Tissue Inhibitor of Metalloproteinase-1 Protects Human Breast Epithelial Cells from Extrinsic Cell Death: A Potential Oncogenic Activity of Tissue Inhibitor of Metalloproteinase-1

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Abstract
Tissue inhibitors of metalloproteinases (TIMPs) inhibit matrix metalloproteinases and some members of a disintegrin and metalloproteinase domain (ADAM) family. In addition, recent studies unveiled novel functions of TIMPs in the regulation of apoptosis. TIMP-1 inhibits intrinsic apoptosis by inducing TIMP-1 specific cell survival pathways involving focal adhesion kinase (FAK). TIMP-3, however, was shown to enhance extrinsic cell death by inhibiting the shedding of the cell surface death receptors mediated by tumor necrosis factor-α converting enzymes (TACE/ADAM-17). Here, we examined whether TIMP-1, an inhibitor of some of the ADAM family members, enhances the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)–induced extrinsic apoptotic pathway. Surprisingly, we found that TIMP-1 effectively protects human breast epithelial cells from TRAIL-induced apoptosis, demonstrating opposite roles of TIMP-1 and TIMP-3 for the regulation of extrinsic apoptosis. TIMP-1 inhibition of TRAIL-induced apoptosis does not depend on its ability to inhibit matrix metalloproteinases or ADAM activities and is unrelated to its ability to stabilize active or decoy death receptors. Importantly, inhibition of PI3-kinase signaling by wortmannin and down-regulation of FAK expression using siRNA significantly diminish TIMP-1 regulation of human breast epithelial cells against TRAIL–induced extrinsic apoptosis. In addition, the in vitro three-dimensional culture studies showed that TIMP-1 inhibits lumen formation and apoptosis during morphogenesis of MCF10A acini. Taken together, these studies suggest that TIMP-1 may exert oncogenic activity in breast cancer through inhibition of both intrinsic and extrinsic apoptosis involving the FAK survival signal transduction pathway. (Cancer Res 2005; 65(3): 898-906)

Introduction
The tissue inhibitor of metalloproteinase (TIMP) family consists of four members (TIMP-1 to TIMP-4) that regulate activities of matrix metalloproteinases (MMPs) and some members of a disintegrin and a metalloproteinase domain (ADAM) family of zinc-dependent endopetidases (1, 2). Matrix degrading enzymes and their inhibitors regulate the integrity and remodeling of extracellular matrix components as well as the cleavage of nonmatrix proteins such as cell surface molecules and growth factors. Thus, these metalloproteinases and TIMPs indirectly regulate diverse cellular processes including cell proliferation, differentiation, migration and apoptosis (3–7). Recent studies also indicate that some TIMPs interact with cell surface proteins and modulate intracellular signal transduction pathways independent of their MMP inhibitory functions (8–10).

During the past several years, investigators have shown critical roles for TIMPs in the regulation of apoptosis. Proapoptotic activity of TIMP-3 and antiapoptotic activity of TIMP-1 have been well documented, whereas both proapoptotic and antiapoptotic activities of TIMP-2 and TIMP-4 were reported (11–26). TIMP-3 enhances extrinsic cell death by inhibiting the shedding of the cell surface death receptors mediated by tumor necrosis factor-α converting enzymes (TACE/ADAM-17; refs. 21, 27). The prodeath domain of TIMP-3 was mapped to the NH2-terminal three loops that coincide with its MMP inhibitory domain (20, 21). In contrast to the proapoptotic activity of TIMP-3, TIMP-1 inhibits intrinsic apoptosis in many cell types, including activated hepatic stellate cells, erythroid cells, Burkitt's lymphoma cell lines, human breast epithelial cells, and mammary epithelial cells in transgenic mice (11–16, 28). Whereas TIMP-1 inhibition of apoptosis was suggested to be dependent on its MMP-inhibitory activity in activated hepatic stellate cells (12, 13), emerging evidence indicated that the antiapoptotic activity of TIMP-1 is mediated by its activation of cell survival pathway independent of its MMP inhibitory activity in other cell types (11, 14–16, 28). Although TIMP-1 does not inhibit TACE, it was shown to inhibit some of the ADAM family members, including ADAM-10 (2), raising the question whether TIMP-1, like TIMP-3, enhances the extrinsic apoptotic pathway by protecting cell death receptors on the cell surface against matrix degrading enzyme-mediated cleavage.

