TRAIL Inactivates the Mitotic Checkpoint and Potentiates Death Induced by Microtubule-Targeting Agents in Human Cancer Cells

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Abstract

Tumor necrosis factor–related apoptosis–inducing ligand (TRAIL) has attracted interest as an anticancer treatment, when used in conjunction with standard chemotherapy. We investigated the mechanistic basis for combining low-dose TRAIL with microtubule-targeting agents that invoke the mitotic checkpoint. Treatment of T98G and HCT116 cells with nocodazole alone resulted in a robust mitotic block with initially little cell death; low levels of cell death were also seen with TRAIL alone at 10 ng/mL final concentration. In contrast, the addition of low-dose TRAIL to nocodazole was associated with maximally increased caspase-3, caspase-8, and caspase-9 activation, which efficiently abrogated the mitotic delay and markedly increased cell death. In contrast, the abrogation of mitotic checkpoint and increased cell death were blocked by inhibitors of caspase-8 and caspase-9 or pan-caspase inhibitor. The addition of TRAIL to either nocodazole or paclitaxel (Taxol) reduced levels of the mitotic checkpoint proteins BubR1 and Bub1. BubR1 mutated for the caspase recognition site, but not wild-type BubR1, was resistant to cleavage induced by TRAIL added to nocodazole, and partially blocked the checkpoint abrogation. These results suggest that adding a relatively low concentration of TRAIL to antimicrotubule agents markedly increases complete caspase activation. This in turn accentuates degradation of spindle checkpoint proteins such as BubR1 and Bub1, contributes to abrogation of the mitotic checkpoint, and induces cancer cell death. These results suggest that TRAIL may increase the anticancer efficacy of microtubule-targeting drugs. [Cancer Res 2008;68(9):3440–9]

Introduction

The tumor necrosis factor (TNF)–related apoptosis–inducing ligand (TRAIL), a member of the TNF family of death ligands, triggers apoptosis through interaction with the death receptors DR4 and DR5 (1–4). Many cancer cell lines express both DR4 and DR5, and each of these receptors can initiate apoptosis independently of the other (5, 6). Evidence suggests that endogenous TRAIL-mediated pathways contribute to anticancer surveillance (7). Although the mechanisms that are involved in this signaling pathway remain a topic of active investigation, one of the most attractive aspects of TRAIL is that it seems to specifically induce apoptosis in certain cancer cells while sparing most normal cells (8, 9). Evidence suggests that binding by TRAIL leads to trimerization of its receptors, which in turn leads to recruitment of Fas-associated death domain, an adaptor molecule, which then recruits and activates caspase-8. Activated caspase-8, an “initiator” caspase, can directly cleave the proenzyme forms of “effector” caspasess such as caspase-3, leading to activation of the latter (this pathway is often referred to as the type I “intrinsic” pathway of apoptosis signaling). Activation of caspase-8 may also induce apoptosis through mitochondrial pathways, referred to as type II “extrinsic” pathways. In an example of the latter, caspase-8 cleaves Bid, which in turn leads to release of cytochrome c from mitochondria, which in turn binds to and activates the adapter protein APAF-1. The resultant “apoptosome” in turn recruits, cleaves, and activates caspase-9, which in turn can also activate caspase-3. It should be noted that activation of caspase-9 may occur independent of caspase-8 activation. The extrinsic pathway of apoptosis signaling is thought to mediate many of the cytotoxic effects of DNA-damaging agents, independent of caspase-8 or receptor-mediated activation. Thus, depending on the cell line or stimulus, either the extrinsic or intrinsic pathway may be activated by TRAIL, but both pathways converge in activating effector caspases such as caspase-3 (reviewed in refs. 6, 10).

The proapoptotic effects of TRAIL have led to interest in whether the anticancer efficacy of conventional chemotherapy, including microtubule-targeting agents such as the Vinca alkaloids or taxanes, might be enhanced when combined with TRAIL (11–14). The taxanes have emerged as part of the standard of care for certain solid tumors such as breast and prostate cancer (15–17), yet treatment resistance remains common and leads to morbidity and death. For other solid tumors such as colorectal cancer or glioblastoma multiforme, antimicrotubule agents have not been proven clinically useful.

