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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, including cell adhesion signaling, regulate apoptosis. It is also unknown whether gene products regulating the apoptosis commitment step have any effect on the upstream survival pathways. Our study demonstrated that the anti-apoptotic gene product bcl-2 greatly induces expression of the tissue inhibitor of metalloproteinase-1 (TIMP-1), but not TIMP-2, 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. TIMP-1 constitutively activates focal adhesion kinase (FAK) independent of cell anchorage or cell-cell interactions and inhibits a classical apoptotic pathway mediated by caspases. Furthermore, we show that exogenous recombinant TIMP-1 protects against apoptosis. Taken together, our study suggests that bcl-2 inhibition of apoptosis involves TIMP-1 induction and TIMP-1 binding on the cell surface induces a cell survival pathway that regulates apoptosis.

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(4) TABLE OF CONTENTS

(5) Introduction

The goals of the Career Development Award application are to understand the role of bcl-2 in breast cancer progression and to investigate the molecular mechanisms of bcl-2 action. We previously reported a potential oncogenic activity for bcl-2 through cyclin D_1 induction (Lin et al., Cell Death and Differentiation, In press). Here, we report our novel observation toward understanding the molecular mechanism by which bcl-2 regulates breast epithelial cell apoptosis.

The signaling pathways critical for cell survival are mediated in part by the composition and integrity of the extracellular matrix and the action of its components on specific cell adhesion receptors (1-5). Withdrawal of anchorage-dependent epithelial cells from their association with ECM results in apoptotic cell death (6, 7). Consistently, matrix degrading enzymes (MMPs) or their inhibitors (TIMPs) have been suggested to regulate apoptosis (8, 9). In this report, we investigated whether bcl-2 inhibition of apoptosis involves regulation of TIMP expression. Here we report that bcl-2 overexpression induces TIMP-1 expression in breast epithelial cell lines (MCF10A, MCF10AneoT.TG3B and MCF-7), while it has no effect on TIMP-2 expression. We demonstrated that TIMP-1 inhibits cell death induced by hydrogen peroxide, Adriamycin or X-ray irradiation. In addition, TIMP-1 overexpression inhibits apoptosis following the loss of cell adhesion (anoikis) in MCF10A cells suggesting that the anti-apoptotic activity of TIMP-1 does not depend on its ability to stabilize cell-matrix interactions. We also showed that TIMP-1 overexpression is associated with a constitutive activation of focal adhesion kinase, a signaling molecule known to be critical for the cell survival pathway.

Taken together, our studies have found that oncogenic activity of bcl-2 involves regulation of gene expression including cyclin D_1 (Lin et al., Cell Death and Differentiation, In press) and TIMP-1 (Li et al., Cancer Research 59:6267-6275, 1999), critical for cell cycle regulation and apoptosis.

(6) Body of Report

Methods and Results

(6-1) Bcl-2 upregulates TIMP-1 expression.

To examine whether bcl-2 inhibition of apoptosis involves regulation of TIMPs expression in human breast epithelial (BE) cells, we introduced a bcl-2 expression vector into MCF10A ("normal" BE cell line), MCF10AneoT.TG3B (preneoplastic BE cell line) and MCF-7 (malignant breast carcinoma cell line). Bcl-2 overexpression in the bcl-2 transfected MCF10A (Fig. 1A), MCF10AneoT.TG3B (Fig. 1B) and MCF-7 (Fig. 1C) clones was confirmed by immunoblot analysis, and several overexpressing clones were identified and selected for further studies. The bcl-2 clones exhibited higher levels of TIMP-1 protein (Fig. 2) and mRNA (Fig. 3) than control cells. Both the intracellular and the extracellular TIMP-1 protein levels were elevated. The intracellular TIMP-1 was detected as a doublet, probably representative of both the precursor and the fully glycosylated (mature) forms (Fig. 2). In contrast to TIMP-1, the levels of TIMP-2 protein and mRNA expression were not altered (data not shown).

