Tissue inhibitor of metalloproteinases-1 signalling pathway leading to erythroid cell survival

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Tissue inhibitors of metalloproteinases (TIMP) are specific inhibitors of matrix metalloproteinases (MMPs) and thus participate in maintaining the balance between extracellular matrix deposition and degradation in several physio-pathological processes. Nevertheless, TIMP must be regarded as multifunctional proteins involved in cell growth, angiogenesis and apoptosis. The molecular mechanisms induced by TIMP remain largely unknown. In the present study, we provide evidence that TIMP-1 induces a significant anti-apoptotic effect in the human erythroleukaemic cell line UT-7 and in the murine myeloid cell line 32D. Using specific kinases inhibitors, we show that TIMP-1-mediated cell survival is dependent upon Janus kinase (JAK) 2 and phosphoinositide 3-kinase (PI 3-kinase) activities. By transient trans-fection of dominant-negative Akt in UT-7 cells, we demonstrate that this kinase is crucial for the TIMP-1 anti-apoptotic effect. Moreover, TIMP-1 enhances specific phosphorylation of both Akt and Bad (Bcl-2/Bcl-X_L-antagonist, causing cell death) in a PI 3-kinase-dependent manner and, besides, controls the level of the anti-apoptotic protein Bcl-X_L. We conclude that TIMP-1 induces haematopoietic cell survival via the JAK2/PI 3-kinase/Akt/Bad pathway.

Key words: anti-apoptotic proteins, Janus kinase 2 (JAK2), phosphoinositide 3-kinase (PI 3-kinase), tissue inhibitors of metalloproteinases-1 (TIMP-1).

INTRODUCTION

Matrix turnover is a process highly regulated by a balance between matrix metalloproteinases (MMPs) and their natural inhibitors, the tissue inhibitors of metalloproteinases (TIMP) [1]. Four TIMP have been characterized, designated TIMP-1, TIMP-2, TIMP-3 and TIMP-4 [2]. Although TIMP act as MMP inhibitors by forming non-covalent complexes, previous investigations [3–6] have pointed out that they also exhibit multifunctional activities distinct from MMP inhibition. Cell-growth-promoting activity was described for TIMP-1 and TIMP-2 on a variety of cultured cell lines, including osteosarcoma cell lines, keratinocytes and haematopoietic cell lines [6]. Recently, TIMP-1 has been shown to display an anti-apoptotic activity on Burkitt's lymphoma cell lines, B cells, human breast cell lines and hepatic cells [7–10]. Survival is induced by a variety of biological, physical or chemical factors that trigger different mechanisms, leading to a common executing system involving the phosphoinositide 3-kinase (PI 3-kinase) pathway [11]. The activation of PI 3-kinase leads to phosphorylation of membrane phosphoinositides at the D-3 position. These 3′-phosphorylated phospholipids can then mediate the diverse cellular functions of PI 3-kinase, leading to the activation of several serine/threonine kinases. The serine/threonine kinase Akt is considered to be one of the major targets of PI 3-kinase involved in cell survival. This kinase is recruited to the inner surface of the plasma membrane and activated by phosphorylation catalysed by phosphoinositide-dependent kinase ('PDK') I and II [12]. Several Akt targets have been identified as promoters of cell survival, such as the pro-apoptotic member of the Bcl-2 family

Bad (Bcl-2/Bcl-X_L-antagonist, causing cell death). Following its phosphorylation at Ser[136] by Akt, Bad is sequestered by 14-3-3 proteins in the cytoplasm, so preventing its heterodimerization with Bcl-X_L and its ability to inactivate this anti-apoptotic protein [13]. The intracellular signalling pathways underlying the anti-apoptotic effect of TIMP-1 remain poorly defined. TIMP-1 and TIMP-2 have been reported to stimulate DNA synthesis in the human osteosarcoma cell line MG-63 through tyrosine phosphorylation and further activation of the mitogen-activated protein kinases (MAP kinases) p42/p44 [14]. TIMP-2 also mediated a mitogenic response in normal dermal fibroblasts and fibrosarcoma cells by stimulating adenylate cyclase and cAMP-dependent protein kinase A [2,15]. We have demonstrated previously [16] that TIMP-1 induced tyrosine phosphorylation of intracellular proteins in the human erythroleukaemic cell line UT-7 and that TIMP-1 induced UT-7 cell erythroid differentiation through the activation of the p38 MAP kinase pathway.

In the present study, we show that TIMP-1 also induces survival of both haematopoietic UT-7 and 32D cells and investigate the intracellular signalling pathway involved. We present evidence that TIMP-1-induced survival is mediated by the PI 3-kinase and the phosphorylation of the downstream signalling proteins Akt and Bad.

EXPERIMENTAL

Materials

[γ-^{32}P]ATP was from Amersham Biosciences (Orsay, France). Human recombinant TIMP-1 and AG490 were purchased from

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Calbiochem-Novabiochem (San Diego, CA, U.S.A.). Human purified recombinant erythropoietin (Epo) (specific activity of 120 000 units/mg), annexin-V-FLUOS and propidium iodide were purchased from Roche Molecular Biochemicals (Meylan, France). α-Minimal essential medium (α-MEM), Iscove's modified Dulbecco's medium and LIPOFECTAMINE™ 2000 were from Life Technologies (Cergy Pontoise, France). Anti-Bcl-$X_{S/L}$ antibodies (S-18) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-phospho-JAK2, -phospho-Akt, -phospho-Bad, -JAK2, -Akt, -Bad, horseradish peroxidase-conjugated anti-(rabbit IgG) antibodies and LY294002 were obtained from New England Biolabs (Beverly, MA, U.S.A.). Anti-p85 antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Renaissance Kit was from NEN Life Sciences Products (Paris, France). All other reagents were purchased from Sigma Aldrich (Saint Quentin Fallavier, France).

