Involvement of the p38 mitogen-activated protein kinase pathway in tissue inhibitor of metalloproteinases-1-induced erythroid differentiation

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Abstract We examined the role of the mitogen-activated protein (MAP) kinase pathway in tissue inhibitor of metalloproteinases-1 (TIMP-1)-mediated cellular effects in a human erythroleukemic cell line UT-7. We show that TIMP-1 induced both UT-7 cell erythroid differentiation and proliferation and tyrosine phosphorylation of many intracellular proteins. Using a panel of phosphospecific antibodies, we also demonstrate that phosphorylation of the p38 and c-Jun N-terminal kinases is increased by TIMP-1 whereas phosphorylation of extracellular signal-regulated kinase 1/2 is not induced. Moreover, inhibition of the p38 activity by SB203580 significantly reduces erythroid differentiation induced by TIMP-1, suggesting that the p38 MAP kinase pathway is involved in TIMP-1-induced erythroid differentiation.

Key words: Tissue inhibitor of metalloproteinases-1; Cell differentiation; p38 mitogen-activated protein kinase

1. Introduction

The extracellular matrix turnover is controlled by the balance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). TIMPs are secreted proteins involved in the maintenance of tissue architecture. Four members have been characterized in a variety of species and designated TIMP-1, -2, -3 and -4. They possess 12 conserved cysteine residues involved in disulfide bonds and they form non-covalent inhibitory complexes with MMPs [1]. Contrasting with their inhibitory activity, TIMP-1 and -2 have been shown to exert mitogenic effects in vitro in a wide range of cells including fibroblasts, chondrocytes, lymphoma cells and several cancer cell lines [2,3]. TIMPs also possess erythroid potentiating activity (EPA) so modulating growth and differentiation of erythroid precursors and of the K562 erythroleukemia cell line [4,5]. These cell growth promoting effects seem to be a direct cellular effect and could be mediating by cell surface receptors [6]. Up to date little is known about the signal transduction pathway induced by TIMPs. In fibroblasts, TIMP-2 stimulates proliferation by directly activating adenylate cyclase so producing cyclic adenosine monophosphate (cAMP) which then activates cAMP-dependent protein kinase A [2]. In human osteosarcoma cell line MG-63, TIMP-1 and -2 stimulate DNA synthesis mediated through activation of mitogen-activated protein (MAP) kinases [7].

In the present study, we report that the erythroid differentiation induced by TIMP-1 is mediated through activation of the p38 MAP kinase and c-Jun N-terminal kinases (JNK) 1/2 in the erythroleukemic cell line UT-7.

2. Materials and methods

2.1. Reagents

Human recombinant TIMP-1 and SB203580 were purchased from Calbiochem. Human purified recombinant erythropoietin (Epo) (specific activity of 120 000 U/mg) was purchased from Roche Molecular Biochemicals. α-Minimal essential (α-MEM) and Iscove's modified Dulbecco's media were purchased from Life Technologies, Inc. Anti-p38 MAP kinase antibodies ((C-20)-G) were obtained from Santa Cruz Biotechnology, Inc. Anti-phospho-p38 MAP kinase, phospho-JNK1/2 and phospho-p42p44 antibodies were obtained from New England Biolabs. Anti-phosphotyrosine antibodies (4G10) were purchased from Upstate Biotechnology, Inc. ECL substrate solution was from Amersham Pharmacia Biotech. All others reagents were purchased from Sigma Aldrich.

2.2. Cell culture and stimulation

UT-7 Epo-dependent cells were cloned from the human leukemic cell line UT-7 [8]. These cells were able to grow in the presence of Epo and express erythroid differentiation markers [9]. UT-7 cells were cultured in α-MEM containing 10% fetal calf serum, 2 mM L-glutamine and 2 U/ml Epo. Before each experiment, cells were serum- and Epo-deprived by incubation overnight in Iscove's modified Dulbecco's medium supplemented with 0.2% deionized bovine serum albumin, 2 mM L-glutamine and 20 μg/ml human holo-transferrin. To study proliferation and erythroid differentiation, cells were incubated for 48 h in the same medium with TIMP-1 at the specified concentrations. Cell viability was determined by trypan blue exclusion and hemoglobin synthesis determinated by benzidine staining [10]. To study stimulation and signal transduction, cells were incubated in Iscove's modified Dulbecco's medium with different concentrations of TIMP-1 and stimulated for 0–30 min at 37°C. Stimulation was stopped by adding ice-cold phosphate-buffered saline (PBS) containing 50 μM Na_2VO_4.