Microtubule-targeting drugs invoke the mitotic checkpoint by disrupting formation of the mitotic spindle. The resultant delay in progression of mitosis is mediated by a protein machinery that includes BubR1 and Bub1 (18). The mitotic checkpoint prevents cells with unaligned chromosomes from prematurely exiting mitosis, thereby ensuring that the daughter cells receive an equal complement of chromosomes. Because this helps ensure that aneuploidy or polyploidy does not occur, the mitotic checkpoint is thought to prevent genomic instability in untransformed cells. In cancer cells (many of which have already become aneuploid), the mitotic checkpoint potentially allows additional time to repair...
Materials and Methods

Cell lines, culture conditions, and reagents. The human glioblastoma cell line T98G and the human colon cancer cell line HCT116 were obtained from the American Type Culture Collection and grown in DMEM (Invitrogen) supplemented with 15% fetal bovine serum (Hyclone) and maintained without antibiotics in 5% CO₂ at 37°C. Taxol and nocodazole were both purchased from Sigma and were prepared as concentrated stock solutions in DMSO and applied at final concentrations of 50 nmol/L and 0.04 μg/mL, respectively. His-tagged TRAIL was prepared as previously described (24). For experiments, TRAIL was diluted in media and used at concentrations noted for each experiment. Pan-caspase inhibitor, caspase-8 inhibitor, caspase-9 inhibitor, and caspase-3 inhibitor were purchased from American Peptide. Transfections (including transient expression of BubR1 and Bub1 fusion proteins) were done with Lipofectamine 2000 (Invitrogen), which allowed transfection efficiencies of 60% to 90%.

Analytic methods. The preparation of cell lysates, the subsequent separation via SDS-PAGE (10 μg of protein per lane), and immunoblotting were done as described (25). Anti-α-tubulin antibody was from Sigma, anti-caspase-3 antibody was from BIOMOL, anti-caspase-8 antibody was from ALEXIS Biochemicals, and anti-caspase-9 antibody was from MBL. The anti-green fluorescent protein (GFP) antibody was from Clontech and anti-phosphorylated histone H3 antibody (“Mitotracker”) was from Upstate. The anti-Bub1 and anti-BubR1 were generous gifts from Drs. Raimundo Freire and Gordon Chan, respectively.

Analysis of cell cycle status and cellular nonviability (sub-G₁, DNA content) were done via fluorescence-activated cell sorting (FACS) and exclusion of propidium iodide was as previously described, with the latter done in the presence of Hoechst 33342 vital dye (Sigma) to stain the nuclei of all cells (20, 25). During FACS analysis, no gating was done on the propidium iodide-stained nuclei to ensure that all cells were included in the data.

Statistical analyses were done with VassarStats (courtesy of Richard Lowry) and Microsoft Excel (Office XP Professional). Comparisons of differences in nonviable cells in the respective treatment groups were done via paired t tests and χ² analyses with the appropriate degrees of freedom. Error bars indicate SD and statistical significance was defined at P < 0.05.

Reverse transcription-PCR. Endogenous total mRNA was isolated using mRNA Isolation Kit (Roche Applied Science). All purification steps were carried out according to the manufacturer’s instructions except for cell shearing, which was substituted with sonication. The isolated mRNA was then assessed via reverse transcription-PCR (RT-PCR). The Titan One Tube RT-PCR system (Roche Applied Science) was used with the following primers: Bub1, 5′-TGGGCGTGTTGGGAGTCAATT-3′, 5′-TGGCTCGACAGGAATGAAATGTC-3′; BubR1, 5′-ATCCGCTGAGGGTGCTCCTCAAG-3′, 5′-ATGGTGCTCATAAAGCGTTGCTGTG-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CAACTTGTGATGTTGAAGAGC-3′, 5′-AGG-GATGATTGCTGAGAGCC-3′. Reactions for all targeted mRNAs were done under similar conditions, with comparatively identical results under a range of cycle times and numbers. The RT-PCR products were analyzed by electrophoresis in 1.5% agarose gel and viewed under UV light. GAPDH was used as an internal standard to confirm equal loading in each experiment.

Results

TRAIL abrogates the mitotic delay induced by nocodazole in HCT116 and T98G cell lines. We chose to study HCT116 colorectal and T98G glioblastoma cell lines because of the relative resistance of the respective tumors to antimicrotubule chemotherapy. In these cell lines, the microtubule-targeting drug nocodazole resulted in a robust mitotic block that persisted for at least 36 h of exposure (Fig. 1A). The cell cycle delay was, however, considerably abrogated when TRAIL was added after nocodazole, such that by 18 h, the proportion of cells that showed sub-G₁ DNA content was increased severalfold (Fig. 1A and B). The increase in cells with sub-G₁ DNA content was concomitant with a reduction in the proportion of cells remaining in mitosis (Fig. 1A and B). The proportion of sub-G₁ cells in both cell lines was further increased by 24 h after the addition of TRAIL to nocodazole, and again accompanied by further abrogation of the mitotic checkpoint such that relatively few cells remained blocked in mitosis. In contrast to these effects, control (mock treated) cells or cells treated with TRAIL only at the same concentration and duration showed little increase in the proportion of cells with sub-G₁ DNA content.