Cell culture and stimulation

The UT-7 Epo-dependent erythroid cells were cloned from the human leukaemic cell line UT-7 [17]. These cells were cultured in α-MEM containing 10 % (v/v) fetal calf serum (FCS), 2 mM L-glutamine and 2 units/ml Epo. The 32D interleukin-3 (IL-3)-dependent myeloid progenitor cells were grown in α-MEM containing 10 % (v/v) fetal calf serum, 2 mM L-glutamine and 3–5 % (v/v) conditioned medium from WEHI cells as a source of IL-3. To study their stimulation and further signal transduction pathways, UT-7 and 32D cells were serum- and growth-factor-deprived before each experiment by incubation overnight and for 4 h respectively, in Iscove's modified Dulbecco's medium supplemented with 0.2 % (v/v) deionized BSA, 2 mM L-glutamine and 20 μg/ml human holo-transferrin. Cells were then incubated in Iscove's modified Dulbecco's medium containing various concentrations of TIMP-1 and stimulated for 0–30 min at 37 °C as indicated. The reaction was stopped by adding ice-cold PBS containing 50 μM Na$_3$VO$_4$. In some experiments, cells were preincubated for 20 min with LY294002 or for 16 h with AG490 before TIMP-1 stimulation.

Preparation of total cell extracts and cell lysates

Following TIMP-1 stimulation as described, cells were washed twice with PBS containing 50 μM Na$_3$VO$_4$, centrifuged and solubilized by adding 80 μl of Laemmli sample buffer to obtain whole-cell extracts. To prepare cell lysates, cells were lysed for 15 min at 4 °C in lysis buffer containing 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 10 % (v/v) glycerol, 1 % (v/v) Brij 98, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM Na$_3$VO$_4$. Insoluble material was then removed by centrifugation (15 800 **g**, 20 min) and 15 μl of supernatant (0.6×10^6 cells) was solubilized by adding 80 μl of Laemmli sample buffer. Samples were then boiled and analysed by SDS/PAGE and Western blotting.

Western blotting

After SDS/PAGE, proteins were transferred on to a nitrocellulose membrane. The membrane was blocked with 5 % (w/v) non-fat dry milk in Tris-buffered saline [TBS; 50 mM Tris/HCl (pH 7.5) containing 150 mM NaCl] and 0.1 % (v/v) Tween 20 (TBST) for 2 h at room temperature. The membrane was then incubated overnight at 4 °C with anti-phospho-JAK2 (1:500), -phospho-Akt, -JAK2, -Akt and -Bad (1:1000), -phospho-Bad (1:2000) and -Bcl-$X_{S/L}$ (1:100) antibodies. After five washings with TBST, the

blot was incubated with horseradish peroxidase-conjugated anti-(rabbit IgG) secondary antibodies (1:4000 for all proteins, except 1:5000 for Bcl-X_L detection). Immunoblotting was detected by adding enhanced chemiluminescence and exposure to Kodak X-Omat film. When necessary, the blot was stripped by incubating the membrane for 30 min at 55 °C in 62.5 mM Tris/HCl (pH 6.7) containing 100 mM 2-mercaptoethanol and 2 % (w/v) SDS, then washed five times with TBST and treated as described above.

Flow cytometry

Cells were incubated with 5 ng/ml TIMP-1 for 24 h, washed with PBS and resuspended for 15 min in 100 μl of labelling solution [10 mM Hepes (pH 7.4), 140 mM NaCl and 5 mM CaCl$_2$] containing 2 μl of annexin-V-FLUOS and 0.1 μg of propidium iodide. The reaction was stopped by adding 400 μl of labelling solution. Cells were then analysed by the FACS Calibur System (Becton Dickinson). The quantitative evaluation of cell populations was performed using a computer-based program (Cell Quest, Becton Dickinson).

PI 3-kinase assay

Following TIMP-1 treatment and cell lysis, the proteins were immunoprecipitated with 2 μg of anti-p85 antibodies. The immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer [25 mM Hepes (pH 7.4), 100 mM NaCl, 5 mM MgCl$_2$ and 200 μM adenosine]. The reaction was initiated by adding 70 μl of phosphatidylinositol and phosphatidylserine (2 mg/ml each), 10 μCi [γ-^{32}P]ATP and 25 μM ATP for 15 min at 30 °C. The reaction was stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 350 μl of chloroform/methanol (1:1, v/v), and the organic phase was washed with 200 μl of methanol/1 M HCl (1:1, v/v). Samples from the chloroform phases (100 μl) were spotted on to oxalate-treated TLC plates and developed in chloroform/methanol/acetone/acetic acid/H$_2$O (40:13:15:12:7, by vol) and analysed by autoradiography.

Transient transfection

The cDNA encoding dominant-negative Akt (K179M-Akt) in pCMV6 expression vector was kindly provided by Dr T. Franke (Department of Pharmacology, Columbia University, New York, NY, U.S.A.). Transfections were performed in UT-7 cells (1×10^6 in 6-well plates) using LIPOFECTAMINE™ 2000 according to the manufacturer's instructions (Life Technologies). pCMV6-HA-K179M-Akt plasmid (1 μg) was co-transfected with 1 μg of enhanced green fluorescent protein (EGFP) plasmid (pEGFP); in control experiments, pEGFP was co-transfected with an empty vector. After incubation for 5 h, growth medium was added to the transfection complexes. Following overnight transfection, cells were washed and incubated for 24 h in Iscove's modified Dulbecco's medium supplemented with 0.2 % (v/v) deionized BSA, 2 mM L-glutamine and 20 μg/ml human holo-transferrin in the presence or absence of 5 ng/ml TIMP-1. Apoptotic cells were assessed by Hoechst 33342 staining. Intact nuclei were characterized by faint chromatin staining, and nuclei at later stages of apoptosis exhibit chromatin condensation and nuclear fragmentation.

