**Angiogenesis, Metastasis, and Tumor Micronenvironment**

### Targeting Cell Spreading: A Method of Sensitizing Metastatic Tumor Cells to TRAIL-Induced Apoptosis

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**Abstract**

TNF-related apoptosis-inducing ligand (TRAIL) is a current focus for the development of new cancer therapies, because of its selective induction of apoptosis in cancer cells. TRAIL has previously been shown to be important for tumor cell clearance from the liver; however, many cancer cell lines show some resistance toward TRAIL, posing a problem for the future use of TRAIL therapies. In this study, we show that interfering with a cell’s ability to attach and spread onto a matrix can sensitize tumor cells to TRAIL-induced apoptosis in vitro. We targeted different members of the integrin signaling pathway using siRNA or inhibitors, including β-integrins, talin, Src, and downstream survival pathways PI3K and MAPK. Targeting any of these molecules could sensitize both MDA-MB-231 human breast cancer cells and TRAIL-resistant 1205Lu melanoma cells to TRAIL-induced apoptosis in vitro. Transcriptionally targeting the cytoskeleton, using myocardin-related transcription factor depletion to disrupt the transcription of cytoskeletal proteins, also caused TRAIL sensitization in MDA-MB-231 cells. We showed that this sensitivity to TRAIL correlated with increased activation of the intrinsic pathway of apoptosis. Manipulation of cell spreading therefore presents a potential method by which disseminated tumor cells could be sensitized to TRAIL therapies in vivo. *Mol Cancer Res;* 9(3); 249–58. ©2011 AACR.

**Introduction**

Metastasis, the spread of disseminated cancer cells, is the main reason for treatment failure in cancer patients. Improving our understanding of the metastatic process at a cellular and molecular level is important to allow us to develop effective therapies, which are currently very few in number. Metastases form when tumor cells gain the ability to break away from the primary tumor, enter and survive in the circulation, and extravasate and colonize at distant sites (1). Despite the frequency of metastasis in cancer patients, it is a very inefficient process, with high numbers of circulating tumor cells being rapidly cleared from the circulation (2). Recent work has highlighted the importance of apoptosis in this tumor cell clearance, suggesting that sensitivity to apoptosis could determine a cell’s metastatic potential (3–5).

TNF-related apoptosis-inducing ligand (TRAIL) is an important molecule implicated in the clearance of disseminated tumor cells from the circulation. This has been shown particularly in the liver, where endogenous NK cells expressing TRAIL are important for clearing metastatic tumor cells (6–8). TRAIL, a member of the TNF family, has been shown to induce apoptosis in a wide range of cancer cell lines with minimal toxicity against normal tissues (9, 10). TRAIL triggers apoptosis by signaling through the extrinsic pathway of apoptosis. TRAIL-induced apoptosis is initiated by the binding of TRAIL to death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5). This binding induces the assembly of adaptor components such as FADD and caspase 8 or 10 into a complex called the death-inducing signaling complex (DISC). Through a cleavage cascade, these upstream caspases activate caspases 3 and 7 that orchestrate the final stages of apoptosis (4).

Cells whose death receptor signaling can generate enough activated caspase 8 to undergo apoptosis are referred to as “type I” cells. “Type II” cells do not cleave enough caspase 8 to undergo apoptosis, and amplify their apoptotic response through activation of the intrinsic pathway of apoptosis (11). The intrinsic (or mitochondrial) pathway of apoptosis, activated by cell stressors such as hypoxia, growth factor withdrawal, and DNA damage, can also be activated by extrinsic signals through cleavage of the proapoptotic molecule bid by caspase 8 (12). Bid is cleaved to a truncated form which can activate the intrinsic pathway of apoptosis by causing mitochondrial permeation, release of proapoptotic molecules, and caspase 9 cleavage.