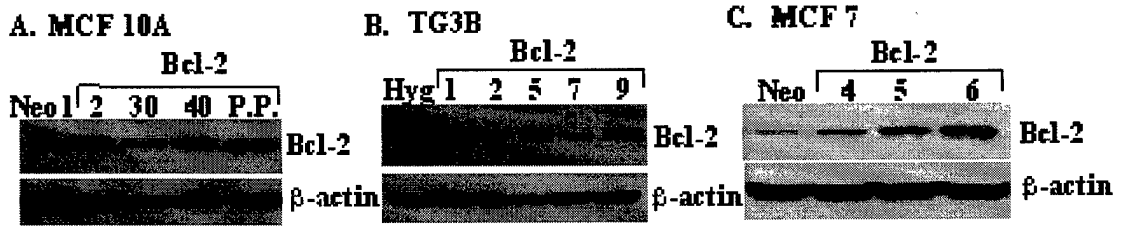


Figure 1. Bcl-2 overexpression in MCF10A, MCF10aneoT.TG3B and MCF7 cells. Lysates (50 μg/lane) of vector-transfected (neo or hygro) or bcl-2-transfected clones of MCF10A (**A**), TG3B (**B**) and MCF-7 (**C**) cells were subjected to immunoblot analysis with an anti-bcl-2 mAb. Detection of the antigen was performed using ECL. The bottom panels in A-C show the β-actin levels of the respective blots reprobed with an anti-human β-actin antibody.

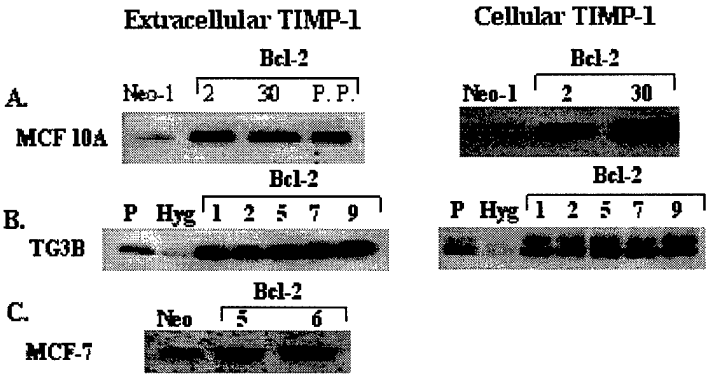


Figure 2. Bcl-2 upregulates TIMP-1 protein expression in human breast epithelial cells. Lysates (cellular; 50 μg/lane) and media (extracellular; 25 μl/lane) of parental (P), vector-transfected (neo or hygro) and bcl-2-overexpressing clones of MCF10A (**A**), TG3B (**B**) and MCF-7 (**C**) cells were subjected to immunoblot analysis with an anti-TIMP-1 antibody followed by detection with ECL.

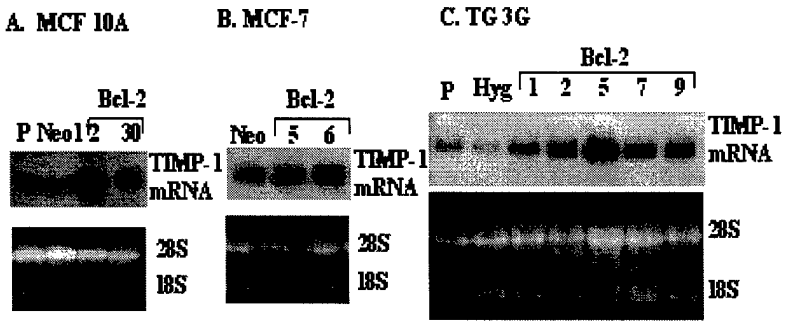


Figure 3. Bcl-2 upregulates TIMP-1 mRNA expression in human breast epithelial cells. Northern blot analysis of total RNA (10 μg/lane) isolated from parental (P), vector-transfected (neo or hygro) and bcl-2-overexpressing clones of MCF10A (**A**), MCF-7 (**B**) and TG3B (**C**). Blots were probed with a human TIMP-1 cDNA probe as described under "Materials and Methods" section. Equal loading of RNAs was confirmed by staining the membranes with ethidium bromide (bottom panels).

(6-2). TIMP-1 inhibits apoptosis in breast epithelial cells.

