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TIMP-1 stimulates proliferation of human aortic smooth muscle cells and Ras effector pathways

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Abstract

Tissue inhibitor of metalloproteinases-1 (TIMP-1) is a multifunctional protein, which is found in most tissues and body fluids. Here, we demonstrated that recombinant TIMP-1 but not the synthetic matrix metalloproteinase inhibitor, GM6001, stimulated proliferation of human aortic smooth muscle cells (AoSMC) in a dose-dependent manner. The mitogenic effect was associated with activation of Ras, increased phosphorylation of ERK, and stimulation of cyclin D1 expression. The phosphatidylinositol 3-kinase (PI3K) signaling pathway was also involved since the PI3K inhibitor, LY294002, abolished the TIMP-1-mediated growth stimulation. These data suggest that TIMP-1 activates Ras, which then turns on the ERK and PI3K signaling pathways to promote cell cycle progression of the AoSMC.

Keywords: Aortic smooth muscle cells; Proliferation; TIMP-1; GM6001; Ras; ERK; PI3K

Smooth muscle cells (SMCs) surround and share a common basement membrane with the endothelial cell layer of the blood vessel wall. There is minimal SMC proliferation in intact arteries but SMC are quickly stimulated to migrate and replicate after vascular injury [1]. A variety of SMC mitogens have been identified, including platelet-derived growth factor, basic fibroblast growth factor, epidermal growth factor, and transforming growth factor β [2,3]. SMC proliferation is a key event in the pathogenesis of important vascular diseases like atherosclerosis [4,5] and SMC are a major source of extracellular matrix (ECM) formation in the atherogenic lesions [6,7]. Stimulation of SMC to migrate and proliferate involves degradation of the ECM, which is mediated through increased production of matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases that are capable of degrading the major components of the ECM [1–3]. MMPs also facilitate the release of growth factors from the ECM and have been shown to be involved in proliferation and outgrowth of SMC from explants of rabbit aorta [8].

MMP-mediated proteolysis is controlled by their endogenous inhibitors, TIMPs [9–11]. TIMP-1 has other functions, which may or may not be dependent on MMP inhibition, including growth stimulation [10,12]. The growth stimulatory function of TIMP-1 was known from the time its sequence was first identified and shown to be identical to that of the erythropoid potentiating activity (EPA) [13]. A number of reports have demonstrated the mitogenic effect of TIMP-1 on a variety of cell types in vitro [14–21]. Vascular SMC express TIMP-1 both in vitro [22,23] and in vivo [24]. TIMP-1 was found to either inhibit [25] or have no effect [26,27] on in vitro proliferation of vascular SMC. In vivo studies showed reduced SMC migration and neointima development associated with overexpression of TIMP-1 [28]. It has been proposed that TIMP-1

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stimulates proliferation through its interaction with a putative cell surface receptor [29].

There is limited knowledge of intracellular signaling associated with TIMP-1-mediated growth stimulation. TIMP-1 was shown to stimulate tyrosine-phosphorylated proteins in breast cancer cells [19] and Ras–GTP complex formation in osteosarcoma cells [30]. In the present study, we asked if TIMP-1 was mitogenic for human AoSMC and if so, whether Ras signaling was involved. Our results showed that TIMP-1 stimulated growth of AoSMC in MMP-independent manner, which involved activation of Ras and signaling through both ERK and PI3K-dependent pathways.