There are suggestions that interfering with cell spreading may be a method of sensitizing tumor cells to TRAIL killing (13–15). Spreading is mediated by the interaction of...
integrins with extracellular matrix components. Integrins, the link between the inside and outside of the cell at focal adhesions, relay signals from the extracellular matrix which can alter the cell cytoskeleton, motility, and signaling. The integrin receptor is a heterodimeric complex made of α- and β-subunits (16). The β1-integrin subunit has been implicated in the control of invasion and migration, and therefore metastasis (17, 18). Integrin-linked kinase (ILK) forms a signaling hub at the β-integrin tail, signaling downstream through parvin proteins, PINCH, and NCK-2 (19, 20). Another adaptor is talin, which binds both to the tail of β-integrins and to actin, relaying signals which aid actin polymerization, leading to cell spreading (21). The nonreceptor tyrosine kinase FAK is activated by integrin-dependent cell attachment, and binds to and activates another nonreceptor tyrosine kinase, Src. These kinases form another signaling hub downstream of integrins and talin. Increased Src activity in cancers has been linked to anoikis resistance, and increased propensity for metastasis (22–24). These oncogenic kinases can signal to downstream pathways such as mitogen-activated protein kinase (MAPK), Akt, and many others, influencing adhesion, motility, invasion, proliferation, and survival (25, 26).

In this study, we show that blocking the efficient spreading and attachment of two tumor cell lines using targets within the integrin signaling pathway can sensitize tumor cells to TRAIL-induced apoptosis in vitro. This suggests a method by which TRAIL-resistant cells could be sensitized to TRAIL signals in vivo.

Materials and Methods

Cells and cell cultures

The WM1789 cell line was originally isolated from a human primary radial growth phase melanoma.1205Lu was derived from the lung metastasis of a vertical growth phase melanoma WM164 (27, 28). Both cell lines were kindly provided by Dr. M. Herlyn (Wistar Institute, Philadelphia, PA) and maintained as described previously (3). SW480, a human colon carcinoma cell line was sourced from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. MDA-MB-231 human breast cancer cells were a kind gift from Prof. Richard Treisman, JCRUK London (CRUK London), along with MDA-MB-231 cells carrying tetracycline-inducible shRNA for myocardin-A (Santa Cruz), MRTF-A and -B (Santa Cruz), bid (Cell Signaling), PARP1 (F-2) (Abcam), caspase 9 (Cell Signaling), 1kB (Abcam), talin1 and talin2 (kind gift from Prof. David Critchley, Leicester), MRTF-A and -B (Santa Cruz), bid (Cell Signaling), PARP1 (F-2) (Santa Cruz), paxillin (Tyr118) and total paxillin (Cell Signaling), Akt (Ser473) (New England Biolab), ERK1/2 (Santa Cruz), caspase 3 (Cell Signaling). Secondary antibodies were caspase 3 (Cell Signaling), caspase 8, caspase 10 (MBL), β-actin (Sigma), caspase 9 (Cell Signaling), 1kB (Abcam), talin1 and talin2 (kind gift from Prof. David Critchley, Leicester), MRTF-A and -B (Santa Cruz), bid (Cell Signaling), PARP1 (F-2) (Santa Cruz), paxillin (Tyr118) and total paxillin (Cell signaling), Akt (Ser473) (New England Biolab), ERK1/2 (Thr202/Tyr204) (Cell Signaling), integrin β1 (Abcam), integrin β3 (Cell Signaling). Secondary antibodies were conjugated to horseradish peroxidase (SouthernBiotech), and signals detected by enhanced chemiluminescence (Amersham). Blots were developed by Xograph Compact X4 (Xograph Healthcare Limited), and processed by Microsoft Office Picture Manager (Microsoft) and Image J software.

siRNA transfection

The siRNA experiments with talin1, ILK1, and control siRNA (all Dharmacon) were performed with a forward transfection protocol using Dharmafect transfection reagent.

Flow cytometry

A total of 5 × 10⁵ cells were suspended in FACS buffer (PBS with 10% FBS) and stained with HS201 and HS202 (Enzo-life sciences), followed by anti-mouse immunoglobulin G (IgG) conjugated to Alexa Fluor 633 (Invitrogen). Measurements were taken by a FACScalibur (BD Biosciences) and analyzed by FloJo software.