To examine whether TIMP-1 plays a direct role in cell survival after exposure to apoptotic stimuli, MCF10A cells were exposed to H_2O_2 in the presence or absence of exogenously added TIMP-1. Recombinant TIMP-1 protein was produced using a vaccinia expression system and purified as previously described (10). Preliminary dose-dependence experiments showed that H_2O_2 (250 to 500 μM) induced apoptosis as determined by nuclear morphological analysis (11). As shown in Table 1, in the absence of TIMP-1, approximately 12% of cells remained viable following 48 hours of H_2O_2 treatment. In the presence of TIMP-1 (500 ng/ml), cell survival increased to 23%. In contrast, similar amounts of TIMP-2 had no effect on H_2O_2-induced cell death in MCF10A cells (data not shown). These results indicate that TIMP-1, but not TIMP-2, increases human breast epithelial cell survival.

Table 1. Exogenous TIMP-1 increases MCF10A cell survival following H_2O_2 treatment. Triplicate culture dishes of MCF10A cells were treated (48 h) with or without H_2O_2 (500 μM) in the presence or absence of TIMP-1 (500 ng/ml). Control cells received no treatment. The number of surviving cells was determined by trypan blue exclusion. Cell survival after each treatment is expressed as a percentage of control cells (100%).

	Experiment Number				
	1	2	3	Mean± SD	% cell Survival
	Cell number X 10^5				
Control	7.2	8.8	10.1	8.70±1.45	100%
TIMP-1	7.7	9.6	8.3	8.53±0.97	97.7%
H_2O_2	1.0	0.9	1.3	1.07±0.21	12.3% [a]
TIMP-1+H_2O_2	1.7	2.3	1.9	1.97±1.45	22.6%[a]

[a] $P<0.02$ (H_2O_2 versus H_2O_2 + TIMP-1)

To further investigate the role of TIMP-1 in apoptosis regulation, we introduced a TIMP-1-expression vector into MCF10A cells. As shown in Fig. 4, expression levels of TIMP-1 increased 3-6 fold in the TIMP-1-transfected MCF10A cells. TIMP-1 expression levels in TIMP-1-transfected MCF10A cells were comparable to those observed in MCF10A cells overexpressing bcl-2. We next investigated whether TIMP-1 overexpression could enhance cell survival against H_2O_2, Adriamycin and irradiation. In addition, we compared the TIMP-1 overexpressing cells with the bcl-2-overexpressing cells. These studies demonstrated similar survival rates after these treatments in MCF10A cells overexpressing TIMP-1 or bcl-2 (Fig. 5). TIMP-1 inhibition of apoptosis was further confirmed by nuclear morphological analysis (Fig. 6). Whereas the control cells showed fragmented nuclei that were consistent with nuclear morphological changes in apoptotic cells (11), no significant changes in nuclear morphology could be observed in either the TIMP-1- or the bcl-2 overexpressing cells. *These studies clearly demonstrated that TIMP-1, like bcl-2, is a potent inhibitor of apoptosis induced by a variety of apoptotic stimuli. Furthermore, both exogenous and endogenous expression of TIMP-1 has anti-apoptotic activity.*

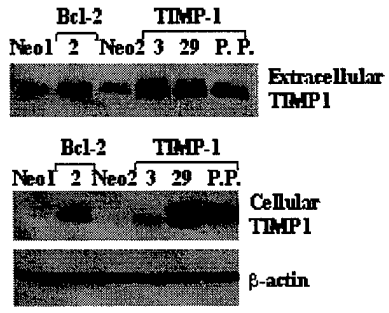
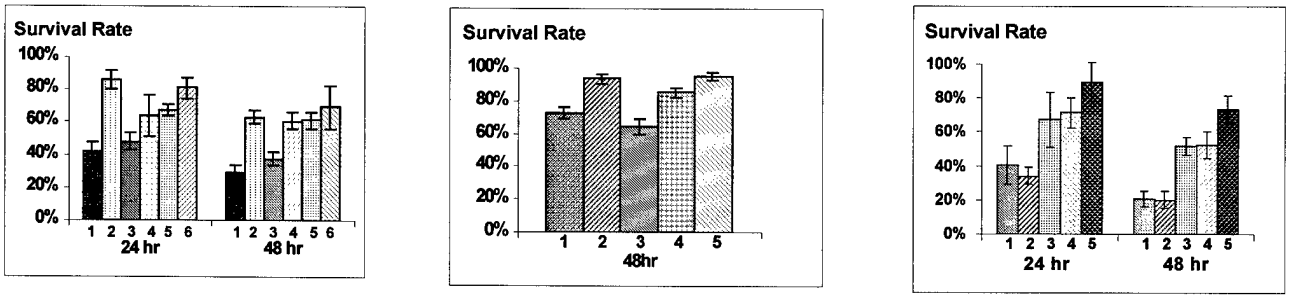