Abrogation of the mitotic checkpoint by TRAIL leads to increased cell death. The proportion of cells with sub-G₁ DNA content as determined via FACS is often considered a measure of nonviable cells (including apoptosis). However, to confirm that the addition of TRAIL to nocodazole led to decreased cellular viability, we conducted cytologic analyses of cells treated in a manner identical to the experiments described in Fig. 1A and B. As expected, most cells treated with nocodazole only were blocked in mitosis, became uniformly round and nonadherent, were of the similar size, and showed nuclei more condensed than control cells, all features characteristic of mitotic cells (Fig. 1C, second row). The majority of cells treated with the relatively low concentration of TRAIL used in these experiments seemed to be similar to control cells; relatively few of the cells treated with only TRAIL appeared irregular and fragmented, and showed chromatin that were more condensed and labeled brighter with Hoechst than that of mitotic cells (third row). In contrast, the appearance of cells treated with TRAIL added after nocodazole was markedly different from either nocodazole or TRAIL alone. These cells treated with combined TRAIL and nocodazole showed membrane blebbing, irregular contours, and sizes and shapes dissimilar from each another, as well as bright Hoechst labeling of chromatin of various sizes—all features suggestive of apoptotic cells (fourth row).

The nuclei of both apoptotic and mitotic cells are condensed, and hence nuclear/chromatin condensation alone may not reliably distinguish the two states, especially during the early stages of apoptosis when nuclear fragmentation is not as obvious. Therefore, to obtain additional evidence that cells treated with TRAIL after nocodazole were truly nonviable, we assessed these cells for propidium iodide exclusion. Whereas Hoechst stains the nuclei of all cells, viable (living) cells exclude propidium iodide while nonviable cells do not. Hence, propidium iodide exclusion is especially useful to distinguish apoptotic/nonviable cells from...
mitotic cells (20, 26). Cells were mock treated, treated with nocodazole only, nocodazole followed by TRAIL, or TRAIL only, and then exposed to Hoechst 33342 concomitant with propidium iodide to determine the percentage of the cells in each treatment group that remained viable and retained the ability to exclude propidium iodide. The cells were counted and imaged immediately after staining. Most of the cells treated with nocodazole only became uniformly round and nonadherent, and showed condensed nuclei as previously noted, but were able to exclude propidium iodide. This is shown by the mitotic cells in Fig. 1D (left), with the arrow pointing to a mitotic cell showing particularly clearly the chromatid condensation and lack of propidium iodide uptake. Cells treated with only TRAIL at this dose and duration were mostly unperturbed and able to exclude propidium iodide, except for a few that showed membrane blebbing and were unable to exclude propidium iodide (Fig. 1D, right). In contrast, the majority of cells treated with TRAIL after nocodazole were unable to exclude propidium iodide (Fig. 1D, middle, with the respective quantification of nonviable cells in each treatment group shown in Fig. 1E). The inability to exclude propidium iodide, interestingly, was characteristic of cells showing membrane blebbing as well as of those that had become flattened. In contrast, cells that were uniformly round, which was characteristic of mitotic cells, excluded propidium iodide well. These observations together support the usefulness of this technique to distinguish a range of apoptotic cells from those remaining in mitosis.

**Combined treatment with TRAIL and microtubule-targeting drugs is associated with efficient caspase-3 activation and decreased levels of BubR1 and Bub1.** To investigate whether the abrogation of the mitotic checkpoint by TRAIL might be regulated through caspase activation, we treated cells with nocodazole only followed by mock treatment (Fig. 2A, Nocodazole) or nocodazole followed by TRAIL (Fig. 2A, Nocodazole > TRAIL) and then assessed for caspase-3 activation. Exposure of either cell line to nocodazole alone for times up to 36 h (12 h followed by 24 h of mock treatment; Fig. 2A, lanes 2–4) induced only modest caspase-3 activation, with only minimally detectable levels of the lower molecular weight activated form of caspase-3 (Fig. 2A, Activated). In contrast, the addition of TRAIL to nocodazole substantially increased levels of the activated cleaved form of caspase-3 by 12 h, which was further increased with the passage of time (Fig. 2A, lanes 5–7). Interestingly, the increase in activated caspase-3 was accompanied by a noticeable decrease in the higher molecular weight, uncleaved, inactive form (Fig. 2A, Proenzyme) and which was especially impressive with shorter exposure times on film.

These experiments and those described in Fig. 1 together suggested that caspase activation and proapoptotic effects of low concentrations of TRAIL might be accentuated when combined with microtubule-targeting drugs. We also found that lethality and mitotic checkpoint abrogation of combined treatment was further accentuated with increased concentration of TRAIL at a given time point (Fig. 2F). The mitotic checkpoint induced by antimicrotubule drugs is mediated by proteins that have been collectively referred to as mitotic checkpoint proteins (26), of which BubR1 and Bub1 have been of particular interest, in part, because of reports of caspase-mediated cleavage of these two checkpoint proteins (20, 23). Thus, caspase activation pathways may potentially influence the level of expression of these mitotic checkpoint proteins, which may in turn influence the progression of mitosis. We therefore assessed BubR1 and Bub1 protein levels after adding TRAIL to cells pretreated with nocodazole or Taxol. As an additional control, we assessed proteins levels after TRAIL alone (i.e., preceded by mock treatment).

