Pediatric Rhabdomyosarcoma Cell Lines Are Resistant to TRAIL-induced Apoptosis and Highly Sensitive to Fas-induced Apoptosis

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ABSTRACT

Seven pediatric rhabdomyosarcoma (RMS) cell lines were resistant to the induction of apoptosis via the Fas death receptor. In contrast, four of seven lines (RD, Rh1, Rh18, and Rh30) were highly sensitive to tumor necrosis factor-α-related apoptosis-inducing ligand (TRAIL). TRAIL induced apoptosis within 4 h and also reduced clonogenic survival, both reversible by caspase inhibitors. DR5 (but not DR4) was expressed at high level in all cell lines. Expression of the decoy receptors DcR1 and DcR2 did not correlate with TRAIL sensitivity. All RMS lines expressed the adapter molecule FADD, and six of seven expressed procaspase-8. Expression of the inhibitory proteins c-FLIPL and c-FLIP were high in three TRAIL-sensitive (RD, Rh1, and Rh30) and two TRAIL-resistant (Rh28 and Rh41) lines. All RMS lines expressed BID and procaspases-3, -6, -7, and -9. Procaspases-8 and -10 were highest in TRAIL-sensitive RMS (RD, Rh1, and Rh30), and procaspase-10 was not expressed in Rh18, Rh36, or Rh41. TRAIL induced loss of mitochondrial membrane potential in TRAIL-sensitive Rh1 but not in TRAIL-resistant Rh41 cells. There was no correlation between expression of members of the Bcl-2 family (Bcl-2, Bcl-xL, Bax, and Bak) and TRAIL sensitivity. TRAIL-sensitive Rh18 expressed procaspase-8 in the absence of procaspase-10 and c-FLIP, and procaspase-10 was not detected in TRAIL-resistant Rh41 in the presence of procaspase-8 and c-FLIP. Data suggest that caspase-8 may be sufficient to deliver the TRAIL-induced apoptotic signal in the absence of both caspase-10 and c-FLIP (Rh18) but not in the presence of c-FLIP (Rh41). In RD, Rh1, and Rh30, the presence of c-FLIP may require amplification of the apoptotic signal via caspase-10.

INTRODUCTION

The cell surface receptor Fas (Apo-1; CD95) comprises a type I transmembrane protein belonging to the tumor necrosis factor receptor superfamily of death receptors and is important in regulating apoptosis in cells of the immune system (1). However, Fas is also expressed in other cells and tissues (1, 2) and in neoplastic disease. Fas has demonstrated functional activity in several malignant cell lines derived from adult and pediatric solid tumors (3–13). Upon ligation of Fas, trimerization of the receptor is followed by rapid recruitment of the adapter protein FADD and procaspase-8 to the cytoplasmic portion of the receptor to form a DISC. Subsequent release of active caspase-8 from the DISC initiates a signaling cascade dependent upon activation of downstream caspases including caspase-3 (14) that cleave specific intracellular proteins and initiate apoptosis. The major determinant of Fas sensitivity in colon carcinoma cell lines was determined to be the level of Fas expressed, which varied by >1000-fold (3).

The only primary cells susceptible to apoptosis induction by the cytotoxic ligand TRAIL are activated T lymphocytes, suggesting a role in limiting the immune response (15, 16). In contrast, several tumors of diverse origin are sensitive to TRAIL. The receptors DR4 and DR5 (expressing two alternatively spliced isoforms), similar to Fas, contain death domains and can transmit a cell death signal. In contrast, two additional receptors (decays), DcR1 (lacking a transmembrane or cytoplasmic domain) and DcR2 (containing a truncated death domain), bind the ligand with comparable affinity to DR4 and DR5, cannot transmit a cell death signal, and may protect normal cells from the cytotoxic action of TRAIL (17, 18). In neoplastic disease, DR4 and DR5 are expressed in primary human brain tumors (19), melanomas (20) including those resistant to FasL (21), and colon carcinomas (22). Further, TRAIL has induced apoptosis in malignant glioma cell lines (23), melanoma cell lines (20), and breast and colon carcinoma cell lines (22). After trimerization of the receptor, the pathway by which TRAIL induces apoptosis remains to be clearly defined.