Figure 4. TIMP-1 overexpression in MCF10A cells. (A) Media (extracellular; 25 μl/lane) and (B) lysates (cellular; 50 μg/lane) of vector-transfected (neo), bcl-2- and TIMP-1-transfected clones of MCF10A cells were subjected to immunoblot analysis with an anti-TIMP-1 antibody followed by detection with ECL. The bottom panel in B shows the β-actin levels of the respective blot reprobed with an anti-human β–actin antibody.

A. H_2O_2 **B. Radiation** **C. Adriamycin**



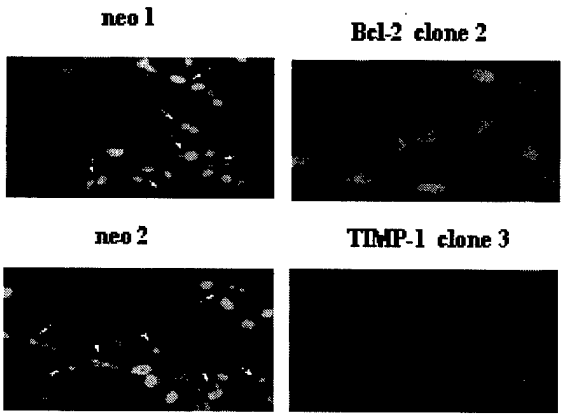
1. MCF10Aneo1
2. Bcl-2 MCF10A #2
3. MCF10Aneo2
4. TIMP-1MCF10A #3
5. TIMP-1 MCF10A #29
6.TIMP-1 MCF10App

1. MCF10Aneo1
2. Bl-2 MCF10A #2
3. MCF10Aneo2
4. TIMP-1MCF10A #3
5. TIMP-1 MCF10A #29

1. MCF10Aneo1
2. MCF10Aneo2
3. TIMP-1 MCF10A #3
4. TIMP-1MCF10A #29
5. TIMP-1 MCF10Aneopp

Figure 5. Effect of TIMP-1 on cell survival following treatments of H_2O_2, radiation, and Adriamycin. MCF10A cells (vector- and bcl-2 or TIMP-1-transfected) were either treated with 500 μM H_2O_2 (**A**), irradiated at 6 Gy with a Cobalt-60 radiation unit (**B**) or treated with 0.5 μg/ml Adriamycin (**C**). At 24 and 48 h post-treatment, the number of live cells was determined by either trypan blue exclusion (**A** and **C**) or by sulforhodamine B staining (**B**) The percentage of cell survival (Survival Rate) was normalized to the respective control cells (no treatment). All experiments were performed in triplicate and the error bars represent the standard deviation.

Figure 6. TIMP-1 inhibits H_2O_2-induced apoptosis in MCF10A cells. MCF10Aneo1, bcl-2 MCF10A #2, MCF10Aneo2 and TIMP-1 MCF10A #3 cells were treated (48 h) with 500 μM H_2O_2 and analyzed for nuclear morphology using bis-Benzimide staining. Arrowheads show apoptotic nuclei.



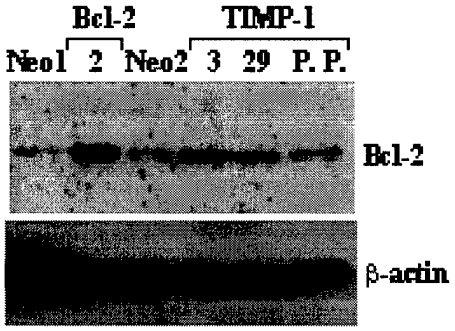
(6-3) TIMP-1 regulation of apoptosis.

TIMP-1 inhibition of apoptosis is independent of its effect on proliferation.