Quantification and statistical evaluation

Quantification of phosphorylated protein bands from Western blotting and PtdIns phosphorylation detected by autoradiography
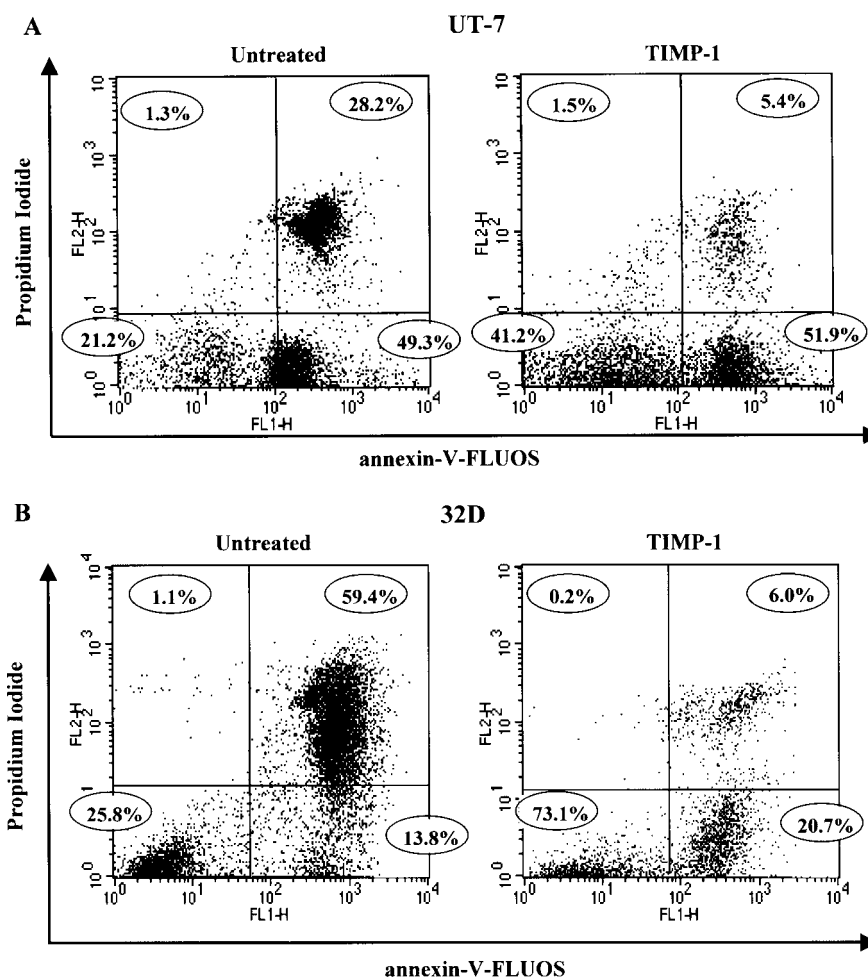
Figure 1   TIMP-1 induces survival of UT-7 and 32D cells

Serum- and growth-factor-deprived cells were incubated for 24 h with 5 ng/ml TIMP-1, and cell survival was determined by flow cytometry using double staining with annexin-V-FLUOS (*x*-axis) and propidium iodide (*y*-axis). Double-stained cells represent cells in later stages of apoptosis (upper-right box). Exclusion of propidium iodide and non-binding of annexin-V-FLUOS indicate viable cells (lower-left box). Data are from a representative experiment out of three performed with UT-7 cells (**A**) and 32D cells (**B**).

was performed by densitometry and subsequent analysis using the program ImageQuant (Molecular Dynamics). Values are means $\pm$ S.E.M. of triplicate determinations. The statistical significance of differences was calculated using Student's *t* test. *P* values referring to corresponding controls are as indicated in Figures and Table.

RESULTS

TIMP-1 enhances survival of UT-7 and 32D cells

To study the potential anti-apoptotic effect of TIMP-1, we used two cell lines: the Epo-dependent human erythroleukaemic cell line UT-7 and the IL-3-dependent murine myeloid cell line 32D. After incubation of cells over a 2 day period with various concentrations of TIMP-1, the anti-apoptotic effect was first determined by Trypan Blue exclusion. Maximal effect was observed after 24 h of incubation with 5 ng/ml TIMP-1 for both cell lines (results not shown). Cell survival was then analysed by flow cytometry using double staining with annexin-V-FLUOS and propidium iodide. As shown in Figure 1(A), TIMP-1 (5 ng/ml) increased the percentage of viable UT-7 cells following 24 h

Table 1   Viable UT-7 and 32D cells after TIMP-1 treatment

Values are expressed as percentages and are means $\pm$ S.E.M. of three separate experiments. ** $P < 0.01$, *** $P < 0.001$.

	Untreated		TIMP-1	
Cell condition	UT-7	32D	UT-7	32D
Viable cells	21.2 ± 1.7	25.8 ± 3.7	41.2 ± 3.5**	73.2 ± 2.8***
Cells in later stage of apoptosis	28.2 ± 3.1	59.4 ± 2.2	5.4 ± 1.9**	6.0 ± 1.8***

incubation (41.2 % as compared with 21.2 % in untreated cells) and simultaneously decreased the percentage of cells at later stage of apoptosis (5.4 % as compared with 28.2 % in untreated cells). The effect of TIMP-1 was highly significant and a similar effect was seen with 32D cells (Figure 1B and Table 1). To eliminate the possibility that the effect of TIMP-1 was attributable to a mitogenic activity, cell proliferation was evaluated by Uptiblue fluorescence, an indicator of cell growth. Under our culture conditions, TIMP-1 did not exhibit any proliferative effect (results not shown).

