The prognosis for patients with chemo-refractory rhabdomyosarcoma remains poor. The tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a hopeful candidate for new strategies in chemotherapy. The effects of TRAIL and melphalan (Mel) in the rhabdomyosarcoma cell line TE-671 were investigated by colorimetric caspase assays and flow cytometry. TRAIL induced the activation of caspases-2, -3 and -8, but not the activation of caspase-9, in the Mel-resistant TE-671 cells. Inhibition of caspase-2 with the caspase-2 inhibitor z-VDVAD-fmk significantly down-regulated the TRAIL-induced caspase-3 activation, as well as the TRAIL-induced cytotoxicity. When TE-671 cells were treated with a combination of Mel and TRAIL, a significant synergism of drug-induced cytotoxicity was obtained. The inhibition of caspase-2 could completely abolish caspase-3 activation, suggesting that TRAIL sensitises TE-671 cells for Mel-induced cytotoxicity via a caspase-2- and -3-dependent mechanism. In conclusion, it was shown, for the first time, that TRAIL could sensitis Mel-resistant tumour cells to melphalan.

Rhabdomyosarcoma is the most frequent soft-tissue sarcoma in children. Despite intensive therapy, including surgery and high-dose chemotherapy combined with radiotherapy, the outcome of patients with chemotherapy-refractory disease remains poor (1, 2).

It has been shown that the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in various malignant cells, in both in vitro and in vivo tumour models (3-6). TRAIL seems to be selectively toxic for tumour cells but not for the majority of non-malignant human cells (7-9) or TRAIL-treated animals (10).

Because of its tumour-selective effect, TRAIL is a possible candidate for new strategies in chemotherapy.

TRAIL is a member of the tumour necrosis factor super family and induces apoptosis via the death receptor extrinsic apoptosis pathway (11). TRAIL interacts with a complex system of cell surface receptors, including TRAIL-R1 and TRAIL-R2. These signal-transducing receptors transmit the pro-apoptotic signal by formation of the death-inducing signal complex (DISC), which comprises of the Fas-associated death domain (FADD) and pro-caspase-8, resulting in caspase-8 activation (12). Caspase-8 can activate caspase-3 directly (13) or via Bid cleavage, providing a truncated Bid (tBid) fragment, which promotes the mitochondrial pro-apoptotic factors cytochrome c and Smac (14). In TRAIL-induced apoptosis, caspase-2 seems to be involved in caspases-8- and -3-mediated pathways (15, 16).

The different pathways involved in TRAIL-mediated apoptosis might explain how TRAIL can amplify the effect of chemotherapeutic drugs. However, it has not yet been shown that TRAIL can overcome drug resistance and sensitis the same cells to this drug. In this report, the modulating effect of TRAIL on melphalan resistance in a rhabdomyosarcoma cell line was analysed.

Materials and Methods

Cell line. The human rhabdomyosarcoma cell line TE-671 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cell line was derived from the cerebellar tumour of a 6-year-old Caucasian female in 1977 (17), and was later identified as a rhabdomyosarcoma (18, 19). The TE-671 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (PAA Laboratories, Cölbe, Germany), containing 15% heat-inactivated foetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin (all additives from Biochrom Seromed, Berlin, Germany). The TE-671 cells were cultured in a humidified atmosphere at 37°C and 5% CO2.

Drug. Melphalan (Mel) was provided as a stock solution by the central pharmacy of the University of Leipzig Medical Centre, Germany. Aliquots were prepared and stored until use at –20°C.

Determination of apoptosis. Apoptosis and cell death were quantified by using an Annexin V-FITC-staining kit (Immunotech,
Coulter Company, Marseilles, France) and flow cytometry. The TE-671 cells were plated at a density of 10⁴/well in 24-well plates. After stimulation with the corresponding amounts of TRAIL (Alexis, Grünberg, Germany) or Mel for 24 h, the cells were harvested using trypsin-EDTA (Biochrom Seromed, Berlin, Germany). For stimulation with TRAIL, 1 µg/ml of ligand enhancer and various concentrations of TRAIL (Alexis) were used. The cells were stained with Annexin V-FITC (AV, 25 µg/ml) and propidium iodide (PI, 250 µg/ml). The fraction of vital cells, i.e., AV- and PI-negative cells, were determined by flow cytometry, as described in detail previously (20).