RMS is the most common soft-tissue sarcoma in children

1 The abbreviations used are: FADD, Fas-associated death domain; DISC, death-inducing signaling complex; TRAIL, tumor necrosis factor-α-related apoptosis-inducing ligand; FasL, Fas ligand; RMS, rhabdomyosarcoma; ERMS, embryonal RMS; ARMS, alveolar RMS; MoAb, monoclonal antibody; RT-PCR, reverse transcription-PCR; FACS, fluorescence-activated cell sorter.

Received 3/15/00; revised 8/2/00; accepted 8/2/00.

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1 Supported by NIH Awards PO1 CA 23099, the Cancer Center Support (CORE) Grant CA 21765, and by the American Lebanese Syrian Associated Charities. I. P. was supported in part by a scholarship from the Fulbright Program and by the First Institute of Pathology and Experimental Cancer Research, Semmelweis University of Medicine, Budapest, Hungary.

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under the age of 15, and despite aggressive approaches incorporating surgery, dose-intensive combination chemotherapy, and radiation therapy, the outcome for patients with metastatic disease remains poor (24, 25). Histologically, the vast majority of tumors demonstrate ERMS or ARMS histology, the latter being considerably more refractory to therapy. Hence, coupled with the relative rarity of this tumor in neoplasia, model systems become essential in delineating novel targets that may be important in developing new therapeutic strategies for treatment of RMS. In this study, only two of seven human RMS cell lines were partially sensitive to Fas-mediated apoptosis at high concentrations of anti-Fas (200 ng/ml). In contrast, four of seven RMS lines were highly sensitive to the cytotoxic ligand TRAIL at low concentrations of the cytotoxic ligand (1 ng/ml). TRAIL-sensitive lines were of both embryonal and alveolar histology. Upon examination of potential determinants of Fas sensitivity, levels of Fas expression were low, and expression of c-FLIP, an inhibitory protein that prevents recruitment of procaspase-8 to the DISC (26), was high in five lines. In contrast, all RMS cell lines expressed high levels of the TRAIL receptor DR5, and TRAIL sensitivity was best correlated with the expression of procaspases-8 and -10. Data suggest that caspase-8 may be sufficient to deliver the TRAIL-induced apoptotic signal in the absence of both caspase-10 and c-FLIP, whereas in the presence of c-FLIP, amplification of the apoptotic signal may be required via caspase-10. These models of pediatric RMS may be important in the exploration of determinants of the TRAIL signaling pathway and apoptosis and in elucidating the role of TRAIL in the development of new therapeutic approaches for the treatment of RMS.

MATERIALS AND METHODS

Cell Lines. The ERMS (RD, Rh1, and Rh36) and ARMS (Rh18, Rh28, Rh30, and Rh41) cell lines used in this study were established at St. Jude Children’s Research Hospital with the exception of RD, which was obtained from American Type Culture Collection. Characteristics of the four cell lines have been described (27–29). Rh36 and Rh41 were derived and obtained from Dr. Peter Houghton. RMS cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum (Life Technologies, Inc.).

Growth Inhibition and Clonogenic Assays. For growth inhibition assays, cells were plated at a density of 100,000 cells/well in six-well plates in 2 ml of medium. After overnight attachment, cells were treated for 72 h or 7 days (Rh28) with the cytolytic anti-Fas MoAb CH-11 (50–200 ng/ml; MBL International Corp.) or the cytotoxic ligand TRAIL (Alexis Biochemicals; 0.5–100 ng/ml, in the presence of 0.25 μg/ml of an anti-FLAG enhancer MoAb). At the beginning of and after treatment, cells were enumerated using a Coulter particle counter. Inhibition of cell growth compared with control growth was determined, and the percentage of cells remaining at the end of treatment was also evaluated (% time 0).

For clonogenic assays, Rh1 and RD cell lines were plated at a density of 2000 or 3000 cells/well, respectively, in 2 ml of medium. After overnight attachment, cells were treated with TRAIL (1–100 ng/ml) for 72 h either in the absence or presence of a chimeric fusion protein, DR5-Fc (50 ng/ml; Alexis Biochemicals), that prevents the binding of TRAIL to its receptors. Clonogenic survival was determined after a further 7 days, as described previously (4). Additionally, cells were treated for 16 h with TRAIL (25 ng/ml) either in the absence or in the presence of the caspase inhibitors (10 μM, 20 μM; Enzyme Systems Products). These included Z-VAD-FMK (general inhibitor), Z-DEVD-FMK (inhibitor of caspases 3, 7, 8, 10), Z-IETD-FMK (caspase-8 inhibitor), or a negative control (Z-FA-FMK), with subsequent determination of clonogenic survival as described.

