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t-PA stimulates VEGF expression in endothelial cells via ERK2/p38 signaling pathways

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Vascular endothelial growth factor (VEGF) plays an essential role in the initiation and regulation of angiogenesis, which is a crucial component of wound healing and vessel growth. Tissue-type plasminogen activator (t-PA) could stimulate angiogenesis but the precise mechanisms of their proangiogenic actions remain unclear. We investigated whether t-PA can induce VEGF expression in ECV304 and further explored the underlying signaling pathway(s) involved. Through adenovirus mediated overexpression of t-PA in ECV304 cells, we demonstrated that t-PA significantly increased both VEGF mRNA and protein expression. A further mechanistic study showed that both ERK and p38 MAPK activation were involved in this process. Incubation of RVEC with PD 98059 (MEK kinase inhibitor) significantly reduced t-PA-induced ERK2 activity, VEGF mRNA and protein expression. Furthermore, PD 98059 treatment almost completely abolished p38 activation. Our data suggest that t-PA-stimulates VEGF expression in RVEC *via* transactivation of p38 by ERK. One potential implication of this finding is that increased t-PA levels in thomb could facilitate vessel growth by stimulating VEGF synthesis and angiogenesis.

1. Introduction

Angiogenesis, formation of new capillary blood vessels from pre-existing vasculature, which enables delivery of oxygen and nutrients, is essential for wound and ulcer healing and growth and metastasis of solid tumors (Folkman and Shing 1992). Angiogenesis is a multi-step process that includes endothelial cell proliferation and migration, capillary tube formation, and extracellular matrix degeneration and remodeling. The process of angiogenesis is tightly regulated by the balance between angiogenic stimulators and angiogenic inhibitors. As previous reports confirm that vascular endothelial growth factor (VEGF) is the key factor in stimulating angiogenesis, more and more attention is directed at the study of angiogenic inhibitors (Ferrara 2001; Leung et al. 1989; Corvol 2008). VEGF is distinct from other growth factors in being a secreted protein, an endothelial cell-specific mitogen *in vitro*, and the only known growth factor with vascular permeability-inducing activity (Houck et al. 1991; Connolly et al. 1989). Activation of VEGF during healing of vessel injury has previously been established (Jones et al. 1999). Endothelial cells serve to regulate the fibrinolytic system in the blood by secreting tissue-type plasminogen activator t-PA or urokinase-type plasminogen activator t-PA as well as type-1 plasminogen activator inhibitor PAI. t-PA can cause vasodilation and stimulate angiogenesis (Mehrabi et al. 2001; Form and Auerbach 1983; Spisni et al. 1992). Recent studies have demonstrated that an elevated tumour tissue extract antigen content of t-PA is associated with increased tumour aggressiveness and poor prognosis. Inhibition of t-PA generation, retards angiogenesis invasion, significantly down-regulates VEGF expression and suppresses tumor growth (Gallo et al. 2001; Liu et al. 2000). However the exact function of t-PA in regulating of VEGF expression and detailed signaling involved are largely unknown. Mitogen-activated protein (MAP) kinases are serinethreonine kinases that are rapidly activated in response to a variety of growth stimuli (Davis 1993; Chiang et al. 2012; Ohki et al. 2010). Here we demonstrate that overexpression of t-PA can significantly stimulate the VEGF expression in endothelial cells, and ERK and p38 MAPK play essential roles in this process. More interestingly, we further revealed that the ERK phosphorylation is located upstream of p38 in mediating the t-PA stimulation of VEGF expression.

2. Investigations and results

2.1. *Adenovirus mediated overexpression of t-PA in ECV304 cells*

In order to study the function of t-PA protein in VEGF regulation, recombinant adenovirus vector overexpressing t-PA protein was constructed and adenovirus was packaged according to previous reports (REF). ECV304 cells were infected with recombinant t-PA adenovirus and control adenovirus, the protein levels of t-PA in t-PA adenovirus infected cells were significantly upregulated at 12 h, 36 h, 48 h and 72 h after infection (Fig. 1A, C), while no obvious changes of t-PA protein level were observed in the control group (Fig. 1B, C). These results demonstrated that the recombinant t-PA adenovirus can successfully mediated the overexpression of t-PA.