We found that TRAIL, by itself, at a range of concentrations (10, 20, and 40 ng/mL), did not appreciably influence protein levels of either BubR1 or Bub1 compared with mock treatment or nocodazole alone (Fig. 2C and D, lanes 1–5) at the time point of harvest. In contrast, the addition of TRAIL to nocodazole led to reduced BubR1, even at the lowest TRAIL concentration of 10 ng/mL (lanes 6–8). The combined treatments also reduced levels of Bub1, albeit to a slightly lesser degree. The substantial decrease in levels of BubR1 and Bub1 was also seen when TRAIL was added after Taxol (lanes 10–12). In these experiments, we also probed for caspase-3 activation. Interestingly, at the time point of harvest, TRAIL alone led to partial activation of caspase-3, marked by the appearance of intermediate forms of caspase-3 (Fig. 2C and D, Intermediate forms; partially cleaved caspase-3 can also be seen in immunoblots in the other figures). However, the lowermost, maximally cleaved, activated form of caspase-3 (Fig. 2A, C, and D, Activated) remained rather minor, even at the highest concentration of TRAIL. This suggested that at the time point of harvest, TRAIL by itself may have induced some, but not all, of the steps leading to caspase-3 activation. The lack of complete caspase activation was likely insufficient for cleavage of BubR1 and Bub1. In contrast, adding TRAIL to nocodazole or Taxol seemed to efficiently complete caspase activation, resulting in increased

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**Figure 1.** TRAIL abrogates the mitotic delay induced by nocodazole and increases cell death. A, TRAIL abrogates the mitotic delay induced by nocodazole in HCT116 and T88G cells in a time-dependent manner. HCT116 or T88G cells were assessed for cell cycle distribution via FACS analysis after mock treatment (Control), treatment with nocodazole for 12 h followed by mock treatment for 24 h (effectively nocodazole treatment for 36 h; Nocodazole only), mock treatment for 12 h followed by TRAIL (10 ng/mL; TRAIL only), or nocodazole for 12 h followed by TRAIL for 12, 18, or 24 h (Nocodazole > TRAIL). The resultant histograms are shown (for each histogram, the y-axis represents the number of cells whereas the x-axis represents DNA content, with the portions reflecting cells with sub-G1 or G2-mitotic DNA content indicated by the brackets). B, bar graphs showing the proportions of the cells shown in A, which showed sub-G1 (black columns) or G2/mitotic DNA content (shaded columns). White and filled stars, significant differences in comparisons between groups (P < 0.005). Experiments were done in triplicate with similar results. C, morphology after treatment. HCT116 cells were mock treated (Control), treated with nocodazole followed by mock treatment for 24 h (effectively nocodazole for 36 h; Nocodazole only), mock treated for 12 h followed by TRAIL (10 ng/mL) for 24 h (TRAIL only), or treated with nocodazole for 12 h followed by TRAIL for 24 h (Nocodazole > TRAIL). The cells were subsequently exposed to Hoechst 33342 (1 ng/mL for 10 min) and imaged for cellular (Phase) or nuclear (Hoechst) morphology. Images on the right and left sides correspond to the same cells, and the cells of all treatment groups were imaged at the same time (24 h after addition of TRAIL). Cells treated with nocodazole only shows morphology consistent with viable mitotic cells. Many cells treated with TRAIL after nocodazole shows membrane blebbing, fragmentation, and hypercondensation of chromatin, morphologic changes that are seen in only a few of cells treated with only this concentration of TRAIL. D, assessment of viability by propidium iodide exclusion. Cells were treated as in C, but exposed to propidium iodide (1 μg/mL) simultaneously with Hoechst 33342, and counted for the percentage of cells unable to exclude propidium iodide (and therefore nonviable). Images in each column correspond to the same cells. Images to the left of each group were captured immediately after addition of propidium iodide exclusion. Most of the cells treated with TRAIL combined with nocodazole shows membrane blebbing, fragmentation, and hypercondensation of chromatin, morphologic changes that are seen in only a few of cells treated with only this concentration of TRAIL. In contrast, whereas occasion nonviable cells are notable (right, arrowheads) most of the cells treated with TRAIL alone or nocodazole alone retain the ability to exclude propidium iodide. Arrow indicate viable mitotic cells (which exclude PI) E, quantitation of nonviable cells after treatment. Cells in the experiment described in D were assessed for the percentage of all cells that show uptake of propidium iodide (PI), with the average percentages plotted in the bar graphs. At least 300 cells in each treatment group were assessed for each experiment, with experiments repeated in triplicate. Bars, SD. Filled stars, significant differences in comparisons between groups (P < 0.005).
prominence of the lowermost, maximally cleaved, activated form of caspase-3 (Fig. 2C and D). The fully activated caspase in turn likely contributed to BubR1 and Bub1 cleavage. Consequently, at each concentration of TRAIL, pretreatment with either nocodazole or Taxol led to considerably higher levels of fully activated caspase-3 and decreased levels of BubR1 and Bub1.

