S-7 [15:50~16:20]

TIMP-1 in the regulation of ECM and apoptosis

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Abstract

The importance of apoptosis in normal development and pathogenesis has been well recognized, and explosive progress towards dissecting its commitment step has been made during the past decade. Mitochondria, Apaf-1, caspase, and bcl-2 family members play central roles in the commitment step. However, it is still unclear how upstream cell survival pathways regulate apoptosis. It is also unknown whether the bcl-2 family members have any effect on the upstream survival pathways. We have demonstrated that the anti-apoptotic gene product bcl-2 greatly induces expression of the tissue inhibitor of metalloproteinase-1 (TIMP-1) in human breast epithelial cells. Surprisingly, we found that TIMP-1, like bcl-2, is a potent inhibitor of apoptosis induced by a variety of stimuli. Functional studies indicate that TIMP-1 inhibits a classical apoptotic pathway mediated by caspases, and that focal adhesion kinase (FAK)/PI 3-kinase and mitogen activated protein kinase (MAPK) are critical for TIMP-1-mediated cell survival. We also showed specific association of TIMP-1 with the cell surface. Consistently, a 150-kDa surface protein was identified in MCF10A cells that specifically binds TIMP-1. Taken together, we hypothesize that TIMP-1 binding on the cell surface induces a cell survival pathway that regulates the common apoptosis commitment step. The results of these studies will address a new paradigm in the regulation of apoptosis by an extracellular molecule TIMP-1, and also greatly enhance our understanding of TIMP-1's pleiotropic activity in many physiological and pathological processes. This information may also be useful in designing more rational therapeutic interventions aimed at modulating the anti-apoptotic activity of TIMP-1.

Introduction

Previous studies in our laboratory examined the role of bcl-2 in apoptosis using breast epithelial cells genetically engineered to overexpress bcl-2. Since ECM is critical for apoptosis regulation, particularly in breast epithelium, it was hypothesized that the death-suppressing activity of bcl-2 is partly mediated by a complex regulation of matrix-degrading enzymes and/or their inhibitors, the TIMPs. We showed that bcl-2 overexpression is associated with enhanced levels of TIMP-1 expression suggesting a role for TIMP-1 in apoptosis. Indeed, apoptosis studies with breast epithelial cells show that administration of recombinant TIMP-1 or endogenous overexpression of TIMP-1 protects against a variety of apoptotic stimuli including anoikis and free radicals (1).

These results raise important basic questions regarding the biological role of TIMP-1 in breast epithelial cells. For example, in breast cancer, TIMP-1 expression has been associated with poor prognosis, an unexpected finding considering that TIMP-1 is a known MMP inhibitor. Previous studies clearly suggest a role for matrix-degrading enzymes such as MMPs on tumor cell invasion, metastasis formation and angiogenesis. The activities of MMPs are regulated by a family of specific inhibitors called tissue inhibitors of metalloproteinases (TIMPs) including TIMP-1. *In vitro* and mice studies demonstrated that TIMP-1 reduces tumor cell invasion through MMP inhibition. Based on these studies, synthetic inhibitors of MMPs including Marimastat, AG3340, COL-3, Neovastat, and BMS-275291, are currently in NCI-sponsored clinical trials. Our preliminary evidence provides new clues about TIMP-1 function in breast neoplasia, as a novel regulator of apoptosis, and may provide an explanation for unexpected results in clinical studies. For these reasons, it is important to understand the molecular mechanisms by which TIMP-1 exerts "oncogenic"

activity in breast epithelium through apoptosis inhibition.

Results

TIMP-1 inhibits apoptosis independent of cell adhesion or cell-cell interaction.

Epithelial cell survival is dependent on interactions of the cells with the ECM (2, 3). Following loss of cell anchorage, epithelial cells undergo anoikis, an apoptotic process caused by loss of substrate adhesion (2, 3). TIMP-1 inhibition of apoptosis may result from its ability to stabilize cell-ECM interactions by inhibiting MMPs. To test whether TIMP-1 inhibits apoptosis in an anchorage dependent manner, we examined the role of TIMP-1 during anoikis. To induce anoikis, control and MCF10A cells overexpressing TIMP-1 or bcl-2 were cultured in dishes coated with polyHEMA, which prevents cell adhesion. After twenty-four hours, cell survival was determined by trypan blue exclusion assay. These studies showed that <20% of control MCF10A cells remained viable in polyHEMA-coated dishes (suspension culture) consistent with induction of anoikis, as previously described (2). In contrast, ~ 80% of bcl-2- or TIMP-1-overexpressing cells remained viable under the same conditions (**Fig. 1A**).