2.2. *Both the mRNA and protein level of VEGF were up-regulated in t-PA overexpressed ECV304 cells*

To illustrate the effects of t-PA overexpression on VEGF expression regulation, we then analyzed both the mRNA and protein

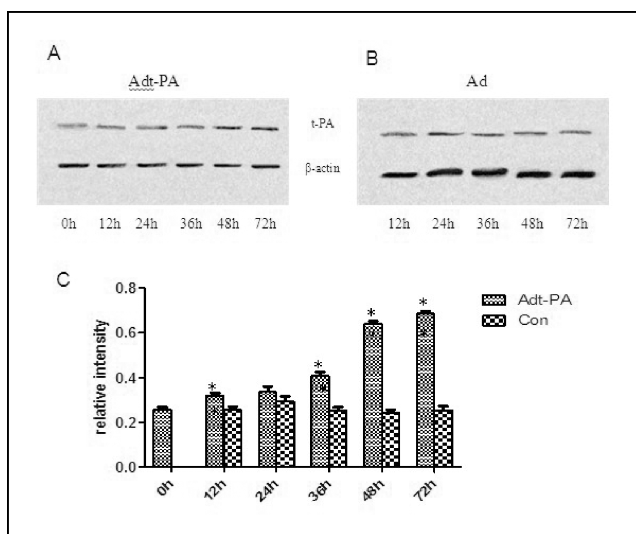

Fig. 1: Adenovirus mediated overexpression of t-PA in ECV304 cells. ECV304 cells were infected with recombinant t-PA adenovirus and control adenovirus, samples were collected at 12 h, 36 h, 48 h and 72 h after infection for western blot analysis for t-PA expression. Representative figures for recombinant t-PA adenovirus (A) and control adenovirus (B) and statistics data (C) were shown from three independent experiments. * $p < 0.05$ *versus* control (Student's t-test).

level of VEGF in t-PA overexpressed ECV304 cells, as indicated in Fig. 2, the mRNA level of VEGF was 1.8 fold that of the control group 24 h after virus infection (Fig. 2), a more significant enhancement of VEGF mRNA expression was observed at 48 h post infection (Fig. 2).

Following analysis of the protein level of VEGF by western blot further confirmed the mRNA expression changes, as com-

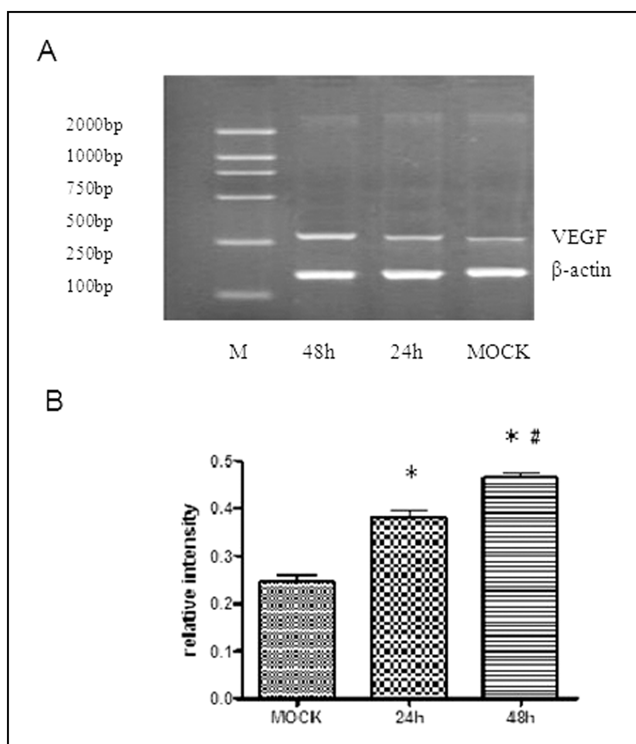

Fig. 2: Up-regulation of VEGF mRNA in t-PA overexpressed ECV304 cells. ECV304 cells were infected with control adenovirus and recombinant t-PA adenovirus, samples were collected at 24 h and 48 h after infection, total RNA were extracted and cDNA were synthesized, RT-PCR were perform for VEGF mRNA level quantification. Representative figure (A) and and statistics data (B) were shown from three independent experiments. * $p < 0.05$ *versus* mock control, # $p < 0.05$ *versus* 24 h sample (Student's t-test).