TIMP-1-induced survival involves the PI 3-kinase

PI 3-kinase and its downstream targets, such as the kinase Akt, are key control points in signalling leading to cell survival. To gain an insight into the role of PI 3-kinase in TIMP-1-induced cell survival, we used LY294002, a specific PI 3-kinase inhibitor. Survival of UT-7 cells incubated with 5 ng/ml TIMP-1 for 24 h in the presence of 20 μM LY294002 was evaluated by flow cytometry using double staining with annexin-V-FLUOS and propidium iodide, and results are expressed as the percentage of viable cells. Inhibition of PI 3-kinase by 20 μM LY294002 completely abolished the effect of TIMP-1 on cell survival (Figure 2A). To assess further the involvement of this enzyme, PI 3-kinase activation was carried out by PtdIns phosphorylation in the presence of [γ-^{32}P]ATP. The generation of PtdInsP resulting from PI 3-kinase activation was enhanced in TIMP-1-stimulated cells and totally inhibited by 20 μM LY294002 (Figure 2B). Quantification of PtdInsP spots revealed a 4-fold increase of PI 3-kinase activity by TIMP-1 compared with a 5-fold increase by Epo, and confirmed that the effect of TIMP-1 was highly significant ($P <$ 0.01; Figure 2C).

The tyrosine kinase JAK2 is involved in TIMP-1-induced survival

Janus kinases (JAKs), and particularly JAK2, play a crucial role in the initial step of Epo signalling. Following Epo stimulation, receptor-associated JAK2 is transphosphorylated and activated in UT-7 cells [18,19]. To determine whether JAK2 was involved in TIMP-1 signalling, UT-7 cells were incubated with 5 ng/ml TIMP-1 in the presence of AG490, a specific JAK2 inhibitor, and cell survival was examined by flow cytometry using double staining with annexin-V-FLUOS and propidium iodide. Results are expressed as the percentage of viable cells. Inhibition of JAK2 by 50 μM AG490, a concentration that did not exhibit cell toxicity, completely suppressed TIMP-1-induced cell survival (Figure 3A). To confirm the involvement of JAK2, we studied its phosphorylation by Western blotting using specific anti-phospho-JAK2 antibodies. TIMP-1 induced JAK2 tyrosine phosphorylation in a time-dependent manner (Figure 3B). Moreover, 50 μM AG490 completely inhibited TIMP-1-enhanced PI 3-kinase activity as evaluated by PtdInsP generation (Figure 3C). Quantification of the PtdInsP spot confirmed that AG490 inhibitory effect was highly significant ($P <$ 0.001; Figure 3D).

TIMP-1-induced Akt phosphorylation and activation are regulated by PI 3-kinase

The role of PI 3-kinase and its downstream targets, such as Akt, is now well established in cell survival. To assess their implication in TIMP-1 signalling, we first studied the role of Akt by cell transfection with a cDNA encoding dominant-negative Akt (K179M). Co-transfection with pEGFP was performed in order to detect transfected UT-7 cells. The EGFP-positive cells were numbered and chromatin condensation was evaluated by Hoechst staining. The results are expressed as the percentage of EGFP-positive cells exhibiting no chromatin condensation. In cells transfected with the empty vector, 5 ng/ml TIMP-1 totally abolished chromatin condensation, whereas, in dominant-negative Akt-transfected cells, chromatin condensation was not blocked by 5 ng/ml TIMP-1 (Figure 4A). These results indicate that Akt is crucial for the TIMP-1-induced anti-apoptotic effect. We next examined Akt phosphorylation by using specific antibodies directed against Akt phosphorylated at Thr^{308}. TIMP-1 induced Akt phosphorylation in a dose- and time-dependent
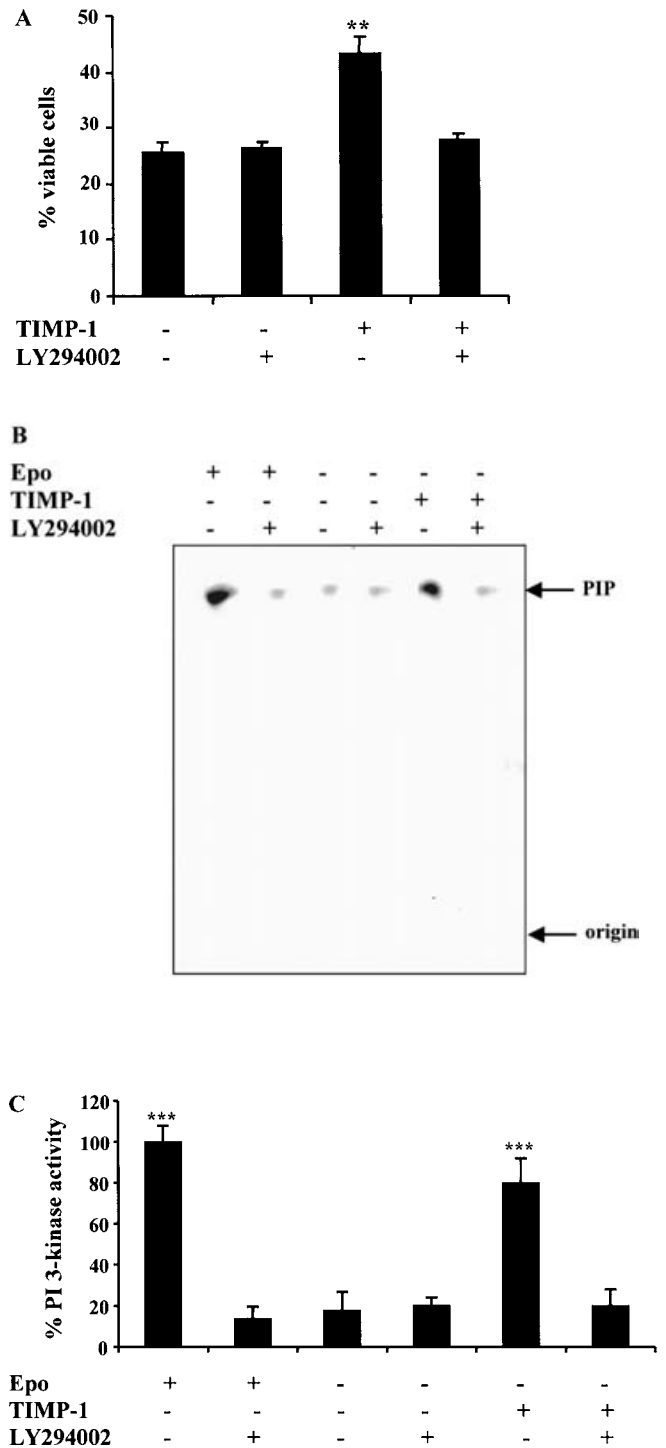