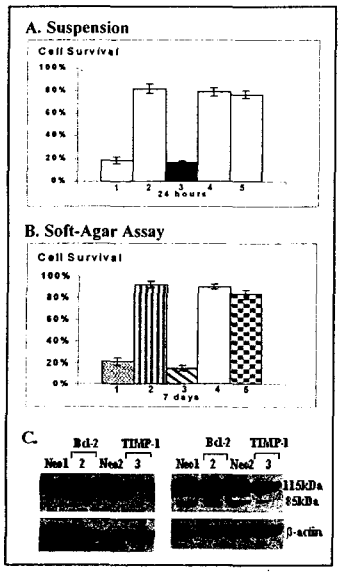

Figure 1. TIMP-1 inhibits anoikis (apoptosis induced by loss of cell anchorage). MCF10Aneo1 (bar 1), bcl-2 MCF10A #2 (bar 2), MCF10Aneo2 (bar 3), and TIMP-1 MCF10A #3 and #29 (bars 4, 5 respectively) cells were cultured in either polyHEMA-coated dishes for 24 h (**A**) or in soft agar for 7 days (**B**). The number of live cells was then determined by trypan blue exclusion. Cell survival is expressed as a percentage of control cells (no treatment). The error bars represent standard deviation of the mean of triplicate samples. Immunoblot analysis of PARP (top panel) and β–actin (bottom panel) from lysates (50 μg/lane) of MCF10Aneo1, bcl-2 MCF10A #2, MCF10Aneo2, and TIMP-1 MCF10A #3 cells cultured on monolayer or in suspension (polyHEMA) (**C**).

Cleavage of poly (ADP-ribose) polymerase (PARP) is an early event in apoptosis, resulting from the activation of caspase/Ced-3 family members (4). We therefore examined PARP cleavage in the control, bcl-2, and TIMP-1-overexpressing cells cultured in polyHEMA-coated dishes. As shown in **Fig. 1C**, apoptosis-specific proteolytic cleavage of PARP (85-kDa fragment) was readily detected in suspension cultures of control cells, whereas it was significantly inhibited in the bcl-2- or TIMP-1-overexpressing cells.

To further test whether TIMP-1 enhanced cell survival resulted from stabilization of cell-cell interactions, anchorage-independent cell survival was also evaluated by a soft agar assay. Cells were trypsinized into single cells and immobilized in soft agar. As shown in **Fig. 1B**, > 80% of bcl-2- or TIMP-1-overexpressing cells remained viable even after 7 days of culture in soft agar, while < 20% of the control MCF10A cells survived. Thus, both bcl-2 and TIMP-1 can prevent anoikis in MCF10A cells.

Taken together, these studies suggest that TIMP-1 inhibits a classical apoptotic pathway mediated by caspases that is independent of its ability to stabilize cell-substrate or cell-cell interactions.

Downregulation of TIMP-1 expression in MCF10A cells and its effects on apoptosis.

To further study TIMP-1-regulation of human breast epithelial cell survival, we established MCF10A clone in which endogenous TIMP-1 expression is downregulated using an anti-sense construct of TIMP-1 cDNA (AS TIMP-1 MCF10A). Immunoblot analysis of TIMP-1 confirmed significant downregulation of TIMP-1 expression in AS TIMP-1 MCF10A cells as shown in **Fig. 2A&B**. To test whether downregulation

We then examined whether TIMP-1 can inhibit caspase activity induced by staurosporine, an apoptotic agent that rapidly decreases the transmembrane potential of the mitochondria, resulting in activation of the intrinsic caspase cascade (5, 6). Downregulation of TIMP-1 expression significantly enhanced staurosporine-induced DEVDase activity in MCF10A cells, and TIMP-1 overexpression effectively prevented DEVDase activity (**Fig. 3B**).

These studies indicate that TIMP-1 regulates classical apoptotic pathways involving caspases following a variety of apoptotic stimuli.