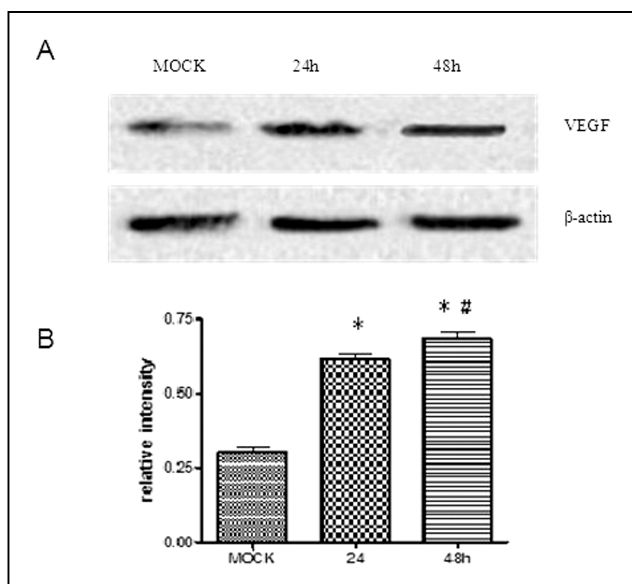

Fig. 3: Up-regulation of VEGF protein level in t-PA overexpressed ECV304 cells. ECV304 cells were infected with control adenovirus and recombinant t-PA adenovirus, samples were collected at 24 h and 48 h after infection, protein sample were prepared for VEGF protein level quantification. Representative figure (A) and and statistics data (B) were shown from three independent experiments. * $p < 0.05$ *versus* mock control, # $p < 0.05$ *versus* 24 h sample (Student's t-test).

pared to the mock control, the VEGF protein level was increased about two fold in the 24 h t-PA-virus infected group (Fig. 3). More significant up-regulation of VEGF was indicated after 48 h infection (Fig. 3).

2.3. *ERK and p38 but not JNK phosphorylation were increased in t-PA overexpressed ECV304 cells*

Mitogen-activated protein kinase (MAPK) such as p38, ERK, JNK were extensively involved in the regulation of gene expression, and VEGF has also been reported under the control of MAPKs (REF). In order to reveal the underlying mechanism how t-PA regulates the expression of VEGF, we analyzed the MAPK phosphorylation in the control virus and t-PA virus infected cells. Firstly, we detected the total ERK and its phosphorylated form expression by western blot, as shown in Fig. 4. The phosphorylated ERK was obviously increased after 24 h infection by the t-PA virus, further significantly enhancement of ERK phosphorylation was observed 48 h and 72 h post t-PA virus infection (Fig. 4A and C). However the total ERK expression was not affected by t-PA (Fig. 4B and D). At the same time, we also detected the expression of JNK and p38 phosphorylation, similar results were observed in p38 (Fig. 5). The phosphorylated p38 was obviously increased after 24 h infection by the t-PA virus, further significant, enhancement of p38 phosphorylation was observed 48 h and 72 h post t-PA virus infection (Fig. 5A and C). However the total ERK expression was not affected by t-PA (Fig. 5B and D). Interestingly, expression of both the total JNK and its phosphorylated form were not affected by t-PA overexpression (data not shown).

2.4. *Both ERK and p38 phosphorylation induced by t-PA overexpressed were necessary for VEGF up-regulation*

Although the above results demonstrated that t-PA overexpression can enhance VEGF expression, at the same time t-PA can also increase ERK and p38 phosphorylation, while it is unknown if the VEGF enhancement is dependend on the MAPK phospho-