In order to demonstrate that TIMP-1-enhanced cell survival following H_2O_2 treatment was not due to TIMP-1 mitogenic activity, the following experiment was performed. MCF10A cells were incubated with ^{3}H-

thymidine for 48 hours, followed by serum-free culture for 24 hours without ^{3}H-thymidine, and then examined for survival after H_2O_2 treatment in the presence or absence of TIMP-1. If TIMP-1 does not inhibit cell death but increases cell number through induction of cell proliferation, the rate of ^{3}H-thymidine loss would be similar regardless of TIMP-1 treatment. Cells treated with H_2O_2 in the presence of TIMP-1 had 1.5-2 fold more ^{3}H-thymidine incorporated cells as compared to H_20_2-treated cells without TIMP-1 (data not shown). *These studies showed that the increased cell survival rate following H_2O_2 treatment in the presence of TIMP-1 (Table 1) results from an effect of TIMP-1 on cell survival, not on proliferation.*

Figure 7. TIMP-1 overexpression has no effect on bcl-2 protein levels. Lysates (50 μg/lane) of vector-transfected (neo), bcl-2- or TIMP-1-overexpressing MCF10A clones were subjected to immunoblot analysis with an anti-bcl-2 mAb. Detection of the antigen was performed using ECL. The bottom panel shows the β-actin levels of the respective blots reprobed with an anti-human β–actin mAb.



TIMP-1 inhibits apoptosis in the absence of bcl-2 overexpression.

To determine whether the anti-apoptotic effects of TIMP-1 were related to the level of bcl-2 expression, we examined the effects of TIMP-1 overexpression on bcl-2 expression level. As shown in Fig 7, TIMP-1 overexpression had no effect on the basal levels of bcl-2 expression. *Thus, TIMP-1 inhibition of apoptosis does not result from upregulation of bcl-2 expression, but may involve a novel anti-apoptotic pathway.*

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

Epithelial cell survival is dependent on interactions with the ECM (6, 12). Following loss of cell anchorage, epithelial cells undergo anoikis, an apoptotic process caused by loss of substrate adhesion (6, 12). 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 anchorage dependent manner, we examine 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 (6). In contrast, ~ 80% of bcl-2- or TIMP-1-overexpressing cells remained viable under the same conditions (Fig. 8A).

Cleavage of poly (ADP-ribose) polymerase (PARP) is an early event in apoptosis, resulting from the activation of caspase/Ced-3 family members (13). We therefore examined PARP cleavage in the control, bcl-2, and TIMP-1-overexpressing cells cultured in polyHEMA-coated dishes. As shown in Fig. 8C, 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. 8B, > 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. Consistently, analysis of gelatinase (MMP-2 and MMP-9) expression in the bcl-2 or TIMP-1 overexpressing cells showed no correlation with bcl-2 or TIMP-1 expression; therefore, gelatinase expression could not be associated with the apoptosis sensitivity (data not shown).