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Abbreviations: TIMP-1, tissue inhibitor of metalloproteinases-1; Epo, erythropoietin; p38 MAP kinase, p38 mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase

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2.3. Whole cells extracts

Prior to stimulation, cell concentration was adjusted to 1×10^6 cells/ml. After stimulation as described above, cells were washed twice in PBS containing 50 μM Na_2VO_4. Cells were pelleted and solubilized by adding 80 μl 2× Laemmli buffer. Samples were boiled and analyzed by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting.

2.4. Immunoprecipitation

After cell stimulation and washing as described above, cells (10×10^6) were lysed for 15 min at 4°C in lysis buffer containing 10 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 10% Brij 98, 1 mM phenylmethylsulfonide fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM Na_2VO_4 pH 7.4. Insoluble material was then removed by centrifugation ($15\,800\times g$, 20 min) and the supernatant was incubated with the specific antibodies for 1 h at 4°C. The mixture was then transferred to protein G-Sepharose pellets and rocked for 1 h at 4°C. The Sepharose beads were washed once with lysis buffer containing 1% Brij 98 and four times with lysis buffer containing 0.1% Brij 98 and 1 mM Na_2VO_4. Then, the Sepharose beads were boiled in 1× Laemmli sample buffer, the proteins were separated by SDS–PAGE and analyzed by Western blotting.

2.5. Western blotting

After SDS–PAGE, the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline pH 7.5 containing 0.1% Tween 20 (TBST) for 2 h at room temperature. The blot was then incubated with primary antibody overnight at 4°C. After five washes with TBST, the blot was incubated with horseradish peroxidase-conjugated secondary antibody. Immunoblotting was detected by adding ECL substrate solution and exposing to Kodak X-Omat film. When necessary, 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 β-mercaptoethanol and 2% SDS. The blot was then washed five times with 50 mM Tris–HCl pH 7.5 containing 150 mM NaCl, 0.1% Tween 20 and reprobed as described above.

2.6. Bacterial expression of GST-fusion protein ATF2

Glutathione S-transferase (GST) fusion protein ATF2 was expressed in *Escherichia coli*. The *E. coli* strain transformed with the pGEX2-ATF2 plasmid was a gift from F. Porteu (INSERM U363, Hôpital Cochin, Paris, France). Overexpression of the protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Then, cells were pelleted, resuspended in ice-cold PBS (containing 1% Triton X-100 and 1 mM PMSF) and lysed by sonication. The fusion protein was isolated by adding glutathione-Sepharose beads to the lysate. The GST-fusion protein was eluted from the beads by incubation with 20 mM reduced glutathione in 100 mM Tris–HCl pH 8 containing 100 mM NaCl, 1 mM PMSF and 2 mM dithiothreitol (DTT).

2.7. In vitro protein kinase activity

The cell lysates obtained as described above were immunoprecipitated with p38 MAP kinase antibodies and protein G-Sepharose beads. After rocking 2 h at 4°C, the beads were washed extensively with lysis buffer containing 1 mM Na_2VO_4 and once with kinase buffer (25 mM HEPES pH 7.4, 25 mM $MgCl_2$, 2 mM DTT, 0.5 mM Na_2VO_4, 25 mM glycerophosphate and 25 mM ATP). Then, immunoprecipitates were incubated with 40 μl kinase buffer containing 5 μg ATF2 and 10 μCi [γ-^{32}P]ATP for 30 min at 30°C. The reaction was stopped by addition of Laemmli sample buffer. The samples were analyzed by 10% SDS–PAGE and autoradiography.

3. Results

3.1. TIMP-1 promotes cell growth and erythroid differentiation

Since TIMP-1 has been described to stimulate growth of a wide range of cells, we studied TIMP-1 effects on UT-7 cell proliferation and erythroid differentiation. Experiments were realized in the absence of serum to rule out the effect of TIMP-1 present in serum. Addition of human recombinant TIMP-1 in the medium significantly induced cells to differentiate (Fig. 1A) and grow (Fig. 1B). TIMP-1 effect was dose-dependent and reached a maximum at 5 and 10 ng/ml for proliferation and differentiation, respectively.