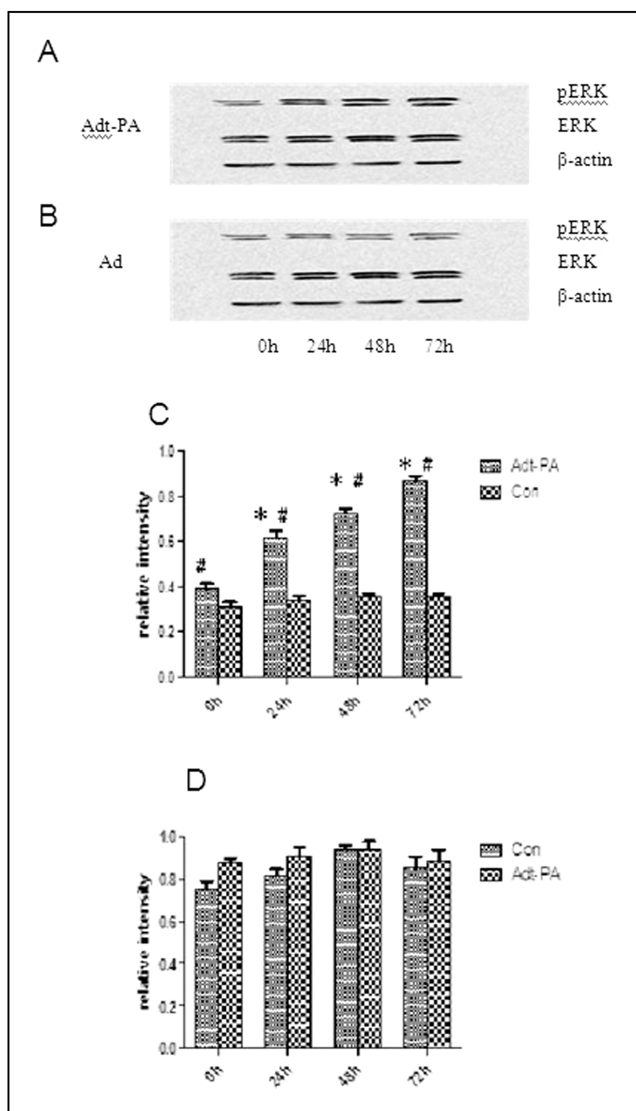

Fig. 4: ERK phosphorylation were increased in t-PA overexpressed ECV304 cells. ECV304 cells were infected with control adenovirus and recombinant t-PA adenovirus, samples were collected at 24 h, 48 h and 72 h after infection, protein sample were prepared for ERK phosphorylation analysis. Representative figure (A, B) and statistics data (C,D) were shown from three independent experiments. # $p < 0.05$ *versus* 0 h sample, * $p < 0.05$ *versus* 24 h sample (Student's t-test).

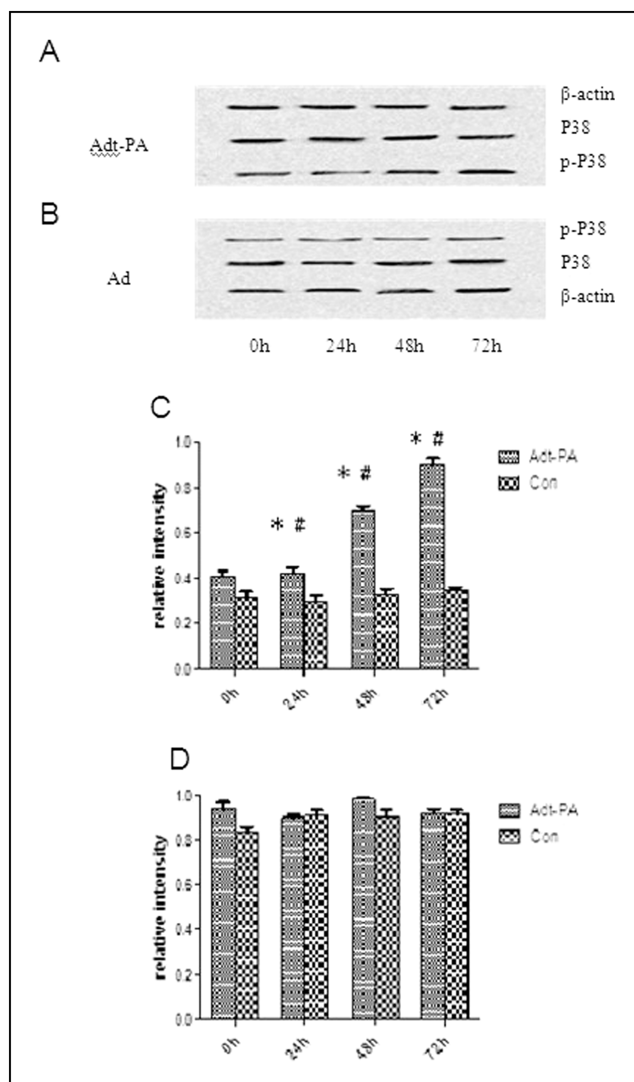

Fig. 5: p38 phosphorylation were increased in t-PA overexpressed ECV304 cells. ECV304 cells were infected with control adenovirus and recombinant t-PA adenovirus, samples were collected at 24 h, 48 h and 72 h after infection, protein sample were prepared for p38 phosphorylation analysis. Representative figure (A, B) and statistics data (C,D) were shown from three independent experiments. # $p < 0.05$ *versus* 0 h sample, * $p < 0.05$ *versus* 24 h sample (Student's t-test).