**BubR1 protein levels after TRAIL and nocodazole do not closely correlate with mRNA levels.** Whereas caspase-mediated cleavage of mitotic checkpoint proteins would indicate a post-translational mechanism of action, we also tested but excluded the possibility that the decreased BubR1 protein levels noted after combined TRAIL and nocodazole treatment were also associated with decreased BubR1 mRNA. Whereas BubR1 mRNA levels were substantially increased by ~3- to 4-fold after nocodazole treatment in either cell line, BubR1 protein levels were not increased (contrast the first and second lanes in Supplementary Fig. S1A and B). TRAIL alone also resulted in increased BubR1 mRNA compared with mock treatment, but BubR1 protein levels were essentially unchanged (contrast the first and third lanes in Supplementary Fig. S1A and B). Finally, treatment with nocodazole followed by TRAIL led to maximally diminished BubR1 protein levels, consistent with experiments previously described here, whereas a corresponding effect on BubR1 mRNA levels was lacking (contrast the first and last lanes in Supplementary Fig. S1A and B). These results together indicated that the decreased BubR1 protein levels seen after combined treatment were not related to transcription but most likely due to a translational or posttranslational mechanism.

**Combined treatment with TRAIL and nocodazole accentuates caspase activation.** Much of the effects of TRAIL have been attributed to the activation of upstream caspases such as caspase-8 and caspase-9, which then funnel toward the activation of effector caspases that include caspase-3. We therefore assessed whether caspase-8 or caspase-9 activation was affected by the combined treatments, compared with that seen with either nocodazole or TRAIL alone. Nocodazole alone induced very little activation of caspase-8 or caspase-9 in both cell lines (Fig. 3A and B, lanes 2 and 3), and which is consistent with lack of caspase-3 activation under these conditions (Fig. 2A). The response to TRAIL alone was heterogeneous: In HCT116 cells, TRAIL resulted in a degree of caspase-8 and caspase-9 activation, manifested by the appearance of the lower molecular weight cleaved forms of caspase-8 and caspase-9 and diminution of levels of the higher molecular weight inactive proenzyme (Fig. 3A, lanes 4 and 5). In contrast, in T98G cells, the TRAIL had no effect on caspase-9 activation and only a minor effect on activation of caspase-8 (Fig. 3B, lanes 4 and 5). The addition of TRAIL to nocodazole led to dramatic yet consistent effects in increasing caspase-8 and caspase-9 activation in both cell lines: The cleaved activated forms of caspase-8 and caspase-9 were both clearly evident, which correlated with substantial reduction of levels of the inactive proenzyme in both cell lines (Fig. 3A and B, lanes 6 and 7).

We next conducted inhibitor studies to verify the roles of caspases in determining the durability of the mitotic block when TRAIL was added after nocodazole. We assessed the effects of the caspase inhibitors on cell cycle progression and the mitotic checkpoint. As before, TRAIL substantially abrogated the mitotic checkpoint induced by nocodazole (compare second and third histograms and bar graphs of Fig. 3C and D). However, when inhibitors of caspase-8 and caspase-9 or pan-caspase inhibitor was added concomitantly with the TRAIL, the proportion of cells in mitosis in each case was considerably preserved, concomitant with fewer nonviable cells with sub-G₁ DNA content (as seen in the fourth through sixth FACS histograms and bar graphs in Fig. 3C and D). We assessed phosphorylation of histone H3 as an additional verification of the abrogation by TRAIL of the mitotic checkpoint induced by nocodazole. Treatment with nocodazole alone substantially increased phosphorylated histone H3 (Ser₁₀), a standard marker of mitotic cells (Supplementary Fig. S2), whereas the addition of TRAIL to nocodazole decreased phosphorylated histone H3 to levels approaching the mock-treated control, consistent with abrogation of the mitotic checkpoint by TRAIL. In contrast, when inhibitors of caspase-8 and caspase-9 or pan-caspase inhibitor were added simultaneously with TRAIL, the levels of phosphorylated histone H3 were partially restored (lanes 3-6 of Supplementary Fig. S2). Together, these results indicate that caspase inhibition blocked the TRAIL-induced abrogation of the mitotic checkpoint. These studies further suggest that the dramatic increase in caspase activation induced by combined low TRAIL and nocodazole was likely attributable to activation of upstream initiator caspases, including activation of both caspase-8 and caspase-9.