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay buffer (Thermoscientific) with protease inhibitor cocktail (Roche) and phosphatase inhibitors (Sigma). Whole cell lysates (25 μg) were resolved on 10% SDS-polyacrylamide gels and transferred to Immobilon-P membrane (Millipore). Antibodies for immunoblotting were against caspase 8, caspase 10 (MBL), β-actin (Sigma), caspase 9 (Cell Signaling), 1kB (Abcam), talin1 and talin2 (kind gift from Prof. David Critchley, Leicester), MRTF-A and -B (Santa Cruz), bid (Cell Signaling), PARP1 (F-2) (Santa Cruz), paxillin (Tyr118) and total paxillin (Cell signaling), Akt (Ser473) (New England Biolab), ERK1/2 (Thr202/Tyr204) (Cell Signaling), integrin β1 (Abcam), integrin β3 (Cell Signaling). Secondary antibodies were conjugated to horseradish peroxidase (SouthernBiotech), and signals detected by enhanced chemiluminescence (Amersham). Blots were developed by Xograph Compact X4 (Xograph Healthcare Limited), and processed by Microsoft Office Picture Manager (Microsoft) and Image J software.

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Statistics
Statistical analysis was done by either a 2-tailed Student’s t test or ANOVA. Significance was defined as \( P < 0.05 \).

Results
TRAIL-induced apoptosis in human cell lines is increased on cell detachment
To study TRAIL sensitivity, we used 3 human cancer cell lines. 1205Lu was derived from a highly metastatic vertical phase melanoma, whereas WM1789 was derived from a poorly metastatic radial phase melanoma (28, 30). Figure 1A shows that the metastatic 1205Lu cell line was more resistant to TRAIL-mediated apoptosis than its poorly metastatic counterpart, using the colon carcinoma cell line SW480 as a TRAIL sensitive control. The metastatic human breast cancer cell line MDA-MB-231 had intermediate TRAIL sensitivity (29). Apoptosis is indicated by a reduction in viability over 18 hours following TRAIL treatment, with an increase in caspase 3–like activity (Fig. 1B).

To confirm reports that detachment could sensitize cells to TRAIL, the 3 cell lines were cultured on low attachment

![Figure 1](https://www.aacrjournals.org/molcanres/article-figures/10.1158/1541-7786.MCR-11-0021.pdf)

**Figure 1.** TRAIL sensitivity. A, viability of cell lines MDA-MB-231, 1205Lu, WM1789, and SW480-positive control, following 18 hours of TRAIL treatment at indicated concentrations. Values expressed as percent reduction in viability of TRAIL-treated cells compared with untreated. B, caspase 3–like activity measured by Caspase-Glo 3/7 assay at indicated time points following 200 ng/mL TRAIL. C, cells were cultured either on a normal cell culture plate (attached) or ultra low attachment plate (detached) for 16 hours and then assayed by Caspase-Glo assay (top) or PARP immunoblot (bottom). Whole cell lysates were used to measure PARP cleavage with or without 200 ng/mL TRAIL treatment for 4 hours. D, increased apoptosis on low attachment plates by WST-1 assay 18 hours after 200 ng/mL TRAIL (top) or 3 hours after TRAIL by Caspase-Glo assay (bottom). Data are shown as mean ± SD and represent 3 individual experiments. Error bars for 1205Lu and WM1789 in D (top) are too small to be seen. Error bars, SD, *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \); ****, \( P < 0.0001 \).
plates, whose hydrophilic surface prevents attachment. Detachment alone did not lead to increased caspase 3–like activity, or PARP cleavage (Fig. 1C) in these cells. However, when detached, all cell lines showed increased PARP cleavage following TRAIL treatment. This increased TRAIL sensitivity could also be seen by a greater reduction in viability 24 hours after TRAIL treatment and a matching increase in caspase 3–like activity (Fig. 1D).

Targeting β1-integrin and the adaptor protein Talin1 sensitizes tumor cells to TRAIL-induced apoptosis, whereas ILK1 does not

To investigate the signaling pathways which lead to attachment-based TRAIL sensitivity, we targeted specific components of the integrin signaling pathway, shown in Figure 2. For these experiments, we used the TRAIL-resistant 1205Lu melanoma cell line, along with the breast cancer cell line MDA-MB-231.