Materials and methods

Reagents and antibodies. Human recombinant TIMP-1, GM6001, LY294002, and FPT inhibitor III were from Calbiochem (San Diego, CA). Human recombinant FGF-2 was from Sigma Chemical (St. Louis, MO). Phosphorylated ERK (p-ERK), phosphorylated Akt (p-Akt), and Akt were from Cell Signaling (Beverly, MA). ERK and actin antibodies were from Chemicon (Temecula, CA). Cyclin D1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and proliferation assay. Human AoSMC were purchased from Clonetics (Walkersville, MD) and expanded in Clonetics regular smooth muscle growth medium (SmGM-2). AoSMC were cultured in Clonetics smooth muscle basal medium (SmBM) without serum or growth factors and used at passages 5–10 in the cell proliferation assays. They were seeded at 5000 cells/well in 96-well culture plates, incubated in the SmGM-2 for 6 h, and then switched to SmBM without serum or growth factors for 24 or 48 h, which is sufficient to accumulate the cells in the G0/G1 phase of the cell cycle [31]. Five wells were incubated in the presence or absence of FGF-2 (50 ng/ml), TIMP-1 (20, 100, or 500 ng/ml), or the synthetic MMP inhibitor, GM6001, (2.5, 12.5, or 62.5 μM) for 48 h with a change of SmBM at 24 h. In the experiments testing the FPT inhibitor III and LY294002, the cells were seeded at 10,000 cells/well and the inhibitors were added 20 min before TIMP-1. The BrdU proliferation assay was performed according to the manufacturer's instructions (Roche, Indianapolis, IN), using five wells for each sample. Nuclear incorporation of BrdU into a newly synthesized DNA identifies cells in the S phase of the cell cycle.

Ras activity assay. The cells were seeded in 6 cm culture dishes at a density of 8×10^4 cells/cm^2 and incubated in SmGM overnight. The cells were then starved in SmBM for 24 h and treated with FGF-2 (50 ng/ml), TIMP-1 (100 ng/ml) or GM6001 (12.5 μM) for 15 or 30 min. Ras activity assay was performed using Ras activation assay kit (Upstate, Charlottesville, VA) according to manufacturer's directions. Briefly, cells were washed twice with cold PBS and lysed in 500 μl of 1× MLB lysis buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, and 2% glycerol) containing complete protease inhibitor cocktail. The cell lysates were centrifuged at 14,000*g* for 5 min at 4 °C and incubated with 10 μg Raf-1 RBD agarose for 45 min at 4 °C. The precipitated Ras–GTP was detected by Western blot analysis using 1 μg/ml anti-Ras antibody (clone RAS10).

Western blot analysis. The cells were seeded in 12-well culture plates at 8×10^4 cells/cm^2, incubated for 6 h in SmGM, starved in SmBM for 24 h, and treated with FGF-2 (50 ng/ml), TIMP-1 (100 ng/ml), or GM6001 (12.5 μM) for 15 min, 1, 6, 18, and 24 h. The cells were then rinsed twice in PBS, extracted with M-PER mammalian protein extraction reagent (Pierce, Rockford, IL) containing complete protease inhibitor cocktail (Roche, Indianapolis, IN), 1 mM Na_3VO_4, and 25 mM NaF, and then centrifuged at 10,000*g* for 10 min at 4 °C. The protein concentrations were determined using MicroBCA protein assay reagent (Pierce, Rockford, IL). The proteins (10 or 20 μg) were separated by SDS–PAGE under reducing conditions and transferred to a PVDF membrane (Invitrogen, Carlsbad, CA). The membrane was treated with 5 or 10% (w/v) skim milk at room temperature for 1 h and then incubated at 4 °C overnight or at room temperature for 2 h with primary antibodies to p-ERK (1:1000 dilution) and total ERK (1:5000 dilution). Immunoreactive bands were visualized using horseradish-peroxidase-conjugated secondary antibody (Dako A/S, Denmark) and ECL reagents (Amersham–Pharmacia Biotech, England). Cyclin D1 expression was also evaluated after 6 and 24 h treatment with the same concentrations of TIMP-1 and GM6001, using cyclin D1 antibody at 1:500 dilution. Actin served as a loading control.

It was examined if the Ras FPT III inhibitor and the PI3 kinase inhibitor, LY294002, affected cell proliferation and expression of p-Akt, p-ERK, and cyclin D1. The concentrations of Ras FPT III inhibitor (5 μM) and LY294002 (10 μM) used have been reported to suppress cell proliferation [32]. The cells were incubated with or without the Ras FPT III inhibitor or LY294002 for 20 min prior to 48 h incubation with or without TIMP-1 (100 ng/ml). BrdU was added 4 h before the end of the 48 h TIMP-1 incubation period and the proliferation assay was performed as described above. Western blot analyses used antibodies to p-Akt (1:1000 dilution), total Akt (1:1000 dilution), p-ERK (1:1000 dilution), and total ERK (1:5000 dilution). Scanning densitometry and Scion Image software were used to calculate the p-Akt/Akt and p-ERK/ERK ratios.