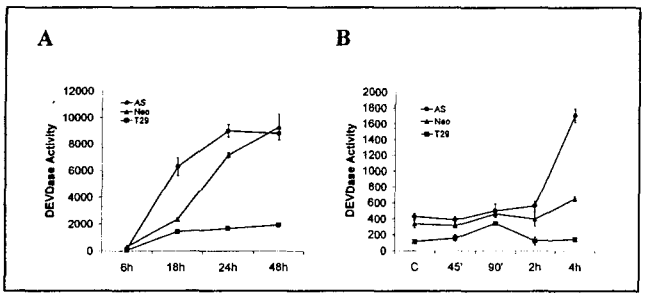

Figure 3. TIMP-1 inhibits DEVDase activity in human breast epithelial cells. Apoptosis was induced in MCF10Aneo (Neo), AS TIMP-1 MCF10A (AS) and TIMP-1 MCF10A #29 (T29) cells by culturing on polyHEMA-coated dishes (**A**), or by treatment with 0.5 μM staurosporine (**B**). At indicated time points, the cells were washed with PBS and lysed with 200 μl lysis buffer as described (**Appendix 3**). After lysates were centrifuged at 16,000 g for 10 min, DEVDase activity in 50 μl cytosol was assayed and the activity was normalized per μg protein. Three independent experiments were performed and the error bars represent standard deviation of the mean of triplicates.

Overexpression of TIMP-1 is associated with constitutive activation of focal adhesion kinase (FAK) in an anchorage-independent manner.

Increasing evidence indicates that cell interactions with the ECM transduce biochemical signals mediated, in part, by focal adhesion kinase (FAK) activation (3, 7-9). Constitutively activated forms of FAK (tyrosine phosphorylated form) protect cells against anoikis (10) and free radical-induced cell death (11), suggesting that FAK activity is critical for cell survival. Therefore, we examined whether TIMP-1 anti-apoptotic activity is involved in modulating FAK activity. Expression levels of FAK were not altered by TIMP-1 overexpression, as determined by immunoblot analysis using an anti-FAK mAb (**Fig. 4A**). We next examined whether TIMP-1 modulates FAK activity. To this end, FAK protein was immunoprecipitated with an anti-FAK mAb and the active form was detected by immunoblot analysis using an anti-phosphotyrosine antibody. As shown in **Fig. 4B**, FAK is more efficiently activated in TIMP-1-overexpressing cells than in the control cells. Since FAK has been shown to require cell anchorage (3, 7-9), we asked whether TIMP-1 upregulation of FAK activation was an anchorage-dependent process. To this end, we cultured control and TIMP-1-overexpressing cells in suspension for 12 hours and examined tyrosine-phosphorylated FAK. As shown in **Fig. 4C**, FAK was constitutively activated in the cells overexpressing TIMP-1 regardless of cell anchorage.

These results suggest that TIMP-1 may regulate apoptosis through constitutive activation of cell survival signaling pathways including FAK activation.

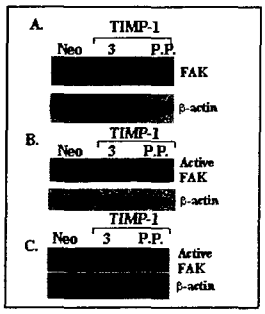

Figure 4. Constitutive activation of FAK in TIMP-1-overexpressing MCF10A cells. A; Lysates (50 μg/lane) of MCF10Aneo, TIMP-1 MCF10A #3 and TIMP-1 MCF10A pooled populations (P.P.) were subjected to immunoblot analysis using an anti-FAK mAb and detection by ECL. The same blot was reprobed with anti-β-actin antibody (bottom panel). **B&C;** MCF10Aneo, TIMP-1 MCF10A #3 and TIMP-1 MCF10App cells were cultured (12 h) in monolayer (**B**) or in suspension (**C**) and solubilized in lysis buffer. The lysates (200 μg in panel B, 400 μg in panel C) were then immunoprecipitated with an anti-FAK mAb and protein G Sepharose beads. The immunoprecipitates were resolved by reducing SDS-PAGE followed by immunoblot analysis with

an anti-phosphotyrosine mAb (top panels). To confirm the amount of immunoprecipitated FAK protein in each sample, the same blot was reprobed with the anti-FAK mAb (bottom panels).