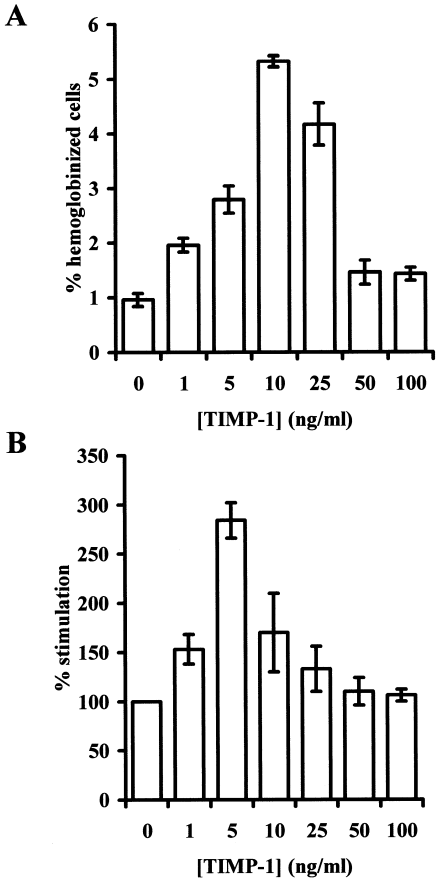

Fig. 1. Effect of TIMP-1 on UT-7 cell differentiation and proliferation. Cells (0.5×10^6) were incubated with various concentrations of TIMP-1 (0–100 ng/ml) for 2 days. A: Cell differentiation was evaluated by benzidine staining and expressed as a percentage of hemoglobinized cells. B: Cell proliferation was evaluated by trypan blue exclusion and expressed as a percent of control without TIMP-1 ($100\% = 0.5\times10^6$). The results were the means ± S.E.M. from three separate experiments.

3.2. TIMP-1 induces tyrosine phosphorylation of intracellular proteins

Little is known on signal transduction pathways induced by TIMPs. To analyze the signaling of TIMP-1, we examined the pattern of tyrosine-phosphorylated proteins after stimulation for 5 min with various concentrations of TIMP-1. Fig. 2 showed that TIMP-1 induced tyrosine phosphorylation of several proteins with molecular masses ranging from 36 to 212 kDa. The effect of TIMP-1 was dose-dependent. Tyrosine phosphorylation of proteins induced by TIMP-1 was effective as soon as 1 min, reached a maximum between 5 and 10 min and returned to the basal level after 30 min (not shown).

3.3. TIMP-1 induces activation of the p38 MAP kinase but not extracellular signal-regulated kinase (ERK) 1/2

We have demonstrated that TIMP-1 is able to promote growth and differentiation of UT-7 cells. So, we investigated the possible involvement of the MAP kinases ERK1/2 and p38 in TIMP-1 signaling. Cells were stimulated with various concentrations of TIMP-1 and whole cell extracts were ana-

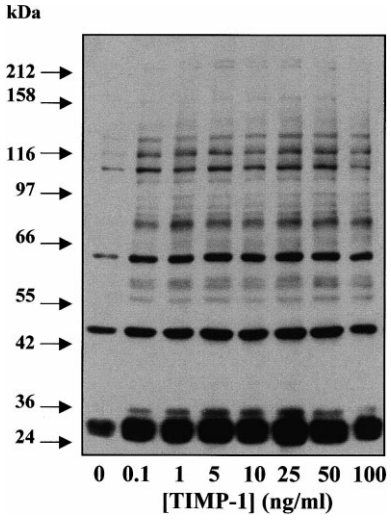

Fig. 2. Tyrosine phosphorylation induced by TIMP-1. Cells (0.5×10^6) were stimulated for 5 min with various concentrations of TIMP-1 (0–100 ng/ml). Whole cell extracts were analyzed by SDS–PAGE and Western blotting using anti-phosphotyrosine antibodies.