Figure 2   TIMP-1-induced cell survival involves the PI 3-kinase

(**A**) Serum- and Epo-deprived UT-7 cells were incubated for 24 h with 5 ng/ml TIMP-1 and 20 μM LY294002. Cell survival was determined by flow cytometry using double staining with annexin-V-FLUOS and propidium iodide. Results are expressed as the percentage of viable cells and are means $\pm$ S.E.M. of three separate experiments. **$P <$ 0.01 compared with control. (**B**) Serum- and Epo-deprived UT-7 cells were preincubated in the absence or presence of 20 μM LY294002 for 20 min at 37 °C and stimulated for 5 min with or without 5 ng/ml TIMP-1 or 10 units/ml Epo. Anti-p85 immunoprecipitates were subjected to PI 3-kinase activity as determined by PtdIns phosphorylation in the presence of [γ-^{32}P]ATP. The phosphorylated product, PtdInsP (PIP), was separated by TLC and analysed by autoradiography. (**C**) Quantification of PtdIns phosphorylation is expressed as the percentage of signal obtained with Epo. Values are means $\pm$ S.E.M. of three separate experiments. ***$P <$ 0.001 compared with control.
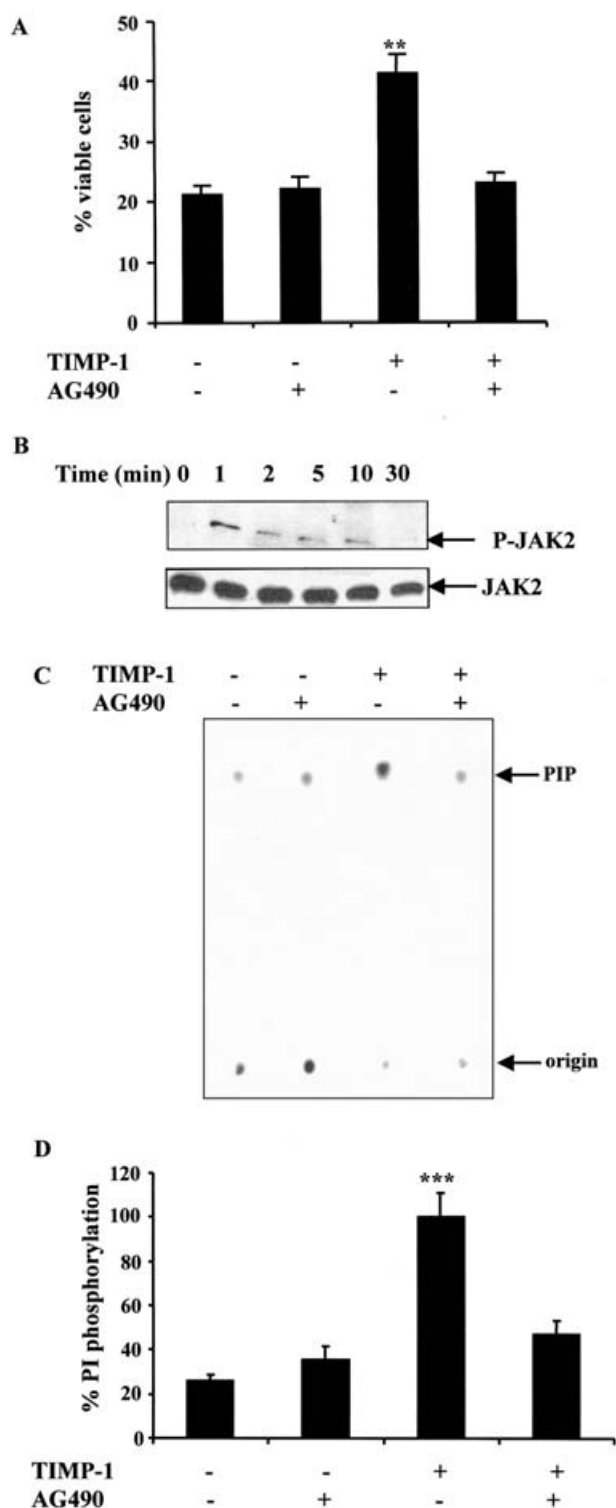
manner, confirming the role of Akt in TIMP-1 signalling (Figures 4B and 4C). Moreover, 20 μM LY294002 significantly and maximally inhibited TIMP-1-induced Akt phosphorylation (Figure 4D). The effect of TIMP-1 was highly significant and a similar finding was observed in 32D cells (results not shown).