PI 3-kinase and MAPK survival signaling pathways are critical for TIMP-1 inhibition of cell death. FAK is the upstream regulator of the phosphotidylinositol 3-kinase (PI 3-kinase)- serine-threonine kinase/Akt survival pathway (12). Phosphorylated FAK binds to PI 3-kinase and activates its activity, which in turn activates Akt (12, 13). To investigate the role of PI 3-kinase for TIMP-1 mediated cell survival, we examined TIMP-1-mediated cell survival in the presence and absence of wortmannin, an inhibitor of PI 3-kinase (Calbiochem, CA). As shown in **Fig. 5A**, TIMP-1 mediated cell survival was almost completely abolished by wortmannin in TIMP-1 overexpressing MCF10A cells. Similarly, exogenously added TIMP-1-mediated cell survival in AS TIMP-1 MCF10A cells was abolished by the wortmannin treatment (**Fig. 5B**). Since studies suggest significance of cross-talk between the dual signaling pathways, PI 3-kinase-Akt and MEK1-MAPK pathways, for the regulation of cell survival/death (14, 15), we also examined the effect of PD98059 (an inhibitor of MEK1, Calbiochem) on TIMP-1 inhibition of cell death. Inhibition of MEK1/MAPK pathway reduced TIMP-1 overexpressing cell survival following growth-factor withdrawal (**Fig. 5A**). Similarly, PD98059 treatment significantly reduced exogenous TIMP-1-mediated cell survival in AS MCF10A cells, while PD98059 or wortmannin treatment alone had no cytotoxicity in these cells (**Fig. 5B**).

These results suggest that PI 3-kinase and MAPK signaling pathways are critical for transfected TIMP-1- or exogenously added TIMP-1-mediated human breast epithelial cell survival.

Indeed, the levels of active ERKs and PI 3-kinase are significantly higher in TIMP-1 overexpressing MCF10A cells (TIMP-1 MCF10A #29) compared to vector-transfected cells (MCF10Aneo), while TIMP-1 overexpression has little effect on JNKs and p38 (**Figure 6A&C**). More importantly, exogenously added recombinant TIMP-1 proteins activate ERKs and PI 3-kinase in MCF10A cells (**Figure 6B&D**). *These results further support our working hypothesis that extracellular TIMP-1 induces cell survival pathways involving ERKs and FAK/PI 3-kinase in human breast epithelial cells.*

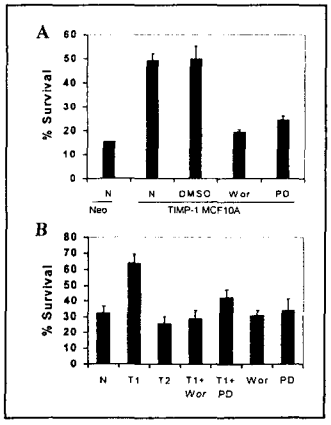

Figure 5. PI 3-kinase and MAPK are critical for TIMP-1 mediated cell survival. A; The control vector transfected (Neo) and TIMP-1 overexpressing MCF10A cells (TIMP-1 MCF10A) were cultured in serum-free medium without (N), or with vehicle only (DMSO), 200 nM wortmannin (wor) or 10 μM PD98059 (PD) for 48 hours. The percentage of cell survival was determined by MTT assay, normalized to the MTT signals in the respective cells cultured in serum containing medium. Shown are the means ± S.E. of the triplicate experiments. **B;** AS TIMP-1 MCF10A cells were cultured in serum free medium in the absence (N) or presence of 500 ng/ml recombinant TIMP-1 (T1), 500 ng/ml recombinant TIMP-2 (T2), TIMP-1 and 200 nM Wortmannin (T1+Wor), TIMP-1 and 10 μM PD98059 (T1+PD), 200 nM Wortmannin (Wor), or 10 μM PD98059 (PD). After 48 hr, the percentage of cell survival was determined as described above.