**BubR1 protein mutated at caspase cleavage sites resists degradation induced by TRAIL and microtubule-targeting drugs.** We have reported that BubR1 undergoes caspase-mediated cleavage at specific tetrapeptide motifs (primarily at Asp⁶⁷⁷/Asp⁶⁸⁰ and secondarily at Asp⁵⁷⁶/Asp⁵⁷⁹; ref. 20). Targeted mutagenesis of aspartic acids at both sites results in a BubR1 protein that is resistant to caspase-mediated cleavage. The resultant caspase-resistant mutant protein, BubR1 Δ₅⁷⁹ Δ₆₁₀, together with the caspase-sensitive wild-type BubR1 protein, are therefore useful for probing whether caspase activation has a direct role in down-regulating BubR1 protein levels through protein cleavage versus other potential mechanisms. For example, if the primary process invoked by TRAIL involved translational repression, then both mutant and wild-type proteins would be equally affected. On the other hand, if caspase activity is the prime determinant of BubR1 levels, increased caspase activation should affect wild-type BubR1 but not caspase-resistant mutant BubR1.

BubR1 wild-type and mutant (Δ₅⁷⁹ Δ₆₁₀; both fused to GFP to distinguish from the endogenous BubR1 protein) were expressed in cells, which were then mock treated, treated with TRAIL, or TRAIL added to nocodazole. Exposure to combined nocodazole and TRAIL led to decreased levels of wild-type BubR1. In contrast, mutant BubR1 protein levels were unaffected under identical treatment conditions (Fig. 4A, compare lanes 4 and 7). In these experiments, transfection led to a degree of heightened caspase activation, as reflected by cleavage/activation of caspase-3, and which accentuates the effects of TRAIL. Nonetheless, the BubR1 protein mutated for the caspase cleavage sites remains impervious under the identical transfection and treatment conditions. The response to paclitaxel (Taxol) was similar: TRAIL led to almost undetectable levels of wild-type BubR1, whereas mutant BubR1 was much less affected (Fig. 4B). These results indicate that the effects of TRAIL in abrogating BubR1 protein levels were predominantly mediated through the activated caspase cleavage of BubR1 protein.

We next assessed the effects of expressing wild-type or caspase-mutated BubR1 on the mitotic block induced by nocodazole followed by mock treatment (i.e., nocodazole only) versus nocodazole followed by TRAIL. Consistent with previous experiments,
exposure of cells expressing wild-type BubR1 to nocodazole led to an accumulation of cells blocked in mitosis (Fig. 4C, M peak in Noc only cells). The addition of TRAIL to nocodazole considerably abrogated the proportion of cells blocked in mitosis, which was accompanied with an increase in cells with sub-G1 DNA content (left pair of FACS histograms in Fig. 4C and bar graphs in 4D). Cells in which mutant BubR1 was expressed were, as expected, also blocked in mitosis after exposure to nocodazole; the abrogation of the mitotic checkpoint by TRAIL was, however, reduced compared with cells expressing wild-type BubR1 (right pair of FACS histograms in Fig. 4C and bar graphs in Fig. 4D). The increased durability of the mitotic checkpoint was accompanied by a reduced proportion of sub-G1 cells. These results suggested that the caspase-mediated degradation of BubR1 induced by TRAIL contributed to the abrogation of the mitotic block. To obtain further evidence that TRAIL-mediated abrogation of the cell cycle delay contributes to increased cell death, we tested the ability of nocodazole- and TRAIL-treated cells expressing wild-type or mutant BubR1 to exclude propidium iodide, as viable cells are able to exclude and do not take up propidium iodide. We found in wild-type BubR1–expressing cells that the addition of TRAIL to nocodazole substantially increased (by ~4-fold) the percentage of

Figure 2. The addition of TRAIL to antimicrotubule agents markedly enhances caspase activation and abrogation of mitotic checkpoint protein levels. A, combined TRAIL and nocodazole leads to markedly increased caspase-3 activation. HCT116 cells were treated with 0.04 μg/mL nocodazole alone followed by mock treatment (Nocodazole), or treated with nocodazole for 12 h followed by TRAIL (10 ng/mL, Nocodazole > TRAIL), and harvested at the indicated times after mock or TRAIL treatment. Cell lysates were separated via SDS-PAGE, transferred to nitrocellulose, and probed for caspase-3 or α-tubulin (loading control). The inactive uncleaved proenzyme (Proenzyme) and the maximally cleaved activated form of caspase-3 (Activated) are indicated. B, dose-dependent abrogation of mitotic checkpoint by TRAIL. HCT116 and T98G cells were assessed for cell cycle distribution via FACS analysis after mock treatment (Control) or treatment with nocodazole for 12 h (Nocodazole) followed by 0, 10, or 20 ng/mL of TRAIL for 24 h. The bar graphs show quantification of the respective percentages of cells of each treatment group with either sub-G1 (black columns; nonviable cells) or mitotic DNA content (shaded columns). White and filled stars, significant differences in percentage of nonviable cells between the Control and Nocodazole groups (P < 0.005). C and D, TRAIL combined with antimicrotubule drugs leads to maximal activation of caspase-3 and reduced levels of BubR1 and Bub1 mitotic checkpoint proteins. HCT116 (C) and T98G (D) cells were mock treated, treated with nocodazole (Nocodazole), or treated with paclitaxel (Taxol) for 12 h, followed by 0, 10, 20, or 40 ng/mL of TRAIL. Cell lysates of all treatment groups were then separated via SDS-PAGE, transferred to nitrocellulose, and probed for BubR1, Bub1, caspase-3, or α-tubulin (loading control). The inactive uncleaved caspase-3 proenzyme (Proenzyme) and the intermediate cleaved (Intermediate forms) and maximally cleaved activated (Activated) forms of caspase-3 are indicated. Treatment with TRAIL alone increases intermediate forms of caspase-3, but the active maximally cleaved form is seen only at the highest dose of TRAIL. In contrast, when the TRAIL is combined with either nocodazole or paclitaxel, the active maximally cleaved caspase-3 is evident at all concentrations of TRAIL, and this is associated with decreased levels of BubR1 and Bub1.
cells unable to exclude propidium iodide (left pair of bar graphs in Fig. 4E). In contrast, in cells expressing mutant BubR1, the percentage of nonviable cells induced by TRAIL was substantially reduced compared with wild-type BubR1–expressing cells (compare right pair with left pair of histograms and bar graphs).