Statistical analysis. All experiments were performed at least three times. Data are expressed as means ± SEM. One-way ANOVA was used for statistical analysis by Prism software package (GraphPad).

Results and discussion

Stimulation of AoSMC proliferation by TIMP-1 but not by the synthetic MMP inhibitor, GM6001

Vascular SMC proliferate and migrate after vascular injury to form a neointima [33]. Our findings showed that exogenous recombinant TIMP-1 stimulated growth of AoSMC in a dose-dependent manner, using a BrdU cell proliferation assay (Fig. 1). Relative to the

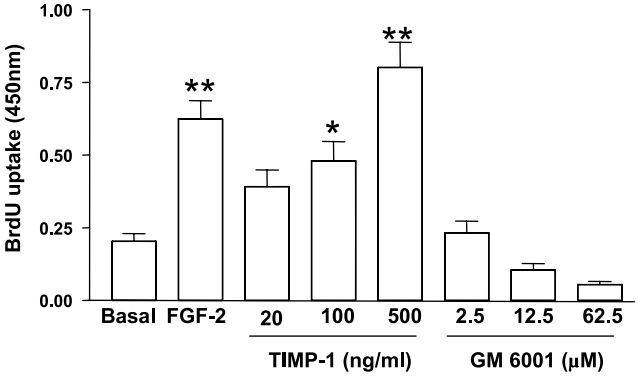

Fig. 1. TIMP-1-stimulated AoSMC proliferation is independent of MMP inhibition. After 48 h incubation with different concentrations of TIMP-1 or the synthetic general MMP inhibitor, GM6001, TIMP-1 produced a dose-dependent increase in BrdU incorporation (*$p < 0.05$; **$p < 0.001$) and GM6001 inhibited BrdU incorporation, although it was not statistically significant. FGF-2 (50 ng/ml) served as a positive control (**$p < 0.001$). The data represent one of four experiments with similar results and are expressed as means ± SE ($n = 5$).

basal proliferation rate of the control AoSMC, it was increased 2.4-fold ($p < 0.05$) and 3.5-fold ($p < 0.001$) by 100 and 500 ng/ml TIMP-1. In the same assay, TIMP-1 did not affect the proliferation of human umbilical vein endothelial cells (not shown). To examine if the growth stimulating activity of TIMP-1 was associated with its MMP inhibitory function, the synthetic MMP inhibitor, GM6001 (12.5 μM), was included in the BrdU proliferation assay. Contrary to TIMP-1, GM6001 suppressed BrdU uptake in a dose-dependent manner although it was not statistically significant. Consistent with our results, others have reported growth suppression of human AoSMC in the presence of GM6001 [34]. On the other hand, a recent study of the MDA-MB-435 breast carcinoma cell line revealed growth stimulation by both TIMP-1 and the GM6001 inhibitor, suggesting MMP-dependent mechanism of the TIMP-1-mediated growth stimulation of the breast carcinoma cells [12].

FGF-2 is released from the ECM of the vascular wall upon injury and is considered to play a key role in vascular SMC proliferation [35]. It has been proposed that TIMP-1 acts as a growth factor in serum [14,36]. When comparing the growth stimulatory capacity of TIMP-1 to that of FGF-2, which served as a positive control, 100 ng/ml of TIMP-1 was 30% less effective than 50 ng/ml FGF-2 (Fig. 1). Interestingly, the mitogenic TIMP-1 concentration of 100 ng/ml is comparable to the reported circulating TIMP-1 level of 94.5 ng/ml in normal individuals [37]. It is therefore plausible that circulating TIMP-1 may contribute to SMC proliferation after vascular injury. Both TIMP-1 and TIMP-2 transcripts have been located in normal blood vessels [24] and vascular SMC produce TIMP-1 in vitro [22,23]. Based on these and our findings, it seems reasonable to speculate that TIMP-1 may act as an autocrine growth factor for SMC. However, others have reported decreased in vitro growth of TIMP-1 overexpressing rat SMC [25] and studies of balloon-injured arteries suggested that TIMP-1 did not affect SMC growth [38]. TIMP-1 knockout mice were employed to elucidate the role of TIMP-1 in the pathogenesis of atherosclerosis, which involves excessive SMC proliferation [39,40]. Both studies showed increased incidence of aortic aneurysms and one study showed reduced size of atherosclerotic lesions, suggesting that TIMP-1 contributes to the buildup of atherosclerotic plaques.