We targeted β-integrin subunits by treatment with β1-blocking, β3-blocking, and β1-stimulating antibodies before seeding onto fibronectin or human collagen I. Expression of these subunits is shown in Supplementary Figure S1A. Preincubation with β1-blocking antibody led to a marked inability of cells to spread after 4 hours on both collagen I and fibronectin (Supplementary Fig. S1B).

β1-Blocking led to TRAIL sensitization of both MDA-MB-231 cells and TRAIL-resistant 1205Lu cells, compared with untreated cells (Fig. 3A and B). Blocking β3-integrin led to mild TRAIL sensitization on fibronectin in 1205Lu cells, but no sensitization on collagen compared with untreated cells. MDA-MB-231 did not express the integrin β3-subunit, and therefore showed no sensitization from integrin β3-blocking. To further dissect this pathway, we targeted the adaptor protein talin1 (31). Talin binds to the intracellular region of β-integrins by a FERM domain on its head region. Its rod domain allows binding to actin, making talin an important player in the transmission of signals from integrins to proteins in the focal adhesion complex. Blotting revealed that the talin1 isoform was more prevalent in these cells (Supplementary Fig. S1C), and knockdown of talin1 by siRNA (Fig. 3C) led to TRAIL sensitization in both cell lines.

Next we targeted ILK1. Recent evidence has shown ILK1 to be a pseudokinase which interacts either directly or indirectly with β-integrin tails and forms a signaling hub at focal adhesions. Knockdown of ILK1 by siRNA was confirmed by immunoblot, but led to no significant change in TRAIL sensitivity 72 hours after transfection (Fig. 3D).

Inhibition of Src kinase signaling sensitizes tumor cells to TRAIL-induced apoptosis in vitro

To inhibit Src family kinase activity, we used the selective Src family kinase inhibitor PP2 using a nonfunctional structural analogue, PP3, as a control. In contrast to PP3, the active inhibitor PP2 induced marked and rapid cell rounding (Supplementary Fig. S1B). PP2 led to reduced phosphorylation of downstream Src target, paxillin in both cell lines (Fig. 4A). Subsequent treatment with TRAIL induced a higher level of apoptosis in all 3 cell lines, measured by a viability reduction (Fig. 4B) and an increase in active caspase 3–like activity (Fig. 4C). The inhibitor alone had no significant effect on viability of any of the cell lines tested (data not shown). To verify this observation, we used a second Src inhibitor, AZD0530, which also caused cell rounding (data not shown) and TRAIL sensitization (Fig. 4D).

Transcriptionally targeting the cell cytoskeleton led to TRAIL sensitization in MDA-MB-231 cells

Cell spreading leads to transcriptional alterations mediated by MRTFs. To investigate a potential mechanism for how cell spreading alterations affect TRAIL sensitivity in vitro, we targeted MRTFs. MRTF-A and -B are transcriptional coactivators for serum response factor, a widely expressed MADS transcription factor responsible for the transcription of many genes with cytoskeletal functions (32). MRTFs are sequestered by monomeric G-actin, but when Rho signaling causes polymerization of G-actin to form F-actin, they move to the nucleus and drive transcription (33). Medjkane and colleagues showed that cells which lack MRTF-A and -B have marked spreading defects (29). We used MDA-MB-231 cells with either control shRNA (C4-GFP) or with inducible shRNA for
MRTF-A/B under the tetracycline promoter (C20-CFP). Figure 5A shows that even in the absence of tetracycline, C20-CFP cells showed a reduction in MRTF-A/B levels, which was fully downregulated after tetracycline treatment (referred to as C20-CFPTET). These cells showed distinct morphologic abnormalities (Fig 5B), previously characterized by reduced numbers of stress fibers and focal adhesions, resulting in reduced adhesion and motility (29). As expected from our previous findings, this phenotype also resulted in marked sensitivity to TRAIL measured by a reduction in viability (Fig. 5C), and an increase in caspase 3–like activity (Fig. 5D).

Blockade of integrin-mediated signaling alters levels of death receptors and causes marked activation of the intrinsic apoptosis pathway

We have explored mechanisms that could account for increased TRAIL sensitivity after blockade of integrin-mediated signaling. MRTF-deficient cells expressed significantly increased levels of surface DR5 (Fig. 6A). Both TRAIL receptors, DR4 and DR5, can trigger activation of caspase 8 and both are members of the DISC complex. However, Src inhibition did not alter DR4 and DR5 levels (data not shown).