Caspase inhibitor assays. The general caspase inhibitor z-VAD-fmk and the caspase-2 inhibitor z-VDVAD-fmk (R&D Systems, Wiesbaden, Germany) were both used at a concentration of 100 µM. The cells were pre-incubated with the respective caspase inhibitor or an equal amount of DMSO, as a solvent control, for 1 h. Next, the cultures were stimulated with or without Mel and/or TRAIL/enhancer. AV/PI staining was carried out 24 h after addition of the drug. The calculations were carried out considering the following definitions: Toxicity = 100% - vital cells (AV-negative/PI-negative); Total specific cytotoxicity = toxicity in the presence of Mel or TRAIL and DMSO-toxicity in the presence of the medium and DMSO; Specific caspase-independent cytotoxicity = toxicity in the presence of Mel or TRAIL and inhibitor-toxicity in the presence of the medium and the inhibitor; Specific caspase-dependent cytotoxicity = total specific cytotoxicity – specific caspase-independent cytotoxicity.

Caspase assays. For the analysis of caspases-2, -3, -8 and -9 activities, colorimetric caspase assay kits (Biocat, Heidelberg, Germany) were used. The cells were stimulated with Mel or TRAIL between 3 and 12 h. Afterwards, cellular protein was isolated by lysis of the cells, according to the manufacturer’s protocol. Protein was quantified by the BioRad protein assay (BioRad, CA, USA). The determination of caspase-3 and -8 activities was carried out with 50 µg of protein.

For caspases-2 and-9, 100 µg of protein were used. To analyse caspase-3 or -8 activities after caspase-2 inhibition, the cells were pre-treated with the caspase-2 inhibitor z-VDVAD-fmk, or equal amounts of DMSO as the solvent control, for 1 h before stimulation with the TRAIL/enhancer or Mel or both. After 6 h, the protein was isolated and the caspase activities were determined, as mentioned above.

Statistical analysis. For statistical analysis, the Student’s t-test for paired samples was used.

Results

Dose-dependent cytotoxicity induced by Mel and TRAIL in TE-671 cells. Mel induced cell death in the rhabdomyosarcoma cell line TE-671 in a dose-dependent manner (Figure 1 A). However, at concentrations below 50 µg/ml, Mel exhibited only marginal cytotoxic effects on TE-671 cells. Thus, the TE-671 cells appeared to be resistant to Mel treatment. In contrast, TRAIL induced a dose-dependent and significant cytotoxic effect, even at low concentrations (Figure 1 B).

Role of caspase activation in Mel- and TRAIL-induced cytotoxicity. The activation of caspases-2, -3, -8 and -9 was analysed after stimulation of the cells with TRAIL or Mel for various time-intervals (3, 6, 12 h). After 6 h of stimulation, TRAIL significantly induced the activation of caspases-2, -3 and -8 in TE-671 cells, whereas caspase-9 activation was not significantly induced (Figure 2). After 3 h of stimulation, TRAIL induced significant caspase-3 activation, while caspases-2, -8 and -9 were not significantly stimulated. After 12 h, no further significant
TRAIL-induced caspase stimulation was observed (data not shown). In contrast to TRAIL, Mel did not induce any significant caspases-2, -3, -8 or -9 activation during the 3- to 12-h incubation period.

To further characterise the selective resistance of TE-671 cells to Mel, whether the cytotoxicity induced by Mel or TRAIL was dependent on caspase activation was analysed. The cytotoxicity induced by TRAIL was observed to be completely caspase-dependent, whereas Mel at high concentrations (100 μg/ml) induced cytotoxicity almost completely by caspase-independent mechanisms (Figure 3). At low concentrations (10 μg/ml), Mel exhibited no relevant cytotoxic effect.

The selective inhibition of caspase-2 almost completely suppressed TRAIL-induced cytotoxicity and caspase-3 activation (Figure 4). In addition, under these conditions, the TRAIL-induced activation of caspase-2 was also significantly reduced (Figure 4 B).

**Synergistic effects of melphalan and TRAIL.** We subsequently investigated whether TRAIL could sensitize TE-671 cells for Mel treatment. The cells were thus stimulated with low concentrations of TRAIL (10 ng/ml plus enhancer) and Mel (10 μg/ml). The combination of TRAIL and Mel, used at suboptimal concentrations, revealed a significant synergistic effect on cytotoxicity (Figure 5). The inhibition of caspase-2 completely suppressed the caspase-3 activation induced by the combination of Mel and TRAIL (Figure 6 A). However, the cytotoxic effects of Mel and TRAIL were only partially suppressed by caspase-2 inhibition, indicating that the synergistic effects of TRAIL and Mel were mediated by both caspase-2-dependent and -independent pathways (Figure 6 B).
Discussion

In our investigations of chemotherapy resistance mechanisms, we used the Mel-resistant rhabdomyosarcoma cell line TE-671. The plasma concentration of Mel in patients undergoing high-dose chemotherapy ranges between 4 and 11 μg/ml (21). However, the drug showed no remarkable effect on TE-671 cells when used at concentrations up to 10 μg/ml. Hazlehurst and co-workers produced the Mel-resistant multiple myeloma cell line 8226/LR5 for investigations of de novo and acquired Mel resistance (22). The dose-cytotoxicity-curve for Mel showed similar courses in the TE-671 cells and the resistant 8226/LR5 cells. Thus, TE-671 cells appear to be resistant to Mel.