TIMP-1 stimulates Ras and ERK activities

It has been proposed that the growth promoting activity of TIMP-1 is mediated through cell a surface receptor although such receptor has not been identified [14,15,17,19]. Of the few studies published on TIMP-1-mediated intracellular signaling, one demonstrated that Ras was activated in TIMP-1 growth stimulated human osteosarcoma cells [30]. Ras is activated by various mitogenic signals through transient binding to GTP [41], which then activates the Raf/MEK/ERK protein cascade [42,43].

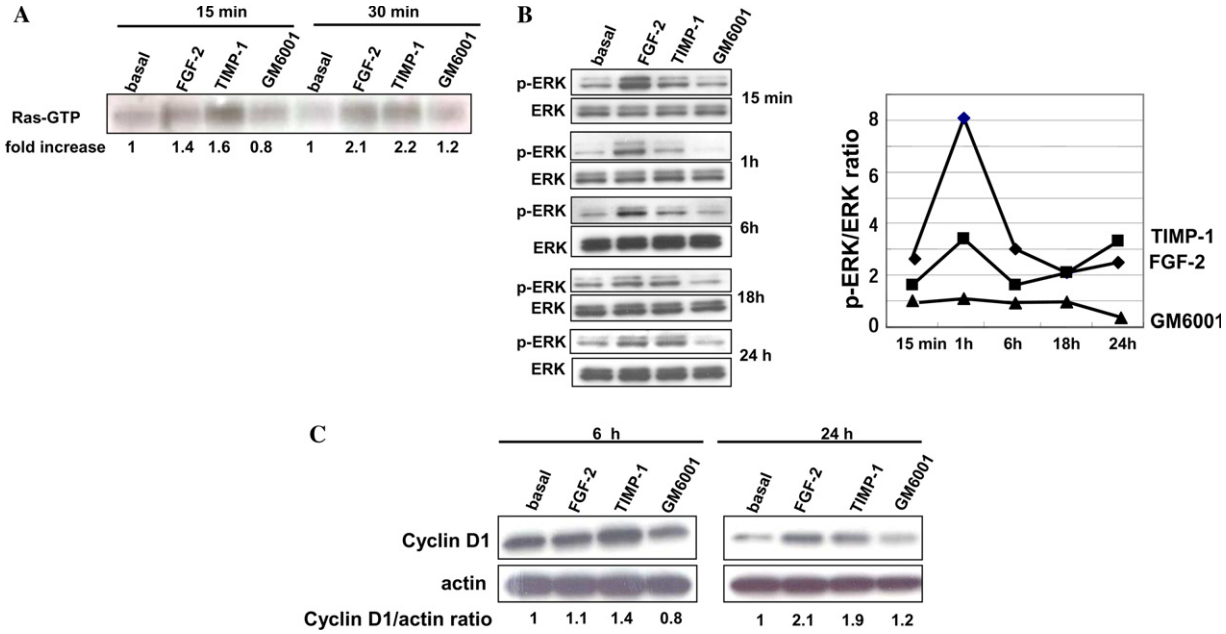

Fig. 2. TIMP-1 stimulates Ras–GTP, p-ERK, and cyclin D1 expression. (A) AoSMC were serum-starved for 24 h and then treated with TIMP-1 (100 ng/ml), GM6001 (12.5 μM), or the positive FGF-2 control (50 ng/ml), for 15 or 30 min. Precipitated Ras–GTP was analyzed by Western blot analysis. (B) The left panel shows Western blot analysis of p-ERK and total ERK expression after 15 min to 24 h exposure to TIMP-1 (100 ng/ml), GM6001 (12.5 μM), FGF-2 (50 ng/ml), or no treatment (basal). The right panel shows the changes in the p-ERK/ERK ratio. (C) Western blot analysis data of cyclin D1 expression after 6 and 24 h exposure to TIMP-1 (100 ng/ml), GM6001 (12.5 μM), or FGF-2 (50 ng/ml).