Treatment of parental MDA-MB-231 cells with TRAIL resulted in cleavage of extrinsic executioner caspases 8
There was also detectable \( \gamma \) activation, indicative of cross-talk between the extrinsic and intrinsic pathway (Fig. 6C). Downregulation of MRTF led to markedly enhanced activation of caspase 9. This was reflected by increased levels of bid before TRAIL exposure and increased amounts of activated t-bid after TRAIL treatment (Fig. 6D). Similar data were obtained after treatment with the Src inhibitor, PP2 (Fig. 6E and F). Thus, interference with the integrin signaling pathway contributed to increased TRAIL killing more generally due to greater activation of the intrinsic pathway of apoptosis.

**Src inhibition causes reduction in levels of phosphorylated ERK and Akt**

Src inhibitor PP2 provides robust sensitization of the tested cell lines to TRAIL-induced apoptosis, and inhibitors to Src are already in clinical trials (25). To investigate how PP2 leads to TRAIL sensitization, we used it in conjunction with PI103 and UO126, PI3kinase and ERK inhibitors, respectively. The ability of these inhibitors to suppress the activity of their targets is shown in Supplementary Figure S2B. As predicted from previously published evidence (34, 35), inhibition of the PI3K/AKT and MAPK pathways led to TRAIL sensitization in both cell lines. This was shown by a reduction in viability of the cell lines, 24 hours after TRAIL treatment (Fig. 7A and B), and was confirmed in the MDA-MB-231 cell line by PARP cleavage as a readout of apoptosis (Fig. 7C and D). Although inhibition of each of these survival pathways alone led to sensitization, a combination of all 3 inhibitors additively enhanced TRAIL-induced apoptosis. Investigation of the downstream survival pathways altered in PP2-treated cells confirmed a reduction in levels of phosphorylated ERK and Akt in both cell lines. Interestingly, there was also an increase in the level of the NF-\( \kappa \)B inhibitor I\( \kappa \)B\( \alpha \) in MDA-MB-231 cells, suggesting a role for NF-\( \kappa \)B survival signals downstream of Src signaling in this cell line (Fig 7E).

**Discussion**

Previous work has highlighted the importance of TRAIL-induced apoptosis in clearance of circulating tumor cells from the liver, particularly through the action of natural killer cells (36, 37). TRAIL therapies are currently in phase I and II clinical trials, having previously shown promising results in animal models. However, some tumor cells seem...
resistant to the killing effect of TRAIL, and resistance could be acquired following therapeutic TRAIL delivery in patients (10). This suggests that TRAIL therapies will be most effective when used in combination with agents which can sensitize tumor cells to TRAIL (38, 39).

Following previous reports that cells which are detached from their matrix show increased TRAIL sensitivity, we show that targeting the integrin signaling pathway (which facilitates spreading) at various levels can sensitize 1205Lu and WM1789 melanoma cells, along with MDA-MB-231 breast cancer cells, to TRAIL-induced apoptosis in vitro.

Signals arise from integrins at the cell surface, and are relayed through a series of adaptor proteins. These adaptors, such as talin, paxillin, and vinculin, relay the signal to many effectors. These can alter the cytoskeleton through proteins complexes such as the Arp2/3 complex or by influencing actin directly. Other effectors are kinases, which can activate survival pathways such as MAPK, PI3K, and NF-kB, among others. Cells which have detached from their matrix can undergo spontaneous cell death (anoikis), or integrin-mediated death (IMD), by which unligated integrins can trigger caspase 8 activation independent of death receptor ligation or DISC formation (40, 41).