In the present study, we investigated TIMP-1 regulation of extrinsic apoptosis in human breast epithelial cells in response to the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, Apo-2L). TRAIL was chosen for this study, because it is among the critical regulators of breast epithelial cell apoptosis during development and has been shown to play key roles in suppressing tumor initiation and metastasis in animal models (29, 30). Recent studies showed that TRAIL-induced apoptosis is essential for the formation and maintenance of the hollow glandular breast epithelial structure (31, 32). For cancer therapy, use of soluble recombinant TRAIL has been of particular interest, because animal experiments showed significant tumor regression with little systemic toxicity (33). Considering that some cancer cells are resistant to TRAIL-induced apoptosis (34, 35), and that TIMP-1 is frequently overexpressed in breast cancer patients (36), it is of particular importance to examine the roles of TIMP-1 in TRAIL–induced extrinsic apoptosis. Here, we show that the levels of endogenous TIMP-1 expression inversely correlate with TRAIL-induced cytotoxicity, and that recombinant...
TIMP-1 protein effectively protects human breast epithelial cells from TRAIL-induced apoptosis. We present evidence suggesting that TIMP-1 inhibits TRAIL-induced apoptosis through its activation of the cell survival pathway independent of its MMP or ADAM inhibition. Importantly, we also show that TIMP-1 inhibits human formation and apoptosis during morphogenesis of MCF10A acini, elucidating a potential oncogenic activity of TIMP-1 for breast cancer development.

Materials and Methods

Cell Culture. Generation of TIMP-1 overexpressing MCF10A clones #3 and #29 (T3 and T29) was previously described (11). Establishment of antisense TIMP-1 construct-transfected MCF10A clone (AS TIMP-1 MCF10A), FLAG-tagged wild-type TIMP-1–transfected MCF10A clones (WT TIMP-1–FLAG #9 and WT TIMP-1–FLAG #10) and FLAG-tagged mutant TIMP-1 transfected MCF10A clones (T2G TIMP-1–FLAG #18 and T2G TIMP-1–FLAG #19) were previously described (28). T2G mutant contains a substitution of the second amino acid residue threonine for glutamine in TIMP-1. Cells were cultured in DMEM/F-12 medium supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml epidermal growth factor, 0.1 μg/ml cholera enterotoxin, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L l-glutamine, and 0.5 μg/ml fungzoin in a 95% air and 5% CO2 chamber at 37 °C.

Reagents. Anti-TIMP-1 Ab-2 (Clone 102 D1) monoclonal antibody (mAb) was purchased from NeoMarkers, Inc. (Fremont, CA), anti-DR4 and anti–focal adhesion kinase (Fak; pY397) from BD Biosciences (San Diego, CA), anti-DR5 and DcR2 from Stress Gen (Victoria, BC, Canada), and anti-DR4 from Upstate Biotechnology (Lake Placid, NY). Anti-human β-actin mAb, anti-mouse IgG peroxidase conjugate and anti-Rabbit IgG peroxidase conjugated rabbit anti-active caspase-3 mAb was purchased from BD Immunocytometry Systems (San Jose, CA) as described before (28).

Production of Recombinant TIMP-1, TIMP-2, and Recombinant TRAIL. Human recombinant TIMP-1 and TIMP-2 were expressed in HEK cells using a vaccinia expression system and purified to homogeneity as described previously (11). To purify soluble TRAIL, a human TRAIL cDNA fragment (amino acids 114-281) generated by reverse transcription-PCR was cloned into a pET-23d vector (Novagen, Madison WI). His-tagged TRAIL protein was then purified using the Ni-NTA, His-Bind Resin Superflow according to the manufacturer’s instructions. Intensity of the bands was quantified using UN-SCAN-IT V5.1 detection system (Silicon Graphics, Fairfield, CA) as described before (28).