Measurement of Apoptosis. Rh1 and RD cells were plated at a density of 400,000 cells/well in six-well plates. After overnight attachment, cells were treated for periods up to 24 h with CH-11 or TRAIL either in the absence or presence of caspase inhibitors. Both the floating cells and attached cells were pooled after trypsinization, fixed in 70% ethanol, and stored at −20°C prior to analysis. Apoptotic cells were detected as a sub-G1 fraction after propidium iodide staining and analysis using a Becton Dickinson FACScan (30, 31).

RNase Protection Analysis. Total RNA was extracted from 2 × 10⁶ cells in RINazol B (Tel-test) using standard procedures. The RiboQuant Multi-Probe RNase protection assay was performed according to the vendor (PharMingen). Three multiprobe template panels were used: hAPO-3 (procaspase-8, Fas, FADD, FasL, FAF, FAP, TNFR60, TRADD, TRAIL, DR3, and RIP); hAPO-3c (procaspase-8, Fas, FasL, DCr1, DR3, DR4, DR5, TRAIL, TNFR60, and TRADD); and hAPO-1c (procaspases-1 to -10).

RT-PCR. The expression of c-FLIP (c-FLIP_L and c-FLIP_S), procaspase-8, procaspase-10, and the TRAIL decoy receptor DCr2 was also determined by semiquantitative RT-PCR, as described previously (3). Complimentary DNA was synthesized from 2 μg of total RNA in a 20-μl reaction using an oligo(dT) primer and a cDNA cycle kit (Invitrogen). β-actin was used as a control to monitor RT-PCR amplification efficiency and quality of the cDNA from 2 μl of the template at 25 cycles, as reported previously (3). PCR products were separated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining and UV light illumination. The primers used for PCR of procaspase-8 (273 bp) were as described (3) and for procaspase-10 (500 bp), c-FLIP_L (501 bp), c-FLIP_S (302 bp), and DCr2 (369 bp) were as follows: CAPS-10F, 5′-CAT-AGATTGTTCCCCAACA-3′; CAPS-10R, 5′-GGACCTACGGCTTCCC-3′; c-FLIP_L, 5′-CTTGCCCATTTGCCTGTA-3′; c-FLIP_S, 5′-CGAGGCAAGATAAGGCAAGA-3′; c-FLIP_R, 5′-CACAGGAACATTTCATTTCC-3′; and c-FLIP_S, 5′-TTCTCAGTGTGGCCAGGG-3′.

Western Analysis. Gene expression was also examined by Western analysis (14). Primary antibodies used were: procaspase-8 (MBL), procaspase-3 (PharMingen), FADD (Transduction Laboratories), Bid (Santa Cruz Biotechnology), and NFX for the detection of c-FLIP_L and c-FLIP_S was a generous gift from Dr. Peter Krammer (German Cancer Research Center, Heidelberg, Germany). Secondary antibodies were horseradish peroxidase-conjugated goat antimouse IgG1 and rabbit antigoat IgG from Southern Biotechnology and sheep-antimouse immunoglobulin and donkey antirabbit immunoglobulin from Amer...
Sham. Signal was detected using the ECL Western blotting system (14).

Flow Cytometric Analysis. The specific binding of TRAIL to cell surface receptors was determined in TRAIL-sensitive RD and TRAIL-resistant Rh36 cell lines by measuring the binding of TRAIL in the presence of an anti-FLAG enhancer mAb and secondary antimouse-IgG1 FITC Ab using flow cytometric analysis as described previously (20).

Measurement of Mitochondrial Membrane Potential (ΔΨm). Rh1 and Rh41 cell lines were plated at a density of 2 × 10⁵ cells/well in six-well plates. After overnight attachment, cells were treated, in duplicate, with 50 ng/ml TRAIL (Alexis) and 500 ng/ml enhancer (Alexis) for 16 h. At the end of incubation, both the floating cells and attached cells were pooled after trypsinization. Subsequently, the cells were incubated with the dye JC-1 (Alexis Biochemicals; 10 μg/ml) in 1 ml of medium for 10 min at 37°C, washed in PBS, and analyzed by flow cytometry (FACS-Scan; Becton Dickinson); 10,000 cells were subsequently collected and analyzed by CellQuest Software as described previously (32–34).