TIMP-1 induces Bad phosphorylation and regulates Bcl-X$_L$ expression

Activation of PI 3-kinase and Akt in cell survival signalling is known to result in the specific phosphorylation of Bad at Ser[136]. Phosphorylated Bad further dissociates from Bcl-X$_L$, thus preventing the cytotoxic interactions of Bad with Bcl-X$_L$. To examine whether the anti-apoptotic effect of TIMP-1 triggered such a pathway, Bad phosphorylation was evaluated using specific antibodies directed against Bad phosphorylated at Ser[136]. TIMP-1 stimulation resulted in significant Bad phosphorylation and this effect was completely abolished by 20 μM LY294002 (Figure 5A). Moreover, TIMP-1 maintained the expression of Bcl-X$_L$ over a period of 24 h and this effect was also completely abolished by 20 μM LY294002 (Figure 5B).

DISCUSSION

The primary action described for TIMP was their ability to inhibit MMPs, but numerous studies have reported a wide variety of other functions some of which are independent of MMP inhibition. TIMP, particularly TIMP-1 and TIMP-2, can modulate cell growth. For instance, TIMP-1 is mitogenic in osteosarcoma cell lines, keratinocytes and haematopoietic cell lines [14,20,21]. Recently, TIMP-1 has been demonstrated to display anti-apoptotic activity on different cell types, including Burkitt's lymphoma cell lines, B cells, human breast cell lines and hepatic stellate cells [7–10].

In haematopoietic cells, Epo protects cultured human erythroid precursor cells from apoptosis by PI 3-kinase activation [22]. Epo induces association of PI 3-kinase with the activated Epo-receptor through a direct interaction or via phosphorylated intermediates [23,24]. In the present study, we demonstrate that TIMP-1 induces survival of both erythroid UT-7 and myeloid 32D cell lines and provide evidence for the first time that TIMP-1 signalling involves the PI 3-kinase pathway. Indeed, TIMP-1 enhances PI 3-kinase activity and inactivation of PI 3-kinase completely abrogates the anti-apoptotic effect of TIMP-1. Furthermore, TIMP-1 is found to induce Akt phosphorylation in a PI 3-kinase-dependent manner. Although PI 3-kinase activation is of crucial importance in survival signalling, alternative pathways, such as the Raf-MAP kinase pathways [25], have also been suggested to promote cell survival and thus could be involved in TIMP-1 signalling. Nevertheless, we have demonstrated previously [16] that p42/p44 MAP kinase phosphorylation was not induced by TIMP-1, and activation of Akt has been demonstrated to be independent of MAP kinases. Taking into account these findings, we can assume that the PI 3-kinase/Akt pathway strictly controls TIMP-1-induced cell survival. One member of the anti-apoptotic machinery phosphorylated by Akt is the protein Bad. Phosphorylated Bad then complexes with 14-3-3 proteins, leading
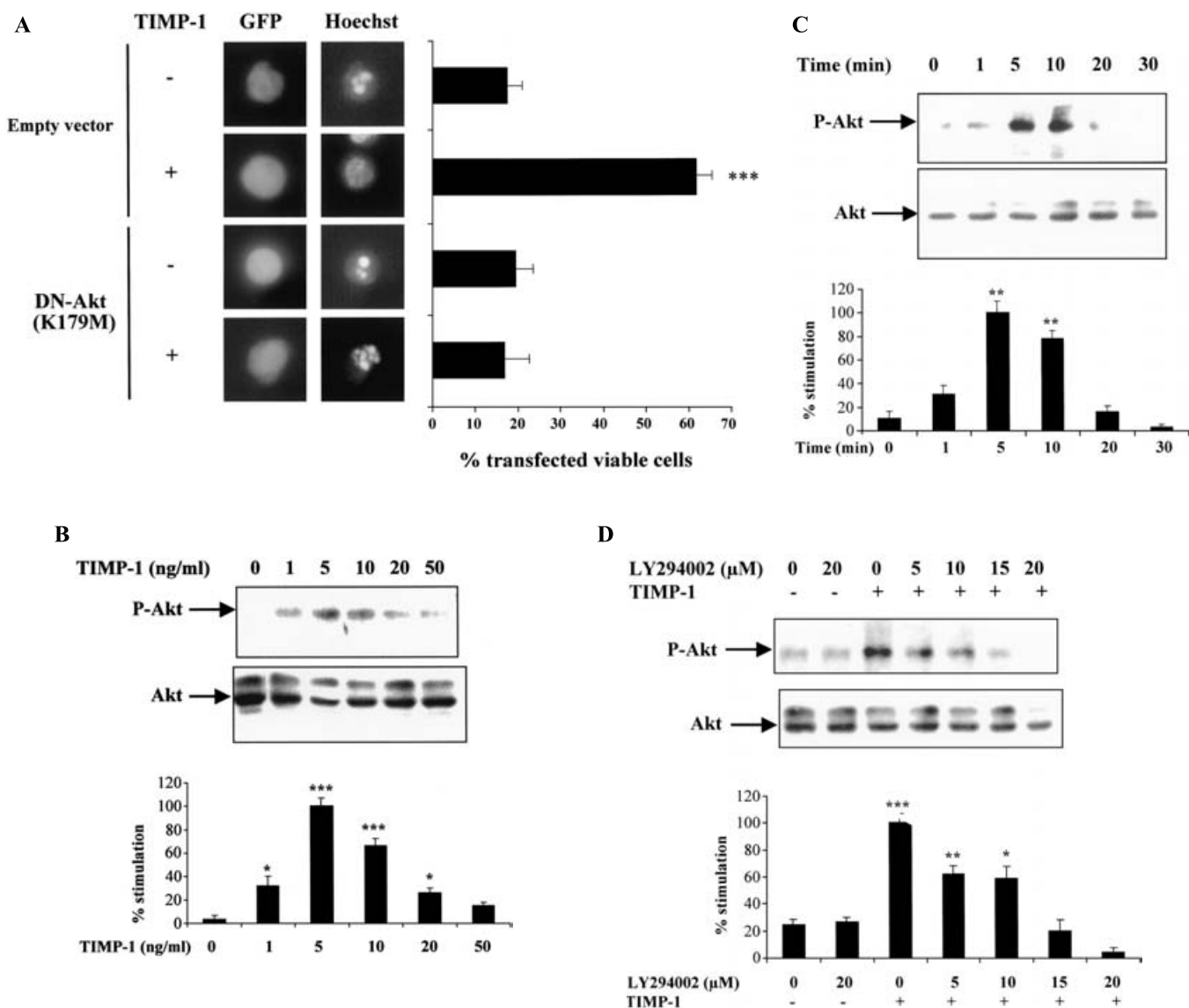
Figure 3   TIMP-1-induced cell survival involves the tyrosine kinase JAK2