Caspase Activity Assay. Cells were lysed in cell extract buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 0.5 mmol/L EDTA, and 0.5% NP40]. Lysates were kept on ice for 30 minutes and centrifuged at 15,000 × g for 10 minutes. Fifty microliters of the cytosolic fraction were incubated for 60 minutes at 37 °C in a total volume of 200 μL of caspase buffer [20 mmol/L HEPES (pH 7.5), 50 mmol/L NaCl, and 2.5 mmol/L DTT] containing 25 μL of Ac-DEVD-AMC for caspase-3-like activity, Ac-IETD-AMC for caspase-8-like activity, or Ac-LEHD-AMC for caspase-9-like activity (BioSource International, Inc., Camarillo, CA). 7-Amino-4-methylcoumarin fluorescence, released by caspase activity was measured at 460 nm using 360 nm excitation wavelength on a Spectra Max Giemini fluorescence plate reader (Molecular Devices, Menlo Park, CA). Caspase activity was normalized per microgram of protein as determined by a bicinchoninic acid protein assay reagent (Pierce).

Detection of Active Caspase-3 by Fluorescence-Activated Cell Sorting Analysis. Active caspase-3 was detected as described previously (39). Briefly, 105 cells were fixed with 1% formaldehyde for 15 minutes at 4 °C, washed twice with PBS, permeabilized with 0.1% Triton X-100 in PBS, and incubated with PBS containing 0.03% horseradish peroxidase (Invitrogen, Carlsbad, CA) at 4 °C for 15 minutes. The cells were then stained with 20 μL FITC-conjugated anti-active caspase-3 mAb at 4 °C for 45 minutes in the dark. Following the incubation, the cells were washed with PBS, fixed with 1% formaldehyde again, and analyzed by flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA). CellQuest (Becton Dickinson) and ModFit LT (Verity Software House, Topsham, ME) were used as software for the analysis.

Down-regulation of FAK by siRNA. FAK (PTK2) siRNA/stAB Kit was purchased from Dharmacon, Inc. (Lafayette, CO). AS TIMP-1 MCF10A and TIMP-1 MCF10A #29 cells grown on 6-well plates were transiently transfected with siRNA SMARTpool FAK (four pooled SMARTselected siRNA duplexes) or its negative control (four pooled nontargeting siRNA duplexes) for 24 hours using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were then replated into 6-well plates (5 × 105 cells per well) or 96-well plates (2 × 104 cells per well) for an additional 24 hours for FAK immunoblot analysis (according to the manufacturer’s protocol). For caspase-3 activity assay or cell survival analysis, the cells were continually cultured in serum-free medium for 24 hours in the absence or presence of TIMP-1 (500 ng/mL). TRAIL (100 ng/mL) was then added to the cells for 4 hours. Caspase-3 activity assay or cell viability assay was done as described above.

MCF10A Morphogenesis Assay in Three-Dimensional Culture. Three-dimensional culture of parental MCF10A cells, control vector transfected (Neo) and TIMP-1 overexpressing MCF10A cells (T3 and T29) was...
carried out as previously developed by the Brugge laboratory (31, 32). Assay medium (DMEM/F12 supplemented with 2% donor horse serum, 10 µg/mL insulin, 1 ng/mL cholera toxin, 100 µg/mL hydrocortisone, 50 units/mL penicillin, and 50 µg/mL streptomycin) containing 5 ng/mL epidermal growth factor and 2% growth factor-reduced Matrigel (BD Biosciences, Bedford, MA) was replaced every 4 days.

At indicated time points, cells were washed four times with PBS containing 1 mmol/L Ca2+ and Mg2+, fixed in 4% paraformaldehyde at room temperature for 20 minutes, and permeabilized in 0.5% Triton X-100 in PBS for 10 minutes at 4°C. Cells were washed thrice with PBS/Glycine buffer (130 mmol/L, 7 mmol/L Na2HPO4, 3.5 mmol/L NaH2PO4, and 100 mmol/L glycine) for 10 minutes followed by incubation with IF buffer (130 mmol/L, 7 mmol/L Na2HPO4, 3.5 mmol/L NaH2PO4, 7.7 mmol/L NaNO3, 0.1% bovine serum albumin, 0.2% Triton X-100, and 0.05% Tween 20) for 1 hour. After three washes with IF buffer, cells were incubated with a rat anti-integrin α6 mAb (Chemicon, Temecula, CA), and a rabbit anti-active caspase-3 polyclonal antibody (Signaling Technology, Beverly, MA) at 4°C overnight. After three washes with IF buffer for 10 minutes, the coverslips were incubated with FITC-conjugated anti-rabbit and Texas Red–conjugated anti-rat secondary antibodies for 1 hour. After three washes with IF buffer for 10 minutes at 4°C, the coverslips were counterstained with 4',6-diamidino-2-phenylindole (Roche Applied Science, Indianapolis, IN) and mounted with antifade solution. Confocal immunofluorescence microscopic analysis was done using a Zeiss LSM 510 confocal microscopy system equipped with a C-Apochromat (NA = 1.2) 63x/1.4 korr objective lenses (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Images for figures were colored and resized with Adobe Photoshop 5.5 software.