**Discussion**

TRAIL has attracted considerable interest as an anticancer agent, in part, because of the specificity of its action in invoking biological pathways leading to the destruction of cancer cells. Indeed, interest in combining biologically targeted interventions with conventional chemotherapies for treating patients with cancer has been stimulated by the recent clinical success of such strategies. For example, bevacizumab may only have modest efficacy when given as monotherapy, yet because of its low toxicity to normal cells and tissues, the agent can often be combined with chemotherapy, which then results in significantly improved overall efficacy (27, 28). The physiologic and molecular basis for the improved efficacy of combined treatment remains a topic of active investigation. It is plausible that the effectiveness of combined treatment is attributable, at least in part, to the effects on multiple pathways that individually might be prone to treatment resistance.

A number of mechanisms have been implicated in the resistance of cancer cells to the cytotoxic effects of TRAIL (6, 29). These include down-regulated expression of ligand receptors (30), expression of decoy receptors, overexpression of antiapoptotic pathway components such as IAP or FLIP, down-regulation of activator caspases, or inactivation of Bax (29, 31–37). Other signaling pathways may also contribute to resistance to TRAIL, including AKT or mitogen-activated protein kinase (38–40). The increased efficacy of combining TRAIL with chemotherapy may lie in the ability to reverse or bypass one or more of these mechanisms to augment cell killing. For example, Taxol has been reported to up-regulate caspase-10 but down-regulate FLIP protein (41), effects which might be useful to augment the sensitivity to TRAIL of a cell line overexpressing FLIP. Taxol has also been
reported to up-regulate DR4 and DR5 receptors, thereby augmenting the binding of TRAIL to cancer cells (4, 11). Through these mechanisms, Taxol and other microtubule-targeting agents may potentially sensitize cancer cells to the lethal effects of TRAIL. Whether the up-regulation of DR4 or DR5 by antimicrotubule agents such as Taxol is cell cycle dependent (e.g., requires entry into mitosis) was not assessed. Interestingly, DR5 is up-regulated during p53-induced apoptosis but not during p53-induced G1-S cell cycle arrest, suggesting that up-regulation of death receptors may not be solely a function of cell cycle arrest (42).

The work described here extends the relevance of TRAIL-mediated caspase activation pathways to other mechanisms that may also influence cancer cell survival, such as control of mitotic progression. The mitotic checkpoint has been postulated to monitor genomic stability and help prevent the deleterious effects of aneuploidy; an increased frequency of cancers has been detected in mice with reduced levels of mitotic checkpoint proteins after exposure to carcinogens (26, 43, 44). The checkpoint may have additional “protective” roles, including preventing early cell death and senescence, perhaps by allowing cells more time during mitosis to repair damaged DNA before mitotic progression, a time when congerison with damaged chromosomes would be lethal to cells (45). Consistent with a role for mitotic checkpoint proteins in promoting survival in normal cells, the cells of mice profoundly