lyzed by Western blotting using specific antibodies which recognized the phosphorylated form of the MAP kinases. Any concentration of TIMP-1 used induced the phosphorylation of the kinases ERK1/2 whereas Epo did (Fig. 3). On the other hand, TIMP-1 (25 ng/ml) induced the phosphorylation of the p38 MAP kinase as soon as 1 min with a maximal effect between 5 and 10 min (Fig. 4A). This TIMP-1 concentration was shown to induce UT-7 cell differentiation. In parallel, the p38 kinase activity assay with GST-ATF2 as a substrate showed that TIMP-1 rapidly and transiently increased the p38 activity (Fig. 4B). The maximal activity was observed at 5–10 min. Furthermore, the TIMP-1-induced phosphorylation of the p38 as well as the p38 kinase activity is completely inhibited by SB203580, a specific inhibitor of the p38 MAP kinase (Fig. 4C).

3.4. SB203580 inhibits TIMP-1-induced UT-7 cell differentiation

To evaluate the role of the p38 MAP kinase pathway during growth and/or differentiation induced by TIMP-1, cells were incubated with TIMP-1 in the presence or the absence of 5 μM SB203580. Proliferation and erythroid differentiation were measured as described in Section 2.2. Fig. 5 showed that SB203580 reduced of 47% and 60% erythroid differentiation

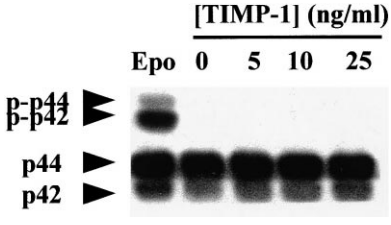

Fig. 3. Effect of TIMP-1 on the phosphorylation of ERK1/2. Cells (1×10^6) were stimulated with various concentrations of TIMP-1 (0–25 ng/ml). Whole cell extracts were analyzed by SDS–PAGE and Western blotting using antibodies which recognized the phosphorylated form of the MAP kinases p42p44 (p-p42, p-p44). The same protein samples were reprobed with anti-p42p44 antibodies.

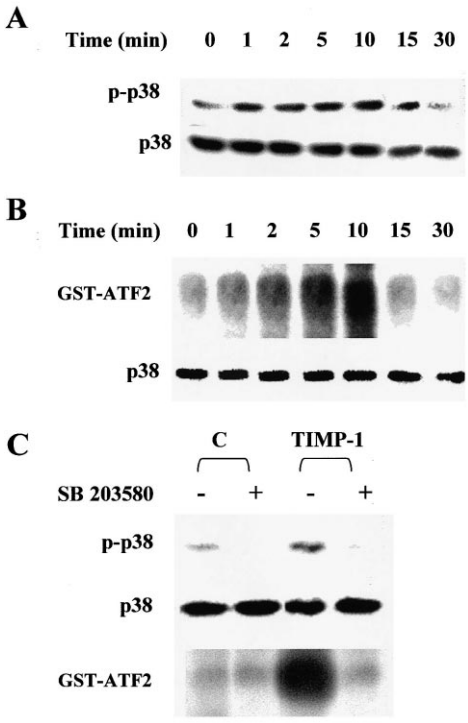

Fig. 4. Activation of the p38 MAP kinase by TIMP-1. Cells were stimulated for various times with 25 ng/ml TIMP-1. A: Whole cells extracts (1×10^6) were analyzed by SDS–PAGE and Western blotting using specific antibodies which recognized the phosphorylated form of the p38 (p-p38). The same protein samples were reprobed with anti-p38 antibodies. B: Cell lysates (10×10^6) were immunoprecipitated with anti-p38 antibodies and the p38 kinase activity was measured using GST-ATF2 as a substrate (as described in Section 2). C: Cells were preincubated for 20 min with 5 μM SB203580, left untreated (C) or stimulated for 5 min with 25 ng/ml TIMP-1. Phosphorylation of the p38 and p38 kinase activity was evaluated as described above.

induced by 5 and 10 ng/ml TIMP-1, respectively, but had no effect on cell proliferation (not shown). This result indicated that the p38 MAP kinase pathway was involved in TIMP-1-induced erythroid differentiation.