**Results**

**TIMP-1 Protects Human Breast Epithelial Cells from TRAIL-Induced Cell Death.** Our previous studies showed that TIMP-1 protects MCF10A cells from apoptosis induced by a variety of intrinsic apoptotic stimuli, including hydrogen peroxide, radiation, anoikis, Adriamycin, staurosporine, and growth factor withdrawal (11, 28). Here, we examined whether TIMP-1 expression modulates TRAIL-induced extrinsic cell death in immortalized, non-malignant human breast epithelial cells (MCF10A). Less than 50% of AS TIMP-1 MCF10A cells (in which TIMP-1 expression was down-regulated by an antisense TIMP-1 construct; ref. 28) were viable, whereas ~70% of control vector-transfected MCF10A cells (Neo MCF10A) remained viable following 6 hours of treatment with 100 ng/mL TRAIL. In contrast, >90% of TIMP-1 overexpressing MCF10A cells (clone TIMP-1 MCF10A #29) survived following the same treatment (Fig. 1A and B). This indicates that the levels of TIMP-1 expression inversely correlate with TRAIL-induced cell death in MCF10A cells.

**TIMP-1 Inhibits TRAIL-Induced Caspase Activity.** To examine whether TIMP-1 modulates TRAIL-induced caspase activity, we measured DEVDase (caspase 3-like), IETDase (caspase 8-like), and LEHDase (caspase 9-like) activities following TRAIL treatment. As shown in Fig. 1C, all of these caspase-like activities were drastically down-regulated in TIMP-1 overexpressing cells (T3 and T29) compared with the neo MCF10A cells. To further examine the role of TIMP-1 in caspase activation, cells expressing active caspase-3 were detected using FITC-conjugated anti-active caspase-3 antibody followed by flow-cytometric analysis (Fig. 1D). Treatment with 100 ng/mL TRAIL for 1 hour increased the ratio of caspase-3 positive cells from ~1.33% to 11.29% (~8.5-fold) in Neo MCF10A cells, whereas it increased from 0.65% to 16.02% (~24.6-fold) in AS TIMP-1 MCF10A cells. In contrast, increase in active caspase-3 positive cells was significantly lower in TIMP-1 MCF10A #29 (T29) cells. It should be mentioned that the percentage of T29 cells expressing active caspase-3 increased from 1.35% to 7.98% (5.9-fold) following 1 hour treatment with TRAIL (Fig. 1D), yet the active caspase-3 levels were not high enough to be detected by DEVDase activity assay during TRAIL treatment for 6 hours (Fig. 1C). These results indicate that TIMP-1 plays a critical role in TRAIL-induced caspase activation in human breast epithelial cells.

We then examined whether exogenously added TIMP-1 could inhibit DEVDase activity in AS TIMP-1 MCF10A cells following TRAIL treatment. As shown in Fig. 1E, recombinant TIMP-1 effectively down-regulated TRAIL-induced DEVDase activity in AS MCF10A cells, demonstrating an inhibitory role for the exogenous TIMP-1 protein in TRAIL-induced caspase activation.

**TIMP-1 Has Little Effect on Stabilization of the TRAIL Receptors.** TRAIL transduces apoptotic signals by binding to its death receptors DR4 and DR5 both of which have a conserved death domain motif. TRAIL also interacts with decoy receptors, which have close homology to the extracellular domains of DR4 and DR5. Decoy receptors lack functional death domains, and therefore are incapable of transmitting an apoptosis signal (40). Previous studies showed that TIMP-3 enhances extrinsic cell death by protecting death receptors from MMP/ADAM–mediated cleavage (21, 27). We investigated whether TIMP-1 regulation of apoptosis is associated with modulation of the TRAIL receptor levels. To test the consequences of different levels of TIMP-1 expression on the levels of TRAIL receptors, we first examined the levels of DR4 and DR5 in Neo MCF10A, AS TIMP-1 MCF10A, TIMP-1 MCF10A #3, and TIMP-1 MCF10A #29 cells. As shown in Fig. 2, the levels of active TRAIL receptors DR4 and DR5 were comparable in these cells. We then studied whether TIMP-1 inhibition of TRAIL-induced apoptosis results from accumulation of decoy receptors. When the levels of TIMP-1 expression on TRAIL decay receptors DcR1 and DcR2 were examined, no significant difference was detected. These results suggest that TIMP-1 modulation of TRAIL-induced apoptosis is not related to its ability to modulate/stabilize active or decoy death receptors.