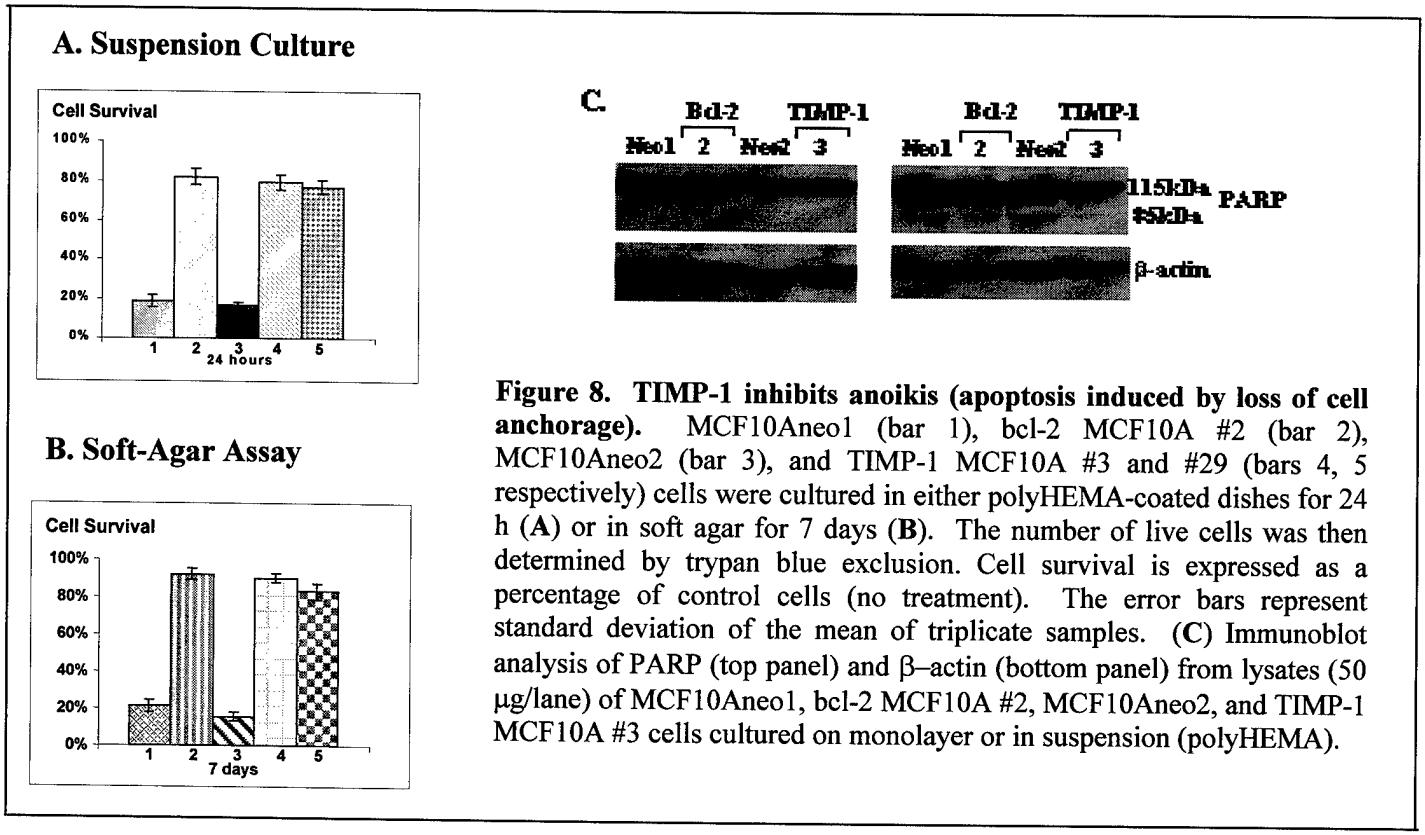


Figure 8. TIMP-1 inhibits anoikis (apoptosis induced by loss of cell anchorage). MCF10Aneo1 (bar 1), bcl-2 MCF10A #2 (bar 2), MCF10Aneo2 (bar 3), and TIMP-1 MCF10A #3 and #29 (bars 4, 5 respectively) cells were cultured in either polyHEMA-coated dishes for 24 h (**A**) or in soft agar for 7 days (**B**). 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. (**C**) 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).

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 (12, 14-16). Constitutively activated forms of FAK (tyrosine phosphorylated form) protect cells against anoikis (14-16) and free radical-induced cell death (17), 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. 9A). 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. 9B, FAK is more efficiently activated in TIMP-1-overexpressing cells than in the control cells. Since FAK has been shown to require cell anchorage (12, 14-16), 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. 9C, 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.

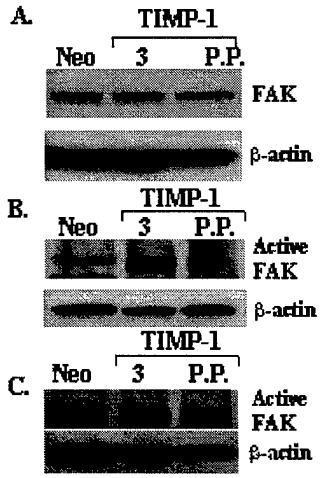


Figure 9. Constitutive activation of FAK in TIMP-1-overexpressing MCF10A cells. (**A**) Lysates (50 μg/lane) of MCF10Aneo, TIMP-1 MCF10A #3 and TIMP-1 MCF10App cells were subjected to immunoblot analysis using an anti-FAK mAb and detection by ECL. The same blot was reprobed with anti-β-actin antibody (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 panle 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).

TIMP-1 inhibits caspases induced by loss of cell adhesion or by staurosporine.