RESULTS

Sensitivity of RMS Cell Lines to Anti-Fas. Sensitivity of the seven RMS cell lines to the cytolytic anti-Fas MoAb CH-11 is shown in Fig. 1. Cells were exposed to anti-Fas at concentrations from 50–200 ng/ml, and data derived at the highest concentration are shown. All cell lines were relatively resistant to induction of apoptosis via Fas. Anti-Fas induced partial growth inhibition in Rh1 and Rh18 after 72 h exposure. This was related to the induction of apoptosis in a fraction of the two cell populations (30%) within 24 h (data not shown). However, the overall effect was growth inhibitory only and did not result in loss of cell numbers at the end of anti-Fas exposure because the surviving cells repopulated (Fig. 1), in contrast to 100% cell kill determined in Fas-sensitive GC/c1 human colon carcinoma cells (3).

Sensitivity of RMS Cell Lines to TRAIL. In contrast, the exquisite sensitivity of RMS cell lines to varied concentrations of TRAIL is demonstrated in Fig. 2. Growth inhibition was determined in RD, Rh1, Rh18, and Rh30, whereas Rh28, Rh36, and Rh41 were resistant to TRAIL. In the four TRAIL-sensitive cell lines, all demonstrated fewer surviving cells at the end of 72 h in comparison with the cell numbers at the start of treatment, indicating that TRAIL was cytotoxic. The exquisite sensitivity of the four cell lines to TRAIL was evident from effects demonstrated at concentrations of ≤1 ng/ml. In TRAIL-sensitive RD and Rh1 cell lines, the effect of 72 h exposure to varied concentrations of TRAIL on clonogenic survival was deter-
mined either in the absence or presence of the chimeric fusion protein DR5-Fc (50 ng/ml; Fig. 3). Loss in clonogenic survival of 90% was demonstrated at 6 ng/ml TRAIL, and significant protection from TRAIL-induced cytotoxicity was demonstrated after coincubation with DR5-Fc.

TRAIL-induced Apoptosis and Effect of Caspase Inhibitors. The time course for induction of apoptosis in Rh1 during treatment with 25 ng/ml TRAIL is shown in Fig. 4. Rapid induction of apoptosis was observed. By 4 h, almost 40% of the cells were undergoing apoptosis, and by 12 h, a plateau was reached where 70% of the cells were apoptotic. The effect of caspase inhibitors on the induction of apoptosis was subsequently examined after 16 h exposure to TRAIL (25 ng/ml; Fig. 5). In TRAIL-treated Rh1 cells, ~80% of the cells were apoptotic at this time. However, in the presence of either Z-VAD-FMK, Z-DEVD-FMK, or Z-IETD-FMK, apoptosis was completely inhibited. Furthermore, coincubation of Rh1 cells with each of the three caspase inhibitors also reduced the loss in clonogenic survival after 16 h of TRAIL exposure (Fig. 6).

Expression of Genes That Regulate Fas- or TRAIL-induced Apoptosis. The expression of Fas and its adapter molecule FADD, receptors for TRAIL (DR4, DR5, DcR1, and DcR2), c-FLIP<sub>L</sub>, and c-FLIP<sub>S</sub> that may attenuate the sensitivity of cells to Fas- or TRAIL-mediated apoptosis, and procaspases-1 through -10 were determined by RNase protection analysis (Fig. 7) and/or by RT-PCR (Fig. 8).

All cell lines expressed FADD, and all except for Rh36 expressed procaspase-8, critical components of the DISC in Fas-mediated apoptosis. With regard to the lack of sensitivity to anti-Fas, two cell lines (Rh30 and Rh41) did not express the receptor, and RD and Rh28 expressed relatively low Fas levels. Of the two cell lines that demonstrated a partial growth inhibitory response to CH-11, Rh1 demonstrated higher levels of Fas than observed in other RMS cell lines but also expressed high levels of c-FLIP. In Rh18, low Fas expression was observed, and c-FLIP was absent.