**TIMP-1 Inhibition of TRAIL-Induced Apoptosis Is Independent of Its Inhibition of MMP or ADAM Activity.** The above results suggest that, unlike TIMP 3, TIMP-1 regulation of TRAIL-induced apoptosis may not depend on its ability to inhibit MMP or ADAM activity. To address this issue, we tested the effects of the T2G TIMP-1 mutant expression in TRAIL-induced apoptosis (28). As shown in Fig. 3A, the T2G TIMP-1 mutant protected MCF10A cells from TRAIL-induced cell death as effectively as wild-type TIMP-1. Consistently, DEVDase activity following TRAIL treatment was greatly inhibited in both WT and T2G TIMP-1 expressing MCF10A cells (Fig. 3B), suggesting TIMP-1 inhibition of TRAIL-induced apoptosis is independent of MMP inhibition. However, the involvement of MMP inhibition in the antiapoptotic effects of TIMP-1 cannot be ruled out by using this mutant only, because the T2G TIMP-1 mutant exhibits selective MMP inhibition (28). To further investigate whether MMP/ADAM inhibition has any antipapoptotic effects in MCF10A cells following TRAIL treatment, we examined the effects of Batimastat (BB-94), a hydroxamate-based broad spectrum MMP inhibitor, and the synthetic peptide hydroxamate metalloprotease inhibitor/tumor necrosis factor-protease inhibitor (TAPI). We also tested TIMP-2, a close homologue of TIMP-1, for its effect on apoptosis. As shown in Fig. 3C, neither BB94 nor TAPI protected AS MCF10A cells from TRAIL-induced cell death, showing that inhibition of MMPs or TACE is insufficient to prevent TRAIL-induced apoptosis in human breast epithelial cells. Similarly, TIMP-2 failed to protect MCF10A cells from TRAIL-induced cell death, suggesting a TIMP-1 specific
regulation of TRAIL-induced apoptosis. More importantly, exogenously added TIMP-1 protein, but not TIMP-2, effectively prevented TRAIL-induced cell death in the presence of BB94 or TAPI. Taken together, these results suggest that TIMP-1 inhibition of TRAIL-induced apoptosis is independent of its MMP or ADAM inhibition.

**TIMP-1–Activated FAK/PI 3-Kinase Is Critical for Its Inhibition of TRAIL-Induced Apoptosis.** We previously showed that TIMP-1 activation of the FAK/PI 3-kinase pathway is critical for TIMP-1 protection of intrinsic apoptotic cell death in human breast epithelial cells (11, 28). In agreement with our previous finding, the levels of active FAK (pY397) and PI 3-kinase were significantly higher in T3 and T29 MCF10A cells compared with neo-MCF10A cells (Fig. 4A and B). To evaluate the significance of TIMP-1-activated FAK/PI 3-kinase in the regulation of extrinsic apoptosis, we tested the ability of TIMP-1 to enhance cell survival in the presence and absence of wortmannin, an inhibitor of PI 3-kinase (28). As shown in Fig. 4C, TIMP-1-enhanced cell survival by ~3.2-fold following TRAIL treatment (39.7% in T29 MCF10A cells versus 12.6% in Neo MCF10A cells). In contrast, when the PI 3-kinase signaling pathway was inhibited by wortmannin, TIMP-1 enhanced cell survival only by ~1.68-fold (24.4% in T29 MCF10A cells versus 14.5% in Neo MCF10A cells), suggesting a role for PI 3-kinase in the TIMP-1–mediated cell survival pathway.