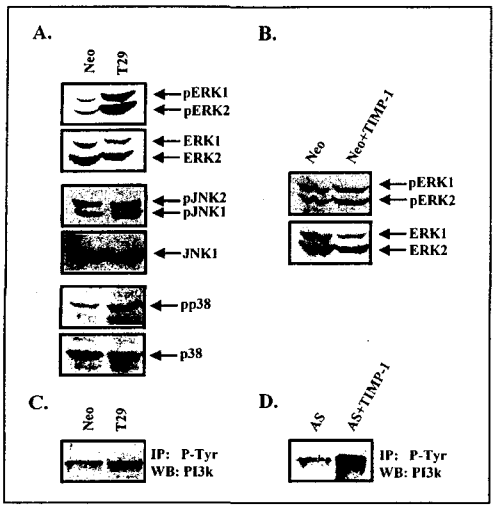

Figure 6. TIMP-1 activates ERKs and PI 3-kinase in human breast epithelial cells. **A;** Cell lysates (80μg/lane) of 48 hr serum-starved MCF10Aneo (Neo) and TIMP-1 MCF10A #29 (T29) cells were subjected to immunoblot analysis with anti-active ERKs (pERK1/2), anti-ERK1/2, anti-active JNK 1/2 (pJNK1/2), anti-JNK1, anti-active p38 (pp38), anti-p38 antibodies. **B;** MCF10Aneo (Neo) cells were treated without or with 500ng/ml recombinant TIMP-1 proteins for 10 min. Cell lysates (80μg/lane) were subjected to immunoblot analysis with an anti-active ERKs (pERK1/2), anti-ERK1/2 antibodies. **C;** Cell lysates (500μg/lane) of 48 hr serum-starved MCF10Aneo (Neo) and TIMP-1 MCF10A #29 (T29) cells were immunoprecipitated with an anti-phosphotyrosine mAb and protein G agarose beads. The immunopreciptitates were subjected to immunoblot analysis with an anti-PI 3-Kinase p85α polyclonal antibody. **D;** Cell lysates (500μg/lane) of AS TIMP-1 MCF10A (AS) cells without or with 500ng/ml recombinant TIMP-1 treatment for 10 min were immunoprecipitated with an anti-phosphotyrosine mAb and subjected to immunoblot analysis with anti-PI 3-Kinase p85α polyclonal antibody.

TIMP-1 binds on the MCF10A cell surface.

The anti-apoptotic effect of the exogenous TIMP-1 suggested that TIMP-1 binding to the cell surface activates cell survival signaling in human breast epithelial cells. To examine whether TIMP-1 localizes on the cell surface, we used immunofluorescence analysis using confocal microscopy. To this end, non-permeabilized MCF10A cells, which express endogenous TIMP-1, were immunostained with a specific rabbit pAb to TIMP-1. As shown in **Fig. 7C**, MCF10A cells exhibit a punctate pattern of TIMP-1 on the cell periphery indicating that TIMP-1 is associated with the cell surface. Addition of exogenous recombinant TIMP-1 to the MCF10A cells resulted in an enhanced signal consistent with binding of TIMP-1 protein to the cell surface (**Fig. 7D**). These stainings were specific since without primary antibody (**Fig. 7A**) or use of preimmune rabbit IgG (**Fig. 7B**) resulted in lack of signals. Taken together, these studies demonstrate that the endogenous TIMP-1 is present on the surface of MCF10A cells and that addition of recombinant TIMP-1 results in association of TIMP-1 with the cell surface. In a control experiment, TIMP-1 was not detected in TIMP-1 -/- murine fibroblasts, as expected (**Fig. 7E**). Consistent with the result shown in **Fig. 7D** (TIMP-1 -/- fibroblasts do not respond to the anti-apoptotic activity of TIMP-1), exogenously added recombinant TIMP-1 proteins failed to associate with cell surface components in TIMP-1 -/- fibroblasts (**Fig. 7F**).

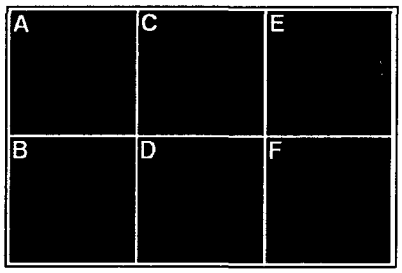

Figure 7. Immunofluorescence study of the TIMP-1 protein on MCF10A cell surface. MCF10A (**A-D**) and TIMP-1 -/- murine fibroblast (**E&F**) cells were incubated with 1% BSA for 1 hr, followed by incubation with 0 (**A,B,C,E**) or 500 ng/ml recombinant TIMP-1 proteins (**D&F**). After washing with PBS for 6 times, cells were incubated without (**A**), with rabbit preimmune IgG (**B**), or with rabbit anti-TIMP-1 antibody (that recognizes both murine and human TIMP-1 proteins) (**C-F**), followed by incubation with anti-rabbit IgG-FITC (**A-F**). After washing with PBS for 6 times, cells were fixed and mounted with anti-fade mounting medium containing 1.5 μg/ml DAPI (Vector Laboratory, CA). The cells were examined with a Zeiss LSM microscope in the confocal mode. The nuclei stained with DAPI were shown in blue, and TIMP-1 proteins in green.

TIMP-1 specifically binds to a 150-kDa surface protein in MCF10A cells.