Of importance to TRAIL-induced apoptosis in RMS cell lines is that all lines demonstrated high levels of expression of DR5 by RNase protection analysis (Fig. 7). In contrast only Rh1 expressed high levels of DR4. Of the two decoy receptors, DcR1 was detected at low levels in Rh1 (TRAIL-sensitive) and Rh28 (TRAIL-resistant; Fig. 7), whereas DcR2 was detected in TRAIL-sensitive lines Rh1 and Rh30 and in TRAIL-resistant Rh28 (Fig. 8). Hence, the expression of either decoy receptor failed to correlate with the cellular resistance of RMS cell lines to TRAIL. Surface expression of TRAIL receptors was examined by flow cytometric analysis by measuring the binding of TRAIL, in TRAIL-sensitive RD and TRAIL-resistant Rh36 cell lines, that expressed DR5 only and no decoy receptors (Fig. 9). Binding of TRAIL to the cell surface was determined to be similar in each of the two cell lines and correlated with expression of DR5 mRNA.

The expression of c-FLIP<sub>L</sub> or c-FLIP<sub>S</sub> also failed to correlate directly with the sensitivity of RMS cell lines to TRAIL-induced apoptosis (Fig. 8). Although TRAIL-resistant Rh28 and Rh41 expressed c-FLIP, similar levels of expression of both isoforms were found in TRAIL-sensitive RD, Rh1, and Rh30. Of the caspases that may be important in the regulation of TRAIL-induced apoptosis, including caspases-3, -6, -7, and -9, these were detected in all of the seven RMS cell lines. However, the initiator procaspases-8 and -10 demonstrated the greatest differential in expression among the seven cell lines (Fig. 7) and were further examined by RT-PCR (Fig. 8). Procaspase-8 was present in all lines except for Rh36 (TRAIL-resistant), was low
in expression in TRAIL-resistant Rh28, and highest in expression in RD, Rh1, and Rh30, which demonstrated the greatest sensitivity to TRAIL. Procaspase-10 was expressed in TRAIL-sensitive RD, Rh1, and Rh30 and in TRAIL-resistant Rh28, using primers that detect all procaspase-10 isoforms as a single PCR product (Fig. 8). Of interest, procaspase-10 was not expressed in TRAIL-sensitive Rh18, although this line expressed procaspase-8 in the absence of c-FLIP. Furthermore, procaspase-10 was not expressed in TRAIL-resistant Rh41 in the presence of procaspase-8 and c-FLIP, and TRAIL-resistant Rh36 failed to express either procaspases-8 or -10.

The expression of procaspase-8, procaspase-3, FADD, c-FLIP L, and c-FLIP S was also examined by Western analysis. Levels of expression of proteins correlated with levels of expression of the respective mRNAs (Fig. 10). Furthermore, Bid (Fig. 11), which is cleaved by caspase-8 prior to mitochondrial membrane insertion, was expressed in all cell lines.

**Mitochondrial Membrane Potential.** Changes in mitochondrial membrane potential were determined by FACS analysis in TRAIL-sensitive Rh1 and TRAIL-resistant Rh41 cells after 16 h incubation with TRAIL (50 ng/ml; Fig. 12). In Rh1, >80% of cells demonstrated low membrane potential after TRAIL treatment, whereas there was no significant change detected in Rh41. To investigate whether the threshold for mitochondrial membrane depolarization may determine sensitivity to TRAIL, the expression of members of the Bcl-2 family with both antiapoptotic (Bcl-2 and Bcl-xL) and proapoptotic (Bax and Bak) function that function at the level of the mitochondria was examined (Fig. 13). The expression of Bcl-2 and Bak was almost identical in the seven RMS lines. Bcl-xL expression was high in cells sensitive to TRAIL, and Bax was expressed in high levels in TRAIL-sensitive lines RD, Rh1, Rh18, and Rh30 but also in TRAIL-resistant Rh41. Thus, there was no direct correlation between changes in mitochondrial membrane potential and sensitivity to TRAIL.

**DISCUSSION**

In this study, we demonstrated that RMS cell lines of both embryonal and alveolar histology, comprising tumor cells derived from skeletal muscle, were exquisitely sensitive to the cytotoxic ligand TRAIL. In contrast, all lines were relatively resistant to ligation of the Fas death receptor and hence to Fas-mediated apoptosis. The signaling pathway that defines Fas-induced activation of programmed cell death and the activation of both initiator and effector caspases is currently better defined than for TRAIL-induced apoptosis. In all of the RMS cell lines except for Rh1, Fas was expressed at very low levels. In a panel of human colon carcinoma cell lines, the level of Fas expressed, which varied by >1000-fold, was found to be the major determinant of sensitivity to Fas-mediated apoptosis (3). The RMS line Rh1 demonstrated only partial growth inhibition in response to anti-Fas, suggesting that additional factors may be involved in the cellular resistance mechanism.