To further evaluate the significance of the FAK/PI 3-kinase for TIMP-1 inhibition of TRAIL-induced extrinsic cell death, FAK expression was down-regulated using a small interfering RNA (siRNA) in both AS MCF10A and TIMP-1 MCF10A #29 cells when compared with the respective control cells. Immunoblot analysis confirmed significant down-regulation of FAK in cells transfected with TIMP-1 siRNA.
with FAK siRNA, compared with the cells transfected with control siRNA (Fig. 5f). We then examined whether FAK is required for TIMP-1 protection of TRAIL-induced extrinsic cell death in AS MCF10A cells. Consistent with our previous study (28), recombinant TIMP-1 protein enhanced cell survival in serum-free conditions (Si-Con versus Si-Con + T1 in Fig. 5b). However, TIMP-1 failed to protect FAK siRNA-transfected AS MCF10A cells against growth factor withdrawal-induced cell death (si-FAK versus si-FAK + T1 in Fig. 5c), consistent with the significance of FAK for TIMP-1 inhibition of intrinsic apoptosis. Recombinant TIMP-1 also failed to protect FAK siRNA-transfected AS MCF10A cells against TRAIL-induced extrinsic cell death (Si-FAK + TR versus Si-FAK + T1 + TR in Fig. 5c), whereas recombinant TIMP-1 significantly reduced TRAIL-induced cell death in control siRNA-transfected AS TIMP-1 MCF10A cells (Si-Con + TR versus Si-Con + T1 + TR in Fig. 5b). It should be mentioned that the cell survival rates in serum-free conditions between control and FAK siRNA-transfected cells were comparable following transient transfection (data not shown). Consistent with cell survival assays, recombinant TIMP-1 down-regulated TRAIL-induced DEVDase activity only in control siRNA-transfected AS TIMP-1 MCF10A cells, but not in FAK siRNA-transfected AS MCF10A cells (Fig. 5e and f).

Next, we investigated whether down-regulation of FAK expression diminishes TIMP-1 overexpression-mediated cell survival following TRAIL-treatment. As shown in Fig. 5d, whereas ~10% of TIMP-1 MCF10A #29 cells underwent cell death following TRAIL treatment, the same treatment effectively killed ~50% of TIMP-1 MCF10A #29 cells with lower levels of FAK expression (Fig. 5d). Concurrently, TRAIL-induced DEVDase activity was greatly enhanced in FAK siRNA-transfected TIMP-1 MCF10A #29 cells compared with the control siRNA-transfected counterparts (Fig. 5g). These results show a significant role for FAK in TIMP-1 protection of human breast epithelial cells against TRAIL-induced extrinsic apoptosis.

**TIMP-1 Inhibits Lumen Formation and Apoptosis during Morphogenesis of MCF10A Acini.** Our previous and the present studies clearly show a potent antiapoptotic activity of TIMP-1 in human breast epithelial cells, suggesting a potential oncogenic activity in breast cancer. One of the hallmarks of breast cancer development/early progression is thought to be filling of the luminal space in the glandular epithelial structure. *In vitro* three-dimensional culture studies showed that MCF10A human breast epithelial cells form hollow acini-like structures (31, 32), and that apoptosis, especially TRAIL-induced apoptosis, is essential for the formation and maintenance of the hollow glandular architecture (31, 32). It was shown that polarized outer layer cells in contact with the basement membrane survive due to activation of cell survival signaling pathways involving Akt, whereas the centrally located cells undergo apoptotic cell death following increased expression of TRAIL (31, 32). Because TIMP-1 constitutively activates cell survival signaling involving FAK/PI3-K and extracellular signal-regulated kinases regardless of cell contacts with matrix, leading to inhibition of intrinsic apoptosis (11, 28) as well as TRAIL-induced extrinsic apoptosis, we asked if TIMP-1 overexpression is sufficient to prevent MCF10A cells from forming a hollow acini-like structure in three-dimensional culture. In agreement with previous reports (31, 32), MCF10A cells underwent morphogenesis, forming a single outer layer of polarized epithelial cells with apoptotic cells in the center after 8 days of culture on Matrigel. However, TIMP-1 overexpressing cells failed to form a luminal structure and displayed no detectable apoptosis even after 19 days of three-dimensional culture (Fig. 6). This indicates an oncogenic activity of TIMP-1 by disrupting the normal breast epithelial architecture.