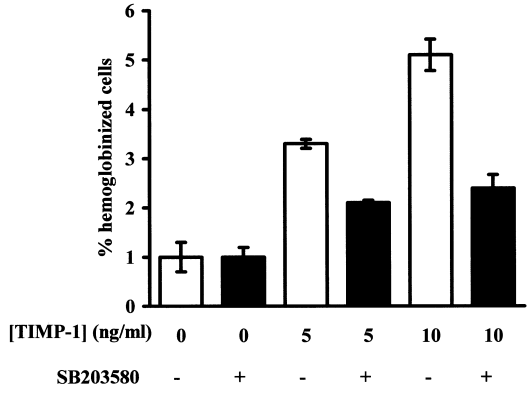

Fig. 5. Effect of SB203580 on UT-7 cell differentiation induced by TIMP-1. Cells (0.5×10^6) were incubated with 5 and 10 ng/ml TIMP-1 in the presence (■) or the absence (□) of 5 μM SB203580 for 2 days. Erythroid differentiation was evaluated by benzidine staining. Results are expressed as a percentage of hemoglobinized cells and are the means ± S.E.M. of three separate experiments.

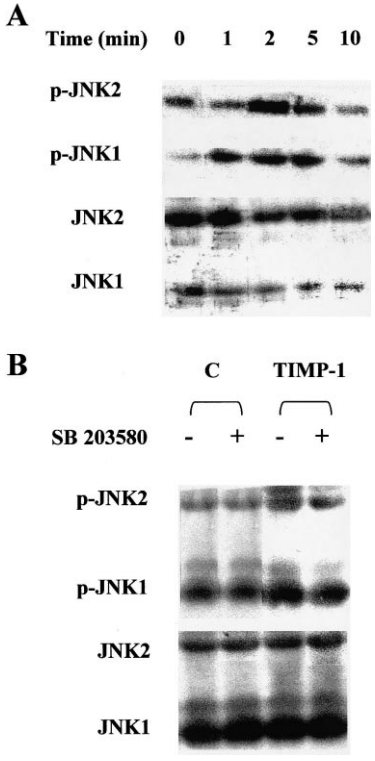

Fig. 6. Activation of JNK1/2 by TIMP-1. A: Cells (1×10^6) were stimulated for various times with 25 ng/ml TIMP-1. B: Cells (1×10^6) were stimulated for 2 min with 25 ng/ml TIMP-1 in the presence or absence of 5 μM SB203580. Whole cell extracts were analyzed by SDS–PAGE and Western blotting using specific antibodies which recognized the phosphorylated form of JNK1/2 (p-JNK1 and p-JNK2). The same protein samples were reprobed with anti-JNK1/2 antibodies.

3.5. TIMP-1 induces phosphorylation of JNK1/2

We further examined the effect of TIMP-1 on JNK1/2 phosphorylation. After cell stimulation for various times with 25 ng/ml TIMP-1, whole cell extracts were analyzed by SDS–PAGE and Western blotting using specific antibodies which recognize the phosphorylated form of JNK1/2. As shown in Fig. 6A, TIMP-1 induced a rapid and transient phosphorylation of the two isoforms of JNK. The JNK phosphorylation was observed as soon as 1 min and returned to the basal level after 10 min. The TIMP-1-induced phosphorylation of JNK1/2 was not inhibited by 5 μM SB203580 (Fig. 6B).

4. Discussion

TIMPs are multifunctional proteins with pleiotropic effects. Although most of the literature pertains to their metalloproteinases inhibitory function, TIMPs and more particularly TIMP-1 and TIMP-2 have been described as being growth-modulating factors. We report here that TIMP-1 alone induces growth and erythroid differentiation of UT-7 cells in a dose-dependent manner. Our results are consistent with others previous reports which demonstrated EPA and cell growth promoting activity for TIMP-1. TIMP-1 stimulated both growth and differentiation of erythroid progenitor cells [11] and human erythroleukemia cell line K562 [4]. TIMP-1 also induced differentiation of the Epo-responsive mouse erythroleukemia cell line ELM-I-1-3 [12]. Moreover, TIMP-1 has been described to be mitogenic for a broad range of human and bovine cells including fibroblasts and keratinocytes [2,6] and the mitogenic activity of TIMP-1 is independent of its metalloproteinase inhibitory activity [13].