The anti-apoptotic factor, c-FLIP, exists in two isoforms, c-FLIP L and c-FLIP S, which appears to be the predominant form expressed in mammalian cells (26, 35). After Fas triggering, FADD, procaspase-8, and c-FLIP isoforms are recruited to the Fas receptor complex. c-FLIP subsequently inactivates the DISC by blocking further recruitment of procaspase-8 into the complex, thereby inhibiting the activation of caspase-8 (14, 26, 35). In cells with high Fas expression, the presence of c-FLIP appears insufficient to block apoptosis. However, in cells with low receptor expression, this is a factor in cellular resistance to the induction of apoptosis via Fas (14). The level of Fas expressed in Rh1 is lower than reported in TS and Thy4 human colon carcinoma cells, highly sensitive to Fas-induced apoptosis (3). Therefore, c-FLIP may be a factor in the Fas-resistance phenotype in Rh1, because procaspase-8 and FADD were both expressed. The second RMS cell line to demonstrate a partial growth-inhibitory response to anti-Fas was Rh18, and although this cell line expressed low levels of Fas, this was in the absence of c-FLIP expression.
In contrast to the general lack of sensitivity of RMS cell lines to Fas-induced apoptosis, four RMS cell lines were highly sensitive to the cytotoxic ligand TRAIL. All lines demonstrated high-level expression of one of the receptors, DR5, that expresses a death effector domain thereby allowing transmission of an apoptotic signal. Four RMS cell lines (RD, Rh1, Rh18, and Rh30) were sensitive to TRAIL at concentrations ≤1 ng/ml, which also reduced clonogenic survival, and three lines were TRAIL resistant (Rh28, Rh36, and Rh41). Expression of the decoy receptors, DcR1 and DcR2, demonstrated no correlation with the sensitivity of RMS cell lines to TRAIL. Thus, the lack of sensitivity to TRAIL-induced apoptosis in Rh28, Rh36, and Rh41 suggested that factors other than expression of receptors determined the resistance phenotype.

C-FLIP can inhibit TRAIL-induced apoptosis (20, 36) in addition to Fas-mediated apoptosis. To date, limited information is available concerning the receptor complexes for the TRAIL receptors DR4 and DR5. Certain studies have reported direct binding of the adapter proteins FADD and TRADD to these receptors and inhibition of TRAIL-induced apoptosis in the presence of DN-FADD and DN-TRADD (20). Furthermore, DISC formation among DR4 or DR5, FADD, and caspase-8 has recently been identified (37–39), which could explain an inhibitory role for c-FLIP in TRAIL-induced apoptosis. However, the exact mechanism of receptor complex formation for DR4 and DR5 including potential adapters other than FADD, and activation of specific caspases in the mechanism of TRAIL-induced apoptosis have yet to be critically identified. All RMS cell lines expressed FADD, and six of seven expressed procaspase-8.

Fig. 7 RiboQuant RNase Multi-Probe Protection assay using hAPO-3 (left panel), h-APO-3c (center panel), and h-APO-1c (right panel) templates as described in “Materials and Methods.”

Fig. 8 RT-PCR analysis of the expression of c-FLIP isoforms, procaspases-8 and -10, and the TRAIL decoy receptor DcR2, as described in “Materials and Methods” using β-actin as an internal control.
Furthermore, expression of c-FLIP did not correlate directly with lack of sensitivity to TRAIL in RMS cell lines, because TRAIL-resistant Rh36 did not express c-FLIP, and TRAIL-sensitive RD, Rh1, and Rh30 cell lines expressed both isoforms. These results are in contrast to those reported by Kim et al. (40), who suggested that DR4 and c-FLIP expression could be potentially useful predictors of TRAIL sensitivity. Of specific interest in RMS cell lines was the observation that in RD, Rh1, and Rh30, procaspase-10 was expressed in addition to procaspase-8 in the presence of high levels of c-FLIP.