**Discussion**

We previously showed that *bcl-2* overexpression is associated with enhanced levels of TIMP-1 expression in human breast epithelial cells (11). Subsequent studies showed that TIMP-1 is a potent inhibitor of apoptosis induced by a variety of intrinsic apoptotic stimuli (11, 28). Here, we show that TIMP-1 also effectively inhibits extrinsic cell death induced by TRAIL in

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**Figure 2.** TIMP-1 has little effect on the expression levels of TRAIL receptors in MCF10A cells. MCF10Aneo (Neo), AS TIMP-1 MCF10A (AS), and TIMP-1 MCF10A #3 and #29 (T3 and T29) cells were cultured overnight and treated with TRAIL (100 ng/mL) for 0, 1, 2, and 4 hours. Cells were harvested and total cell lysates were subjected to immunoblot analysis for TRAIL receptors including specific antifunctional receptors (DR4 and DR5) and decoy receptors (DcR1 and DcR2) antibodies. **Bottom,** β-actin levels of the same blot reprobed with an anti-human β-actin antibody to ensure the equal loading of the samples. Densitometry analysis of intensity of the DR4, DcR1, DR5, and DcR2 bands were presented as a ratio to the respective cells at 0 hour, after normalizing to the respective β-actin signals.
human breast epithelial cells. It has been reported that cleavage of FAK during TRAIL- and Fas-triggered apoptosis contributes to the morphologic changes observed in apoptotic suspension and adherent cells (41). Here we show that TIMP-1–mediated constitutive activation of FAK is critical for inhibition of extrinsic apoptotic pathways. Phosphorylated FAK activates PI 3-kinase, which in turn activates Akt (42). Akt then phosphorylates bad, a proapoptotic member of the bcl-2 family, at the Ser136 residue (43). The phosphorylated form of bad no longer interacts with bcl-2 or bcl-X<sub>L</sub> (antiapoptotic members of the bcl-2 family), resulting in bcl-2 and bcl-X<sub>L</sub> activation (43). Collectively, these studies suggest a positive feedback loop between bcl-2 and TIMP-1 in the regulation of the apoptosis commitment step. TIMP-1 inhibition of extrinsic apoptotic pathway may be associated with TIMP-1 protection of the mitochondrial pathway (28), possibly involving activation of antiapoptotic bcl-2 family members, preventing apoptotic signal amplification between the death inducing signaling complex and mitochondria. Alternatively, TIMP-1–activated FAK may directly inhibit extrinsic cell death through regulation of the death inducing signaling pathway, since the FAK/PI 3-kinase/Akt signaling pathway is also known to exert antiapoptotic effects by up-regulating several apoptosis inhibitors including FLIP which inhibits caspase-8 activation at the death inducing signaling complex (44). Consistently, constitutive activation of FAK was shown to inhibit cell death receptor-mediated extrinsic apoptotic pathway and caspase-8 activation (45).

TIMP-1, but not TIMP-2, BB94, or TAPI, inhibits apoptosis in human breast epithelial cells, suggesting that the antiapoptotic activity of TIMP-1 is independent of its MMP or ADAM inhibitory activity. This is consistent with recent findings that
some TIMP family members regulate cellular processes independent of their protease inhibitory activities. For example, α3β1 integrin-mediated binding of TIMP-2 to endothelial cells results in dissociation of the phosphatase SHP-1 from β1 integrin, which abrogates angiogenic growth factor-mediated endothelial cell proliferation (8). This antiproliferative/angiogenic activity of TIMP-2 was mapped to Loop 6 of the TIMP-2 protein, a dispensable domain for MMP inhibition, demonstrating a novel TIMP-2 activity independent of its MMP inhibition (9). Like TIMP-2, emerging evidence, including ours, indicates that TIMP-1 induces cell survival signaling independent of its MMP inhibition (11, 14, 15, 28). However, ultimate proof of an antiapoptotic activity of TIMP-1 independent of MMP inhibition awaits identification of a TIMP-1 binding cell surface protein which mediates cell survival signaling without involvement in MMPs regulation. Identification of a TIMP-1 binding cell surface protein will also shed light on molecular mechanisms by which TIMP-1 activates FAK and additional survival signaling pathways (if there exist). In light of a recent report that TIMP-1 inhibits human breast carcinoma T-47D cell apoptosis via activation of c-Src and PI 3-kinase (46), TIMP-1–mediated survival signaling pathways depend or independent of FAK need to be further investigated.