Up to date there is little information on intracellular signaling of TIMPs. Our results show that many intracellular proteins are phosphorylated after UT-7 cell stimulation with TIMP-1. The decrease of the phosphorylation observed after 30 min of stimulation is correlated with the kinetics of tyrosine phosphorylation of intracellular proteins and probably corresponds to the termination of TIMP-1 signaling. Similar results were previously described in the human osteosarcoma cell line MG-63 [7]. The signaling of TIMPs was thought to be a direct cellular effect probably mediated by a cell surface receptor. Previous reports evidenced the presence of both high and low affinity binding sites for TIMP-2 on the cell surface of Raji cells [6] and for TIMP-1 on K562 cells and keratinocytes [4,14]. Recently, it was reported that BC-61 cells expressed a transmembrane protein of 80 kDa able to bind TIMP-1 with a high affinity [15] but TIMP-1 receptor has not yet been cloned. TIMP-1 does not induce tyrosine phosphorylation of the Epo receptor (not shown) so suggesting that TIMP-1 signaling is independent of the Epo receptor.

As Epo signaling involves the MAP kinase pathways and biological activity of TIMP-1 was firstly described as an EPA, we investigated the role of the MAP kinases in TIMP-1 signaling. The MAP kinases ERK1/2 are not phosphorylated after UT-7 cell stimulation with either growth or differentiation promoting concentrations of TIMP-1 and our results are somewhat different from a previous study in the osteosarcoma cell line MG-63 [7]. In UT-7 cells, Epo-induced activation of ERKs was described to be correlated with cell proliferation [16] and recent studies indicated that activation of ERKs was essential for Epo-induced SKT-6 cell growth but not for Epo-induced erythroid differentiation [17]. ERKs are activated by various cell growth and differentiation stimuli [18] and these results suggest that the precise role of the MAP kinases ERK1/2 in cell differentiation and/or proliferation is not determinated.

We report here that UT-7 cell differentiation induced by TIMP-1 involves the p38 MAP kinase pathway. Indeed, TIMP-1 induces the specific phosphorylation of the p38 and the time-course of phosphorylation closely paralleled its kinase activity. Both p38 phosphorylation and p38 kinase activity are completely inhibited by SB203580, a specific inhibitor of the p38 MAP kinase. While this compound has been described to inhibit the activity but not the phosphorylation of the p38 MAP kinase, it has also been demonstrated that SB203580 inhibited stimulus-induced activating phosphorylation of the p38 at the Thr-Gly-Tyr activation motif leading to the inhibition of the p38 kinase activity [19]. Moreover, we show that SB203580 significantly inhibits UT-7 cell differentiation induced by TIMP-1. Activation of the p38 MAP kinase has also been evidenced to be required for Epo-induced SKT-6 cell differentiation but not for proliferation [20] and both expression and phosphorylation of the heat shock protein hsp 28, a target of the p38, are correlated with hemin-induced K562 cell differentiation [20].

We also show that TIMP-1 induces the phosphorylation of JNK1/2 which was not inhibited by SB203580. JNKs have been described as essential for cell differentiation in several cell lines [21,22]. Nevertheless, a definitive role for the kinases

p38 and JNK is not clearly established and these kinases appear to be involved in opposite effects. Indeed, in the murine erythroid progenitor cell line HCD-57, sustained activation of p38/JNK is observed after Epo withdrawal leading to cell apoptosis whereas in SKT-6 cells and FD-Epo cells, activation of p38/JNK is induced during Epo-induced differentiation and proliferation, respectively [17,21]. Our results show that TIMP-1 induces activation of the MAP kinases p38 and JNK while ERK1/2 are not phosphorylated after stimulation with TIMP-1. This dynamic balance between ERK and JNK/p38 pathways has been demonstrated as being important in determining the cell fate, whether cell undergoes proliferation, differentiation or apoptosis [23,24].

The present study suggests that TIMP-1 promotes UT-7 cell proliferation and erythroid differentiation. Moreover, TIMP-1 induces the tyrosine phosphorylation of a number of intracellular proteins and TIMP-1-induced erythroid differentiation requires the p38 MAP kinase pathway. Further experiments will be required to identify the upstream regulators of the p38 MAP kinase in TIMP-1 signaling.

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