Caspase-3 activation after Mel treatment has been reported in the promyelocytic leukaemia cell line HL60 (23) and in the multiple myeloma cell line 8226 (22). In contrast, in TE-671 cells, Mel did not induce caspase-3 activation. Since TRAIL induced significant activation of caspases-2, -3 and -8 in these cells, the detection of x-linked inhibitor of apoptosis protein (XIAP) by Western blotting in TE-671 cells (data not shown) was not likely to be a relevant cause for Mel resistance in these cells, in contrast to the findings by Fulda et al., who reported that the presence of XIAP is a major cause of drug resistance in neuroblastoma cells (24). Thus, the resistance to Mel was probably caused by an upstream mechanism.

In the TE-671 cells, TRAIL induced not only caspase-3 but also caspase-2 activation. Mel did not activate either caspases-2 or -3 in TE-671 cells. In contrast, in the Mel-sensitive Ewing sarcoma cell line RD-ES, Mel mediated a significant activation of caspase-3 at a low concentration and Mel-induced cytotoxicity was mediated, at least in part, via a caspase-dependent pathway (25). However, at a low concentration, Mel did not significantly stimulate caspase-2 activation (data not shown), thus indicating that Mel and TRAIL activate different patterns of caspases. Since the inhibition of caspase-2 activation suppressed TRAIL-mediated cytotoxicity, the different patterns of caspase
activation mediated by TRAIL or Mel might explain why the TE-671 cells were resistant to Mel but not to TRAIL.

The role of caspase-2 in TRAIL-mediated apoptosis has been controversially discussed. Wagner and co-workers placed caspase-2 in TRAIL-induced apoptosis upstream of Bid cleavage (15) and assumed its function to be an initiator caspases. Other studies detected caspase-2 activation downstream of caspase-3 activation (26, 27), which led to the assumption that caspase-2 might amplify the signal downstream of caspase-3 activation (28, 29). In our study, the inhibition of caspase-2 significantly down-regulated the TRAIL-induced caspase-3 activation, indicating that caspase-2 probably acts as an initiator caspase in these cells.

Keane et al. showed that TRAIL is able to augment the cytotoxic effects of several anticancer drugs, such as doxorubicin, 5-Fluorouracil, paclitaxel and Mel, in malignant and normal breast cell lines (30). When TE-671 cells were treated with a combination of Mel and TRAIL, a significant synergism of drug-induced cytotoxicity was obtained. However, in contrast to the TE-671 cells, the reported breast cancer cell lines were sensitive to the applied drugs. Thus, we report, for the first time, that TRAIL can sensitise cells to chemotherapy, even if the cells are resistant to these drugs alone.

TNFα, another member of the tumour necrosis factor super family, can sensitise malignant cells, which are resistant to TNFα-induced apoptosis, to chemotherapeutic drugs. Schmelz et al. found that this pathway is independent of caspase-8 activation (31). In our study, we showed that TRAIL sensitises TE-671 cells to Mel. However, the inhibition of caspase-2 completely reduced TRAIL-induced caspase-3 activation and TRAIL-induced cytotoxicity. In addition, caspase-2 inhibition completely inhibited the caspase-3 activation induced by the combination of Mel and TRAIL, but the cytotoxic effect was only partially suppressed. These results suggest that the effect of TRAIL alone is totally dependent on caspase-2 activation, whereas the synergism obtained by stimulation of the cells with a combination of Mel and TRAIL is only partly explained by TRAIL-induced caspase-2 activation; the complete answer may involve a, as yet unknown, mechanism which involves neither the activation of caspase-2 nor that of caspase-3.

In conclusion, we showed that TRAIL induced cytotoxicity in the Mel-resistant cell line TE-671 via activation of caspase-2. In addition, TRAIL sensitised TE-671 cells to Mel, partially via a caspase-2-dependent pathway. Thus, the application of TRAIL might become a therapeutic option to overcome drug resistance in rhabdomyosarcoma.

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