Targeting the direct attachment of cells to a matrix by blocking β1-integrin signaling, or talin1 which bridges the cellular tail of integrins with actin, made cells more sensitive to TRAIL killing. β3-Integrins bind preferentially to fibronectin whereas β1-integrins bind to a much broader range of extracellular matrix substrates (42). Our data are consistent with this, as β1-block led to TRAIL sensitization on both fibronectin and collagen, whereas β3-blocking caused mild sensitization only on fibronectin. The effect of β3-blocking was only evident in 1205Lu cells due to lack of the β3-integrin subunit in MDA-MB-231 cells. There was some unexpected sensitization by β1-stimulating antibody on fibronectin; however, unlike β1 blocking antibody, this sensitization was not significant. Although some spontaneous apoptosis was evident when we targeted β1-integrins, the TRAIL sensitization was also seen when we inhibited downstream targets talin and Src. This shows that although there may be a role for IMD in the spontaneous death of these cells, the TRAIL sensitivity effect we observed was independent of the IMD pathway.

Although some of the changes in sensitivity seem small, it is certainly possible for these changes to have a large effect on a tumor. For example, talin siRNA led to a 30% to 50% increase in viability change, and if this resulted in a comparable change in TRAIL threshold, the result could be substantial.

ILK1 interacts with PINCH1, parvins, and Nck-2 to alter cytoskeletal dynamics. It has also been shown to phosphorylate Akt on Ser473 and interact with FAK (43, 44). Interestingly, targeting ILK1 by siRNA had no effect on TRAIL sensitivity in either cell line.
Targeting Src family kinases, essential for relaying survival signals from the focal adhesion complex, led to a decrease in downstream paxillin phosphorylation, and sensitized these cells to TRAIL-induced apoptosis. This supports findings by Zhang and colleagues that Src signaling can aid metastatic breast cancer cell resistance to TRAIL signaling in the bone microenvironment (23).

We see DR5 expression changes after MRTF depletion in MDA-MB-231 cells. DR5 has been previously shown to be involved in anoikis suppression in human colorectal carcinoma cell lines (14). Although these changes in DR5 may contribute to TRAIL sensitivity in some lines, this may not be a general mechanism, as it could not be seen in Src inhibited cells. We show in this article that reducing a cell’s ability to attach to its substrate inactivates multiple survival pathways downstream of the focal adhesion complex, resulting in TRAIL sensitivity. Previous evidence has shown that alterations in the Akt and ERK pathways can lead to TRAIL sensitivity (34, 35, 45). Although Shradar reported TRAIL sensitization from Akt inhibition in a bladder carcinoma line, and not from ERK inhibition using UO126, we clearly see sensitization in the cell lines tested in this article following UO126 treatment (46). This suggests that the combinations of survival signals controlling TRAIL sensitivity differs between cell lines, and is likely to be dependent on the growth receptor and mutation status. Evidence of this also comes from our observation of Src-induced NF-κB activity in MDA-MB-231 cells, but not in 1205Lu cells. This suggests that targeting specific survival signals to sensitize to TRAIL may only provide cell-type-specific sensitization. We show that Src family kinase inhibition reduces signaling through the PI3K and MAPK pathways, along with NF-κB signaling in MDA-MB-231 cells. By inhibiting combinations of these pathways by inhibitors for
Src, PI103 and ERK, we saw additive increases in TRAIL sensitivity, suggesting each pathway has a different and independent mechanism of sensitization. All of these survival pathways have been shown to control levels of intrinsic apoptosis pathway components, and we report robust “type II” cell activation in both MDA-MB-231 breast cancer and 1205Lu melanoma cells when the spreading pathway was inhibited. Interestingly, a large amount of TRAIL resistance in these cell lines was due to signaling through Src, PI3K, and ERK survival pathways. Therefore, targeting this pathway at the level of Src, or above, may provide a mechanism by which cells are more sensitive not only to TRAIL but also to other apoptosis-inducing cancer therapeutics.

This study identifies a number of targets by which to sensitize circulating or dormant tumor cells to TRAIL by inhibiting components of the integrin signaling pathway. In combination with previous findings stating the importance of TRAIL in the clearance of metastatic tumor cells, this study suggests a method by which circulating tumor cells in vivo may be sensitized to TRAIL signals, either arising from preexisting immune cells, or through therapeutic TRAIL delivery. Future work is needed to investigate the ability of such drug combinations to reduce circulating tumor cell survival in vivo. Screening by libraries of siRNA could also allow the identification of a large number of cytoskeletal targets whose silencing leads to TRAIL sensitization.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interest were disclosed.

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