(**A**) UT-7 cells were incubated for 24 h with or without 5 ng/ml TIMP-1 and in the absence or presence of 50 μM AG490. Cell survival was determined by flow cytometry using double staining with annexin-V-FLUOS and propidium iodide. Results are expressed as the percentage of viable cells and are means $\pm$ S.E.M. of three separate experiments. **$P < 0.01$ compared with control. (**B**) Serum- and Epo-deprived cells were stimulated with 5 ng/ml TIMP-1 for the indicated times. Whole-cell extracts were analysed by SDS/PAGE, followed by Western-blot analysis using an anti-phospho-JAK2 antibody. The blot was then stripped and reprobed with an anti-JAK2 antibody. A representative blot is shown. (**C**) Serum- and Epo-deprived UT-7 cells were incubated for 5 min with 5 ng/ml TIMP-1 and 50 μM AG490. Anti-p85

immunoprecipitates were subjected to PI 3-kinase activity as determined by PtdIns phosphorylation in the presence of [γ-^{32}P]ATP. The phosphorylated product, PtdIns$P$ (PIP), was separated by TLC and analysed by autoradiography. (**D**) Quantification of PtdIns phosphorylation is expressed as the percentage of signal obtained with TIMP-1 Values are means $\pm$ S.E.M. of three separate experiments. ***$P < 0.001$ compared with control.

Figure 4   Akt is essential for TIMP-1 anti-apoptotic effect

(**A**) Serum- and Epo-deprived UT-7 cells were co-transfected with pEGFP and an empty vector or pEGFP and K179M-Akt, and then incubated with TIMP-1 (5 ng/ml) for 24 h. Left: positive EGFP cells were photographed with a charged-coupled device camera and chromatin condensation was evaluated by Hoechst staining (1 cm = 2.5 μm). Right: 100 EGFP-positive cells were counted in three separate experiments and results are expressed as the percentage of viable transfected cells. Values are means $\pm$ S.E.M. of three separate experiments. ***$P < 0.001$ compared with control. Serum- and Epo-deprived UT-7 cells were stimulated with various concentrations of TIMP-1 for 5 min (**B**), 5 ng/ml TIMP-1 for the indicated times (**C**), or 5 ng/ml TIMP-1 for 5 min and various concentrations of LY294002 (**D**). Whole-cell extracts were analysed by SDS/PAGE, followed by Western-blot analysis using an anti-phospho-Akt antibody. The blot was then stripped and reprobed with an anti-Akt antibody. A representative blot is shown. Quantification of Akt phosphorylation is expressed as the percentage of signal obtained with TIMP-1. Values are means $\pm$ S.E.M. of three separate experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control.

to Bcl-X_L release. In erythroid progenitors, Bcl-X_L is described as a critical factor involved in Epo-induced cell survival and constitutive expression of Bcl-X_L is sufficient to protect cells from apoptosis [26]. The involvement of Bcl-X_L was established previously [8] in the anti-apoptotic action of TIMP-1 in Burkitt's lymphoma cells, but the molecular mechanisms involved were not addressed. In the present study, we show that TIMP-1 induces specific phosphorylation of Bad and controls Bcl-X_L expression in a PI 3-kinase-dependent manner, thus confirming the crucial involvement of the PI 3-kinase pathway in TIMP-1 signalling. In erythroid progenitors, Bcl-X_L expression is also controlled by another signalling pathway, the JAK/signal transduction and activators of transcription ('STAT') pathway

[27]. We also investigated whether TIMP-1 maintains Bcl-X_L expression via the JAK/signal transduction and activators of transcription pathway independently or in parallel with the PI 3-kinase pathway, since the anti-apoptotic effect of TIMP-1 is found to involve the tyrosine kinase JAK2. Indeed, JAK2 inhibition leads to complete suppression of the effect of TIMP-1 on cell survival. The tyrosine kinase JAK2 plays a crucial role in the initial steps of Epo signalling and is considered as the primary kinase activated following Epo binding to its receptor. Recent studies [28] demonstrated that TIMP-1 could activate the protein Ras in MG-63 human osteosarcoma cells, but the tyrosine kinase responsible was not identified. We have demonstrated previously [16] that TIMP-1 induced tyrosine phosphorylation
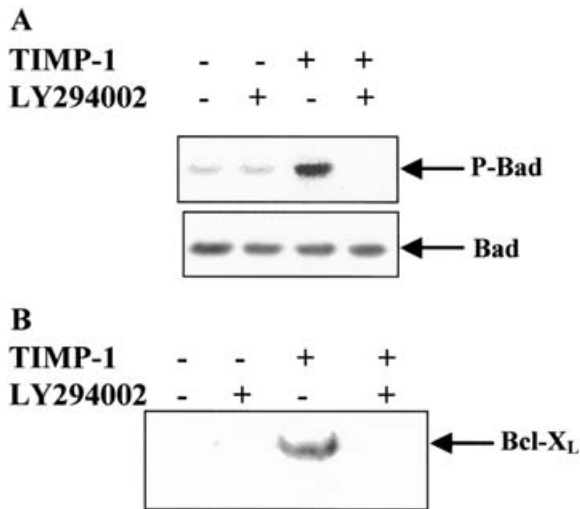
A