TIMP-1 inhibits cleavage of poly(ADP-ribose) polymerase (PARP) following loss of cell adhesion (anoikis) (Fig. 8), suggesting that TIMP-1 downregulates caspase activity. Since caspases including caspase-3 and –7 cleave PARP at the $DEVD^{216}$-G site, DEVDase activity was determined by fluorescence released from the tetrapeptide substrate Ac-DEVD-amc (as shown Fig. 10). DEVDase activity increased 3 fold in the control cells at 24 hr following the loss of cell adhesion. In contrast, DEVDase activity was significantly lower (~3 fold) in the TIMP-1 overexpressing cells during anoikis. 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 caspase activation (18, 19). DEVDase activity rapidly increased ~4 fold following 2 hrs of treatment with staurosporine, while there was no detectable increase in DEVDase activity in TIMP-1 overexpressing cells.

These studies demonstrate that TIMP-1 overexpression results in inhibition of caspases following a variety of apoptotic stimuli.

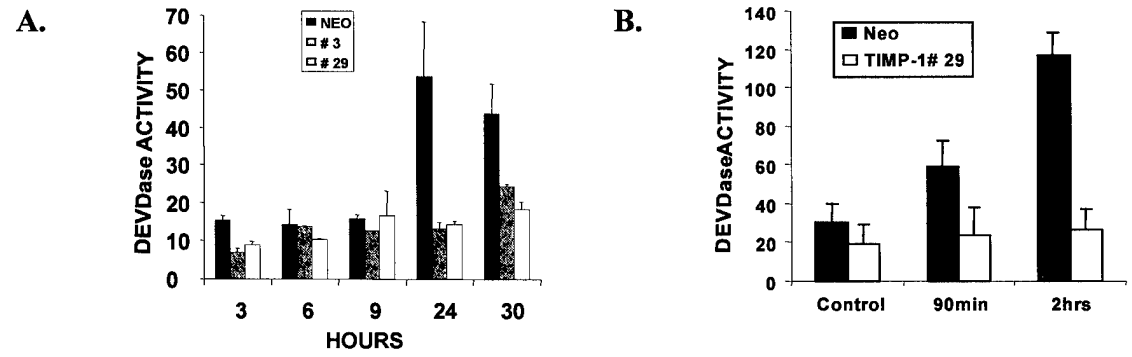


Fig. 10. TIMP-1 inhibits DEVDase activity in human breast epithelial cells. Apoptosis was induced in MCF10Aneo, TIMP-1 MCF10A #3 and #29 cells by culturing on polyHEMA-coated dishes (**A**), or by treatment with 0.5 μM staurosporine (**B**). At indicated time points, the cells were washed with PBS and 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.

(7) Conclusion

An explosive progress towards dissecting the molecular basis for regulation of the apoptosis commitment step has been made during the past decade. Mitochondria, Apaf-1 (CED-4), caspase (CED-3) and bcl-2 (CED-9) family members play central roles in regulation of the apoptosis commitment step. However, it is still unclear how upstream cell survival pathways such as growth factor- and cell adhesion-mediated signaling regulate apoptosis. Also, it is unknown whether CED-4, CED-3, or CED-9 family members have any effect on the upstream survival pathways. Our study supported by DOD CDA clearly demonstrated that bcl-2 regulation apoptosis involves induction of TIMP-1 which activates the FAK survival pathway.

Reportable Outcomes

DOD CDA has been extremely helpful for the PI to establish a breast cancer research program. Support from DOD CDA has been acknowledged in the following publications.

Kim, H.-R. C., Li, G., Kim, H.E., Han, S.J. Rahman, K. H., Liu, H., Waid, D. and Lee, Y. J. Levels of $p21^{WAF1/CIP1}$ do not affect radiation-induced cell death in human breast epithelial cells. Int. J. Oncology 11: 1349-1353, 1997

Akahani S., Nangia-Makker N., Inohara, H., **Kim, H.-R. C.** and Raz, A. Galectin-3: A novel anti apoptotic molecule with a functional BH1 (NWGR) domain of bcl-2 family. Cancer Res. 57: 5272-5276, 1997

Kim, H.-R. C., Lin, H.-M., Biliran, H. and Raz A. Cell cycle arrest and inhibition of anoikis by galectin-3 in human breast epithelial cells. Cancer Res. 59: 4148-4154, 1999

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