In Fas-mediated apoptosis, two signaling pathways have been demonstrated (14, 41). In type I proposed by Scaffidi et al. (14), ligation of Fas leads to strong caspase-8 activation at the DISC, thereby directly activating other caspases including caspase-3 in the absence of mitochondrial involvement. In type II Fas-mediated cell death, only a small amount of the DISC is formed, leading to the activation of mitochondria, which amplifies the signal when caspase-8 cleaves the cytosolic substrate Bid, leading to release of mitochondrial cytochrome c and activation of caspases-3, -7, -8, and -9 (14, 41), a process that can be blocked by antiapoptotic members of the Bcl-2 family (41). RMS cell lines in this study expressed procaspases-3, -6, -7, -8, and -9.

The procaspases activated by TRAIL still remain to be defined, although caspase-8 is activated during formation of a TRAIL-induced DISC (37–39). The recent ordering of the caspase signaling cascade downstream of the mitochondria demonstrates activation of caspase-9, followed by caspase-3 and caspase-6, which further activates caspases-8 and -10 (42). It is evident that TRAIL induced mitochondrial membrane depolarization in TRAIL-sensitive Rh1 but not in TRAIL-resistant Rh41 RMS cell lines. However, the relative expression levels of both proapoptotic (Bax and Bak) and antiapoptotic (Bcl-2 and Bcl-xL) members of the Bcl-2 family that function at the level of the mitochondria did not correlate with the effect of TRAIL on mitochondrial membrane potential. Furthermore, Bid expression was similar among the seven RMS cell lines, suggesting that it may be factors upstream of the mitochondria that may determine the sensitivity of RMS cell lines to TRAIL. Activation of both caspase-8 and caspase-3 have been identified downstream of the receptors in TRAIL-induced apoptosis in human melanoma cell lines (20), although activation of caspase-10 was not examined. Of interest was that expression of procaspases-8 and -10 were highest in TRAIL-sensitive RMS cell lines. Little information is available concerning the function of caspase-10 (43). However, it is known that the functional death effector domain binds to the corresponding domain of FADD (44), suggesting that FADD may be the adapter protein for procaspase-10. Furthermore, procaspase-10 is expressed in high
level in fetal skeletal muscle cells but is low or absent in this tissue in the adult, suggesting a role for caspase-10 in development (43). Hence, procaspase-10 expressed in malignant RMS cells that resemble fetal muscle myoblasts could be activated in response to stress. Of interest in RMS cell lines was that procaspase-8, but not procaspase-10 or c-FLIP, was expressed in TRAIL-sensitive Rh18, and in TRAIL-sensitive RD, Rh1, and Rh30 c-FLIP isoforms were expressed at high level in addition to procaspase-8 and procaspase-10. Furthermore, procaspase-10 was not expressed in two of three TRAIL-resistant lines. It is possible in TRAIL-sensitive lines with high c-FLIP expression that caspase-10 activation is necessary for the induction of apoptosis downstream of caspase-8 activation for amplification of the apoptotic signal, whereas in Rh18, caspase-8 activation in the absence of c-FLIP expression is sufficient to induce apoptosis directly. Caspase activation was clearly important in the mechanism of TRAIL-induced apoptosis in RMS cell lines because all caspase inhibitors blocked not only TRAIL-induced apoptosis but also TRAIL-induced loss in clonogenic survival. The role of caspase-8 in TRAIL-induced apoptosis in RMS cells may be further substantiated by the effectiveness of the caspase-8 inhibitor Z-IETD-FMK in this regard. However, the relative roles of caspase-8 and caspase-10 may be further defined in these models of pediatric RMS because of the differences in their cellular expression and TRAIL sensitivity among the different RMS cell lines.

It has been well documented that RMS with alveolar histology are less sensitive to therapeutic modalities than ERMS. Furthermore, high mortality rates remain in patients with metastatic disease (24, 25). There is, therefore, a need to continue to identify new targets and new therapeutic approaches for the treatment of patients with RMS. Of significance is that both ERMS and ARMS cell lines demonstrated sensitivity to the cytotoxic ligand TRAIL, as well as high DR5 expression, irrespective of the pathological diagnosis. On the basis of the similar sensitivity of ERMS and ARMS cell lines to TRAIL, it will be of importance to determine whether the approach of using TRAIL as a cytotoxic agent may be further exploited in the therapy of RMS.

REFERENCES


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*Clin Cancer Res* 2000;6:4119-4127.

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