We were interested to find out if Ras and ERK were activated by TIMP-1 in the growth stimulated AoSMC. The cells were incubated with 100 ng/ml TIMP-1 or 12.5 μM GM6001 for 15 or 30 min. These concentrations of TIMP-1 and GM6001 were chosen because they possessed comparable MMP-2 inhibitory activity [44]. Using a Ras activity assay, 1.6- and 2.3-fold increase in Ras–GTP was observed after 15 and 30 min incubation with TIMP-1 (Fig. 2A). The increase in Ras–GPT at 30 min was comparable to that produced by the positive FGF-2 control (Fig. 2A). In contrast, the GM6001 inhibitor had minimal effect on Ras–GPT expression, which was consistent with its inability to stimulate growth of the AoSMC.

Mitogen stimulation of quiescent cells causes biphasic pattern of Ras activation [42,45,46]. The first peak of Ras activity is seen upon entry into G1 phase of the cell cycle when the ERK cascade is activated [42]. ERK activity was examined at different time points following exposure to TIMP-1, GM6001, and FGF-2 (Fig. 2B). Low basal expression of p-ERK suggested that ERK was constitutively activated in the cultured AoSMC. After 1 h of TIMP-1 treatment, a peak of p-ERK activity was observed, which was much smaller than the peak produced by the positive FGF-2 control at the same time point. At 24 h, however, the TIMP-1-stimulated p-ERK level was slightly higher than that of the FGF-2 control (Fig. 2B). The GM6001 inhibitor did not affect p-ERK expression, except for a slight decrease at the 24 h time point (Fig. 2B). Cyclin D1 is one of the best characterized cell cycle components targeted by Ras and is stimulated during the second peak of Ras activity by mid-G1 phase [31]. Consistent with the TIMP-1-stimulated Ras activity, cyclin D1 expression was increased 1.9-fold by TIMP-1 at 24 h, compared to 2.1-fold increase by the positive FGF-2 control, whereas the GM6001 inhibitor had negligible effect (Fig. 2C).

TIMP-1 activates the PI3K signaling pathway

The PI3K signaling pathway is important for SMC migration and proliferation [32,47]. Ras-mediated activation of the ERK pathway drives the cells out of quiescence, whereas the PI3K pathway contributes to mitogenic signaling at a later time in the cell cycle [42,48]. To further substantiate the involvement of Ras and PI3K in the TIMP-1-stimulated growth of the AoSMC, the PI3K inhibitor, LY294002, and the Ras FPT III inhibitor were used. They were added to the AoSMC 20 min before TIMP-1 and the effects on growth were measured 48 h later, using the BrdU cell proliferation assay. The Ras FPT III inhibitor blocked growth of the untreated and TIMP-1 treated AoSMC completely and LY294002 almost completely (Fig. 3A), suggesting that both Ras and PI3K were essential for both basal and TIMP-1-stimulated growth of the