The antiapoptotic activity of TIMP-1 in vitro raises important questions regarding the biological role of TIMP-1 in breast epithelial cells in vivo. In breast cancer, TIMP-1 expression has been associated with poor prognosis (47). TIMP-1 expression was shown to be induced at the early stages of carcinogenesis and confined to aggressive tumors (48), an unexpected finding considering that TIMP-1 is a known MMP inhibitor. Our study suggests an oncogenic activity of TIMP-1 through inhibition of apoptosis, thereby providing an explanation for the unexpected results of clinical studies. TIMP-1 may exert its oncogenic activity in breast cancer through its inhibition of TRAIL-induced apoptosis, escaping immune surveillance. Both innate and adaptive components of cellular immunity are thought to mediate immune surveillance against tumor cells. Whereas a key component for the adaptive cellular immunity is CD8+ CTL that recognize tumor cell surface antigens presented by histocompatibility complex (MHC) class I molecules, natural killer cells have been implicated in innate immunity against tumors, especially MHC class I–deficient ones (30, 34, 49, 50). Interestingly, TRAIL was shown to be a key factor mediating cytotoxic activity of both activated natural killer cells and T cells against tumor cells (30, 34, 50). Thus, TIMP-1 inhibition of TRAIL-induced cell death may play a critical role in breast cancer progression in vivo.

The ability of TIMP-1 to prevent MCF10A cells from forming acini-like structures is striking. Unlike TIMP-1, the potent intrinsic apoptosis inhibitors, bcl-2 and bcl-XL, were shown not to prevent, but delay lumen formation by 4 to 5 days (31). Increased cell proliferation by overexpression of cyclin D1 or human papilloma virus 16 E7 was also insufficient to prevent the lumen formation (31). These suggest that oncogenic activity leading to either inhibition of intrinsic apoptosis alone or increased cell proliferation alone is not sufficient to disrupt breast epithelial glandular structures (31). TIMP-1’s ability to constitutively activate cell survival signaling regardless of cell adhesion, preventing both intrinsic and TRAIL-induced extrinsic apoptosis, may be responsible for its inhibition of lumen formation. Additionally, TIMP-1’s ability to exert multiple activities in breast cancer progression.

![Figure 5](image_url) Down-regulation of FAK expression using siRNA abolishes TIMP-1 protection against TRAIL-induced cell death. A. AS TIMP-1 MCF10A (AS) and TIMP-1 MCF10A #29 (T29) cells were transiently transfected with siRNA SMARTpool FAK (FAK) or its negative control (Con) for 24 hours. Cells were then replated in 6-well plates (5 × 10^5 cells per well) for a total of 24 hours and harvested. Total cell lysates were subjected to immunoblotting analysis using anti-FAK mAb. Bottom, β-actin levels of the same blot reprobed with an anti-human β-actin antibody. Intensity of the FAK bands was normalized to the respective β-actin signals, and densitometry analysis was presented as a ratio to the control siRNA-transfected cells. B-D. AS TIMP-1 MCF10A (AS) and TIMP-1 MCF10A #29 (T29) cells, transiently transfected for 24 hours with siRNA SMARTpool FAK (Si-FAK) or its negative control (Si-Con), were replated in 6-well plates. Cells were then cultured in serum-free medium for 24 hours in the absence or presence of 500 ng/mL TIMP-1 (T1). TRAIL (TR, 100 ng/mL) was added to the cells for 4 hours. Cell viabilities were determined by WST-1 assay as described above. Cell survival is expressed as a percentage of the respective control, which was considered 100%. Columns, means of sextuple experiments; bars, ± SE. E-G. AS TIMP-1 MCF10A (AS) and TIMP-1 MCF10A #29 (T29) cells, transiently transfected for 24 hours with siRNA SMARTpool FAK (Si-FAK) or its negative control (Si-Con), were replated in 6-well plates. Cells were then cultured in serum-free medium for 24 hours in the absence or presence of 500 ng/mL TIMP-1 (T1). TRAIL (TR, 100 ng/mL) was added to the cells for 4 hours. Cell viabilities were determined by WST-1 assay as described above. Cell survival is expressed as a percentage of the respective control, which was considered 100%. Columns, means of sextuple experiments; bars, ± SE.
TIMP-1 Activated FAK Prevents TRAIL-Induced Apoptosis

References
Tissue Inhibitor of Metalloproteinase-1 Protects Human Breast Epithelial Cells from Extrinsic Cell Death: A Potential Oncogenic Activity of Tissue Inhibitor of Metalloproteinase-1

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