcl-x_L in MM cells by combined treatment with TRAIL and SBHA sensitizes these resistant cells to the immunological apoptogen.

To get a direct evidence that Bcl-x_L regulates susceptibility to apoptosis in MM cells exposed to TRAIL and SBHA, we stably transfected MM–BI cells with a wild-type and mutant Bcl-x_L (ΔBcl-x_L). While over-expression of wild-type Bcl-x_L has been shown to be anti-apoptotic, the mutant Bcl-x_L, lacking the mitochondria-docking COOH terminus [19], did not offer protection in another apoptosis model [25]. Consistent with this report, Fig. 3 reveals that over-expression of Bcl-x_L but not that of ΔBcl-x_L suppressed apoptosis induced in MM–BI cells exposed to TRAIL in the presence of sub-apoptotic concentration of SBHA.

The data above suggest a cross-talk between the proximal (receptor) and distal (mitochondrial) pathways in induction of apoptosis by combinatorial treatment of MM cells with TRAIL and SBHA. To confirm this link, we knocked down caspase-8 or caspase-9 in MM–BI cells by transfection with caspase-8 or caspase-9 siRNA, respectively, and exposed the cells to the inducers. Fig. 4 shows that blocking the expression of either of the two genes antagonized induction of apoptosis by combinatorial treatment with TRAIL and SBHA, confirming a causal relationship between the two major apoptosis signaling pathways in this model.

In this report, we present data documenting sensitization of MM cells to apoptosis by co-treatment

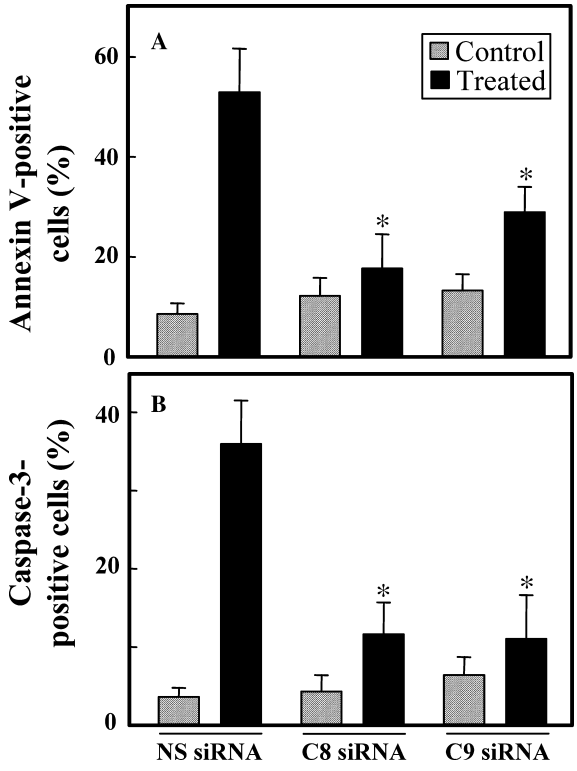

Fig. 4. Receptor and mitochondrial signaling pathways are linked in sensitization of MM cells to TRAIL by SBHA. MM-BI cells were caspase-8 siRNA, caspase-9 siRNA, or non-silencing (NS) siRNA. After 48 h, the cells were exposed to hrTRAIL at 30 ng/ml plus 20 μM SBHA, and the level of apoptosis (annexin V binding) (A) and caspase-3 activation (B) were assessed. The symbol "*" denotes significant difference of cells pre-incubated with caspase-8 siRNA or caspase-9 siRNA, and those pre-incubated with NS siRNA and treated with TRAIL in combination with SBHA.

with SBHA and TRAIL. Our focus was on the possible role of the Bcl-2 family proteins in resistance of MM cells to TRAIL, since TRAIL signals cell death

through both receptor and mitochondrial routes and since proteins like Bcl-x_L and Mcl-1 are elevated in human mesotheliomas [10]. Several reports have suggested that down-regulation of Bcl-2 and Bcl-x_L by anti-sense treatment sensitized MM cells to chemotherapy [26]. Moreover, virus-mediated transfection of MM cells with pro-apoptotic Bcl-2 family proteins, such as Bak, was itself pro-apoptotic [27]. Finally, induction of apoptotic pathways by chemotherapeutic agents was shown to sensitize the cells to TRAIL [6]. We have observed a similar effect in case of the apoptogenic vitamin E analogue α-tocopheryl succinate (unpublished data).

Our results reported here differ from those of Cao et al. [28], who observed a significant decrease in the level of the Bcl-x_L protein in MM cells exposed to apoptogenic levels of the HDAC inhibitor sodium butyrate. They also show that down-regulation of Bcl-x_L occurs at the time when elevated apoptosis was observed, and that clones with higher level of Bcl-x_L expression were more resistant to sodium butyrate. In our experiments, expression of the protein was decreased when the cells were co-treated with SBHA and TRAIL; SBHA alone did not modulate the expression of Bcl-x_L. Moreover, the protein was down-regulated at sub-apoptotic concentrations of SBHA and at times preceding the onset of apoptosis assessed by phosphatidylserine externalization. In agreement with the report of Cao et al. [28], we also observed protection from apoptosis in MM cells over-expressing Bcl-x_L, but not in cells transfected with its mutant lacking the mitochondrial docking sequence.

Our data suggest that co-treatment of MM cells with TRAIL and SBHA facilitates the mitochondrial apoptotic pathways, as can be inferred from the role in the process of modulation of Bcl-x_L expression. Therefore, there is apparently a cross-talk between the receptor- and mitochondria-mediated signaling pathways, as suggested by the caspase-8 and caspase-9 knock-down studies. These data are consistent with recent reports showing that HDAC inhibitors induce apoptosis by stimulating the intrinsic, mitochondrial-dependent signaling [14], and further stress the potential of synergistic effect of TRAIL with chemical inducers of apoptosis [29–31]. We conclude that our results strengthen the emerging paradigm of potential combinatorial treatment of TRAIL-resistant cancers, such as are the fatal human malignant mesotheliomas.

Acknowledgments

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