TIMP-1   -   -   +   +
LY294002   -   +   -   +

← P-Bad

← Bad

B

TIMP-1   -   -   +   +
LY294002   -   +   -   +

← Bcl-X$_L$

Figure 5   TIMP-1 induces Bad phosphorylation and regulates Bcl-X$_L$ expression

(**A**) Serum- and Epo-deprived cells were stimulated with 5 ng/ml TIMP-1 for 10 min in the absence or presence of 20 μM LY29400. Whole-cell extracts were analysed by SDS/PAGE, followed by Western-blot analysis using an anti-phospho-Bad antibody. The blot was then stripped and reprobed with an anti-Bad antibody. (**B**) Serum- and Epo-deprived cells were incubated with 5 ng/ml TIMP-1 for 24 h in the absence or presence of 20 μM LY294002. Whole-cell extracts were analysed by SDS/PAGE and Western blotting using an anti-Bcl-X$_{S/L}$ antibody. Representative blots are shown.

of intracellular proteins in UT-7 cells in a similar manner as shown previously in fibroblasts stimulated by TIMP-1 or TIMP-2 [14]. In the present study, the time course of JAK2 phosphorylation indicates that JAK2 activation could be one of the initial events triggered following TIMP-1 binding to a putative receptor.

Although the TIMP-1-receptor has not yet been characterized, our data suggest that TIMP-1 induces an intracellular signalling pathway independent of Epo and Epo-receptor as follows: (i) TIMP-1 effect on cell survival is observed in the absence of Epo and any growth factor in both UT-7 and 32D cells; (ii) TIMP-1 induces survival of Epo-receptor-lacking 32D cells; and (iii) TIMP-1 does not induce the tyrosine phosphorylation of Epo-receptor in UT-7 cells (results not shown). TIMP-1 binding sites have been identified on human keratinocytes and K562 cells [20,29]. Cross-linking studies in the erythroleukaemic cell line K562 demonstrated the existence of a TIMP-1-receptor with an apparent molecular mass of 32 kDa, and the human breast carcinoma cell line BC-61 expressed a 80 kDa transmembrane protein able to bind TIMP-1 [29,30]. The bell-shaped dose–response curve of Akt phosphorylation induced by TIMP-1 could be explained by either a negative cross-talk or the presence of both high- and low-affinity binding sites as has been demonstrated in Raji cells [31]. Study of the TIMP-1-receptor and identification of the molecular mechanisms involved after TIMP-1 binding need to be investigated further and are currently underway in our laboratory.

TIMP-1 was first described as a growth factor present in serum, exhibiting an erythroid-potentiating activity (EPA) [32]. This EPA was able to increase the number of burst-forming units-erythroid ('BFU-E') colonies derived from erythroid stem cells and to potentiate Epo effects on erythroid differentiation of the mouse erythroleukaemia cell line ELM-I-1-3 [32,33]. TIMP-1 was demonstrated further to display growth-potentiating activity in the absence of Epo in non-erythroid cells, such as

fibroblasts and keratinocytes [14,20]. We demonstrated previously [16] that TIMP-1 induced UT-7 cell erythroid differentiation and, in the present study, we provide evidence that, in serum- and Epo-free conditions, TIMP-1 is also able to induce survival of two haematopoietic cell lines, the Epo-dependent erythroid UT-7 and the IL-3-dependent myeloid 32D cells. Since Epo-stimulated UT-7 cells have been described to secrete TIMP-1, our findings in the present study suggest that secreted TIMP-1 could act as an autocrine factor [34]. Moreover TIMP-1 secreted by non-erythroid cells present in the human haematopoietic microenvironment could also act as a paracrine factor and participate in bone marrow haematopoiesis [35]. Thus secreted TIMP-1 could exert its action either alone or in a synergistic way with Epo, as first described for its EPA. By inducing erythroid cell survival and differentiation, our present results reinforce the idea that the extracellular matrix of the haematopoietic microenvironment plays a pivotal role in the promotion of proliferation and differentiation of haematopoietic stem cells, suggesting a wide role for TIMP-1 in haematopoiesis. The cell-growth-promoting activity of TIMP-1 might depend on the amount of TIMP-1 produced by cells and secreted into the medium. TIMP-1 levels in normal human serum averaged 100 ng/ml, a concentration 20-fold higher than that inducing erythroid cell survival in the present study. Nevertheless, although TIMP-1 level in bone marrow was not determined, it has been demonstrated that supplementation of long-term bone marrow cultures with a TIMP-1 concentration as low as 6.25 ng/ml significantly increased the number of burst-forming units-erythroid colonies [32].

Structure–function studies have distinguished the MMP-inhibitory activity from the growth-promoting effect of TIMP-1, although exceptions were reported [13]. For example, TIMP-1 suppressed B cell apoptosis in an MMP-independent manner, whereas, in hepatic stellate cells, the anti-apoptotic effect of TIMP-1 is mediated through MMP inhibition [8,10]. In UT-7 cells, we observed that reductively alkylated TIMP-1, devoid of MMP-inhibitory activity, exhibited a similar survival effect when compared with unreduced TIMP-1 (results not shown), demonstrating that the anti-apoptotic action of TIMP-1 is independent of MMP inhibition.

Characterization of the signalling pathway from the extra-cellular matrix that impact apoptotic or anti-apoptotic regulatory molecules is an exciting area of study. By inducing both erythroid differentiation and cell survival, our present results suggest that TIMP-1 plays a critical role in haematopoiesis. In the present study, we identify the JAK2/PI 3-kinase/Akt/Bad pathway as being involved in TIMP-1 signalling leading to erythroid cell survival.

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