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**Figure 4.** TRAIL-induced abrogation of the mitotic checkpoint is impeded by mutation of the BubR1 caspase cleavage sites. **A.** TRAIL leads to increased cleavage of BubR1 wild-type, whereas BubR1 mutated for the caspase cleavage sites is unaffected. Wild-type BubR1 fused to GFP (to distinguish from endogenous BubR1; *GFP:*BubR1 wild-type) or BubR1 mutated for caspase cleavage sites (*GFP:*BubR1 Δ579 Δ610) was expressed in T98G cells, which were then mock treated, mock treated followed by exposure to TRAIL for 36 h (*TRAIL*), treated with nocodazole followed by mock treatment for 24 h (i.e., nocodazole for 36 h; *Nocodazole*), or treated with nocodazole for 12 h followed by TRAIL (10 ng/mL final concentration) for 24 h (*Nocodazole > TRAIL*). Cell lysates of all treatment groups were then separated via SDS-PAGE, transferred to nitrocellulose, and probed for the wild-type or mutated fusion BubR1 protein, α-tubulin (as loading control), or caspase-3, followed by exposure to film. **B.** cells were treated and analyzed as in **A,** except that treatment with paclitaxel (Taxol) was substituted for the nocodazole. **C,** histograms resulting from FACS analyses of cells in which BubR1 wild-type (*GFP:*BubR1 wild-type; *left pair*) or BubR1 mutated at caspase-cleavage sites (*GFP:*BubR1 Δ579 Δ610; *right pair*) was expressed and which were treated with either nocodazole for 12 h followed by mock treatment for 24 h (i.e., nocodazole for 36 h; Noc only) or nocodazole for 12 h followed by TRAIL for 24 h (Noc > TRAIL). The portions of the resultant histograms that correspond to cells with sub-G1, and mitotic (M) DNA content are indicated. **D,** bar graphs showing the proportions of the cells shown in **C,** which showed sub-G1 (*black columns*) or mitotic DNA content (*shaded columns*). **E,** assessment of viability by propidium iodide exclusion. Cells were treated as in **C,** but exposed to propidium iodide (1 μg/mL) simultaneously with Hoechst 33342 and counted for the percentage of cells that were unable to exclude propidium iodide. Bar graphs show the respective percentages of cell showing uptake of propidium iodide and therefore are nonviable. Bars, SD. Filled stars, significant differences in the percentages of mitotic cells in comparisons between groups (*P* < 0.01).
References


Figure 5. Model of the effects of TRAIL on abrogating mitotic checkpoint function, leading to increased cancer cell death. The mitotic checkpoint is invoked in the presence of damaged or misaligned chromosomes, potentially allowing more time for repair and proper alignment at the metaphase plate. Abrogation of the mitotic cell cycle delay before chromosomal repair leads to cell death (or the danger of increased chromosome aneuploidy if cell death is not accomplished). TRAIL results in caspase activation, which accelerates degradation of protein components of the checkpoint machinery, such as BubR1 and Bub1, and contributes to abrogation of the mitotic checkpoint and increases cell death.

deficient in BubR1 protein show early senescence and increased apoptosis, which likely contributes to early aging and decreased life span of the mice (46). Whether the mitotic checkpoint serves similar roles in cancer cells, many of which have already become aneuploid, remains unclear. Tumor cells deficient in BubR1 expression have been noted to be more sensitive to rapid killing by microtubule-targeting agents, and knockdown of BubR1 mediated by RNA interference ultimately leads to massive cancer cell death (19, 47). On the other hand, forced overexpression of wild-type BubR1 has been found to increase cancer cell death, whereas others postulate a requirement for the mitotic checkpoint for paclitaxel-induced cell death (48).

More recently, we and others have discovered direct links between caspase activation and control of mitotic checkpoint proteins. Through biochemical, targeted mutagenesis, and induced expression studies, we showed that BubR1 undergoes caspase-3–mediated cleavage at two evolutionarily conserved tetrapeptide motifs, which influenced its ability to maintain a mitotic delay (20). Perera and Freire have separately established that Bub1 likewise undergoes caspase-3–mediated cleavage at specific caspase recognition sequences during apoptosis (23). These studies suggest that pathways leading to activation of effector caspases such as caspase-3 likely also influence expression levels of mitotic checkpoint proteins such as BubR1. Reduced levels of mitotic checkpoint proteins due to caspase-mediated cleavage, in turn, may influence the robustness of the cell cycle delay. Our results suggest that TRAIL may contribute to cancer cell killing by antimicrotubule agents through multiple pathways. By “short-circuiting” this potentially protective mitotic checkpoint cell cycle delay, and by activating effector caspases with diverse downstream targets, TRAIL may help accelerate the execution of defective cells (Fig. 5). In addition, up-regulation of receptors for TRAIL or activation of caspases by Taxol, Vinca alkaloids, or other chemotherapy may in turn independently sensitize cancer cells to being killed by TRAIL or bypass resistance to TRAIL (49).

In summary, the studies here show that levels of mitotic checkpoint proteins are diminished by TRAIL, which results in the abrogation of the mitotic cell cycle delay induced by antimicrotubule agents, and which results in increased nuclear fragmentation in cancer cells. These effects are likely mediated through both caspase-8– and caspase-9–dependent mechanisms and can be blocked by caspase inhibitors. The mechanisms by which TRAIL abrogates the mitotic checkpoint likely include the degradation of mitotic checkpoint proteins mediated through caspase activation. The studies presented here merit confirmation in an animal model, but illuminate the possibility that the efficacy of chemotherapy that invokes the mitotic checkpoint might be increased when administered in combination with TRAIL.

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