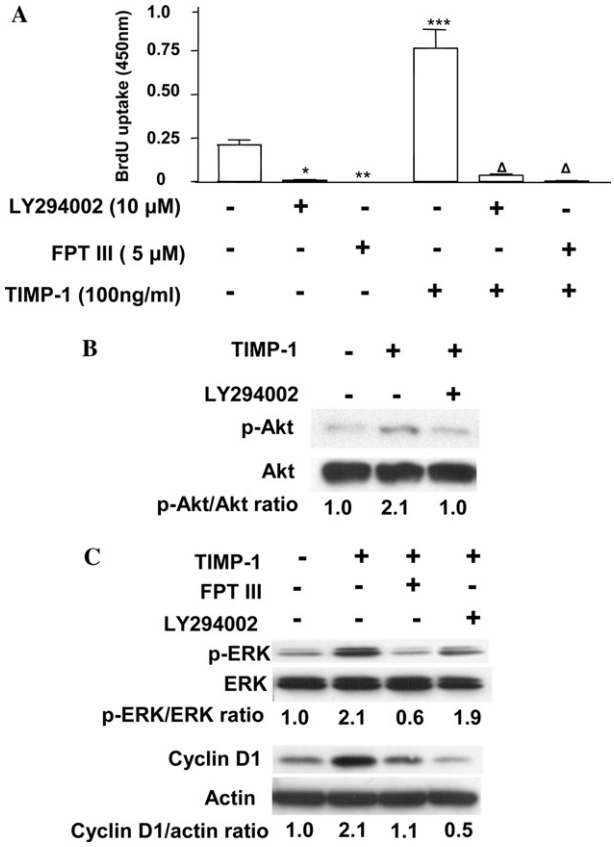

Fig. 3. Ras and PI3K inhibitors abolish TIMP-1-stimulated AoSMC proliferation, p-ERK, and cyclin D1 expression. (A) Serum starved AoSMC were incubated in the presence or absence of TIMP-1 (100 ng/ml), the PI3K inhibitor, LY294002 (10 μM), or the Ras inhibitor FPT III (5 μM). BrdU was added during the last 4 h of the 48 h incubation period. The data represent one of three experiments with similar results and are expressed as means ± SE ($n = 5$) (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. untreated cells, $\Delta p < 0.001$ vs. TIMP-1 only treated cells). (B). Western blot analysis of p-Akt expression in the presence or absence of TIMP-1 (100 ng/ml) or LY294002 (10 μM). (C). Western blot analysis of p-ERK, ERK, and cyclin D1 expression in the presence and absence of TIMP-1 (100 ng/ml), FPT III (5 μM), or LY294002 (10 μM).

AoSMC. We next investigated phosphorylation of Akt, which is a downstream target of PI3K. A faint band of phosphorylated Akt (p-Akt) was observed in the control, suggesting that Akt was constitutively active in the AoSMC (Fig. 3B). The p-Akt expression level was increased 2.1-fold following 15 min TIMP-1 treatment and was returned to the basal level in the presence of the PI3K inhibitor, LY294002. These results stress the involvement of the PI3K pathway in the TIMP-1-stimulated growth of the AoSMC.

The FPT III inhibitor was employed to further substantiate the involvement of Ras in the TIMP-1-stimulated p-ERK expression. In the presence of the Ras FPT III inhibitor, the p-ERK level was suppressed to almost half of that observed for the control (Fig. 3C). This indicated that Ras was critical for both the basal and TIMP-1-stimulated p-ERK expression. However, the

negligible effect of LY294002 on p-ERK expression implies that inhibition of the PI3K pathway did not interfere with TIMP-1 stimulation of Ras and ERK (Fig. 3C). Because cyclin D1 is stimulated during the second peak of Ras activity, which is associated with activation of the PI3K pathway [31], the effects of the Ras and PI3K inhibitors on cyclin D1 expression were examined. Elimination of the TIMP-1-mediated stimulation of cyclin D1 by the Ras FPT III further supports our finding that Ras activity was important for the TIMP-1-mediated stimulation of cyclin D1 (Fig. 3C). In the presence of both TIMP-1 and the PI3K inhibitor, LY294002, cyclin D1 was suppressed to 50% of the basal level (Fig. 3C). This signifies the importance of PI3K in normal cell cycle progression of AoSMC.

In conclusion, we have shown that TIMP-1 stimulates growth of human AoSMC, which is associated with activation of Ras and involves both ERK and PI3K signaling pathways. Our findings raise the questions whether TIMP-1 helps replenishing SMC following vascular injury and contributes to the excessive SMC proliferation associated with atherosclerosis.

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