To identify TIMP-1 binding protein(s) on the surface of MCF10A cells, the cells were surface biotinylated with sulfo-NHS-biotin (Pierce, IL). The lysates were subjected to immunoprecipitation with the pAb to TIMP-1 and the immunoprecipitates were resolved by SDS-PAGE followed by detection of the biotinylated proteins with avidin-HRP. **Fig. 8A** (lane 4) shows that the pAb to TIMP-1 specifically precipitated biotinylated TIMP-1 (~29 kDa), as expected, and an additional biotinylated protein of ~150-kDa (arrow). These proteins were not detected in non-biotinlylated cells (**Fig. 8A**, lane 2) demonstrating that the signal was not due to non-specific binding of the avidin-HRP. Furthermore, preimmune IgG failed to immunoprecipitate these proteins (**Fig. 8A**, lane 3) confirming the specificity of the signals. The ~60 kDa protein (marked with an aterisk) represents a non-specific band as it was also detected with the preimmune IgG. To confirm that the biotinylated ~29 kDa protein (T1 in **Fig 8A**, lane 4) was indeed TIMP-1, and to ensure that the co-immunoprecipitation efficiencies between biotinylated and non-biotinylated lysates were comparable, the same blots were reprobed with the anti-TIMP-1 antibody. This experiment confirmed that the ~29 kDa protein (T1, lane 4) as TIMP-1 protein, and that the efficiencies of the immunoprecipitations were comparable (**Fig. 8B**).

Ligand blot analyses were also used to identify TIMP-1 binding proteins (**Fig 8C**). Briefly, cell lysates of MCF10A cells were subjected to SDS-PAGE analysis and transferred to a nitrocellulose membrane. The filter strips were then incubated without (**Fig. 8C**, lane 1) or with ~500 ng/ml biotinylated recombinant TIMP-1 protein (**Fig. 8C**, lane 2). Proteins interacting with biotinylated TIMP-1 were visualized by avidin-HRP detection. These studies identified a protein of ~150-kDa that was specifically detected in the presence of TIMP-1. The ~70- kDa band (§) represents a non-specific protein since it was observed in the absence of TIMP-1.

These data demonstrate that TIMP-1 can be detected on the surface of MCF10A cells and that a ~150-kDa surface protein (refereed to as p150) forms a specific complex with TIMP-1.

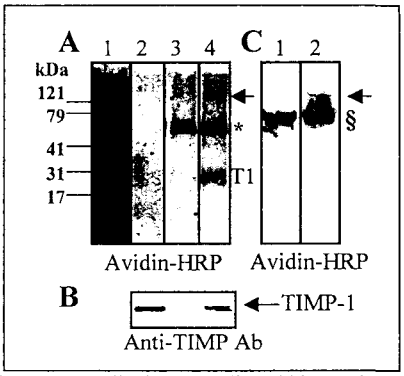

Figure 8. Identification of the putative TIMP-1 binding protein on the cell surface. **A**; Ten μg of surface-biotinylated MCF10A lysates were subjected to SDS-PAGE analysis (lane 1). Five hundred μg of non-biotinylated (lane 2) and cell-surface biotinylated (lane 3&4) MCF10A cell lysates were subjected to a co-immunoprecipitation experiment with anti-TIMP-1 antibody (lane 2&4) or with rabbit preimmune IgG (lane 3). TIMP-1 complexes were detected by streptavdin-HRP. **B**; The same filter of panel A was subjected to immunoblot analysis using anti-TIMP-1 antibody. **C**; A ligand blot analysis: Fifty μg of total cell lysates were subjected to SDS-PAGE analysis, transferred to a nitrocellulose membrane, and the strips of lane 1 and lane 2 were incubated with 5% non-fat milk in TTBS, followed by incubation with 0 (lane 1) and 500 ng/ml biotinylated TIMP-1 proteins (lane 2). TIMP-1 binding proteins were detected by avidin-HRP.

Summary

Our studies demonstrate that (i) TIMP-1 expression is highly induced by the anti-apoptotic gene bcl-2 in breast epithelial cells; (ii) endogenously expressed TIMP-1 or exogenous TIMP-1 can protect against apoptosis induced by a variety of apoptotic insults; (iii) TIMP-1 inhibition of apoptosis does not depend on its ability to stabilize cell-ECM or cell-cell interactions; (iv) TIMP-1 constitutively activates focal adhesion kinase (FAK); (v) FAK/PI 3-kinase and MAPK signaling is critical for TIMP-1 mediated cell survival; (vi) TIMP-1 binds to the cell surface of MCF10A cells; and (vii) a 150-kDa surface protein binds TIMP-1. Based on these results, we hypothesize that TIMP-1 binding on the cell surface, induces a signal transduction pathway leading to FAK and MAPK activation. Active FAK induces PI 3-kinase/Akt and MAPK survival pathway, which regulates the apoptosis commitment step involving bcl-2 family members.

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