rylation, so we further studied this relationship using the MAPK inhibitors. PD98059 (PD) is the most common tool to inhibit the activity of ERK (REF), and we detected if PD can diminish the VEGF enhancement, as shown in Fig. 6. PD treatment inhibits the ERK phosphorylation both of the control and t-PA group, which indicates the drug efficiency (Fig. 6A-C). At the same time, data from VEGF protein level quantification showed that t-PA can enhance the VEGF expression, while this enhancement was significantly inhibited upon PD treatment. These results demonstrated that ERK phosphorylation induced by t-PA overexpressed were necessary for VEGF upregulation. As to p38, a similar analysis was performed, SB203580 (SB) was the specific inhibitor of p38 phosphorylation, the data in Fig. 7 show a similarly important role of p38 in mediating the up-regulation of VEGF by t-PA. SB treatment inhibits the p38 phosphorylation both of the control and t-PA group, which indicates drug efficiency (Fig. 7A-C). At the same time, data from VEGF protein level quantification showed that t-PA can enhance the VEGF expression, while this enhancement was significantly inhibited upon PD treatment. The above results demonstrated that t-PA stimulates VEGF expression in endothelial cells *via* both ERK and p38 signaling pathways.

2.5. *t-PA induced ERK phosphorylation was at the upstream of p38 phosphorylation*

The above results demonstrated the necessary function of both ERK and p38 signaling pathway in mediating the t-PA stimulated VEGF expression, but the relative position of ERK and JNK in this signaling incident, is interesting so we further analyzed this question by applying the ERK and JKN inhibitors. As indicated above, t-PA overexpression can stimulate both ERK and JNK phosphorylation (Fig. 8 A, B, D, E). t-PA stimulated p38 phosphorylation can be blocked by SB treatment (Fig. 8 A, B), however, the t-PA stimulated ERK phosphorylation was almost not influenced by PD treatment (Fig. 8 D, E). These results clear revealed that t-PA induced ERK phosphorylation was upstream of p38 phosphorylation.

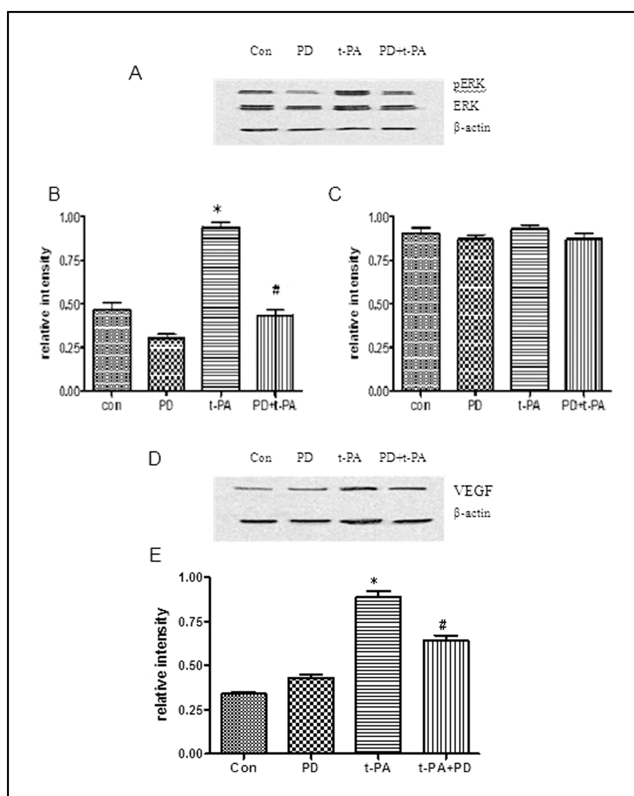

Fig. 6: Up-regulation of VEGF by t-PA overexpression was inhibited upon PD98059 treatment. ECV304 cells were infected with control adenovirus and recombinant t-PA adenovirus, or treated with PD98059, samples were collected, protein sample were prepared for ERK phosphorylation and VEGF protein level analysis by western blot. Representative figure (A, D) and statistics data (B, C, E) were shown from three independent experiments. $*p < 0.05$ *versus* 0 h sample, # $p < 0.05$ *versus* t-PA overexpressed group (Student's t-test).

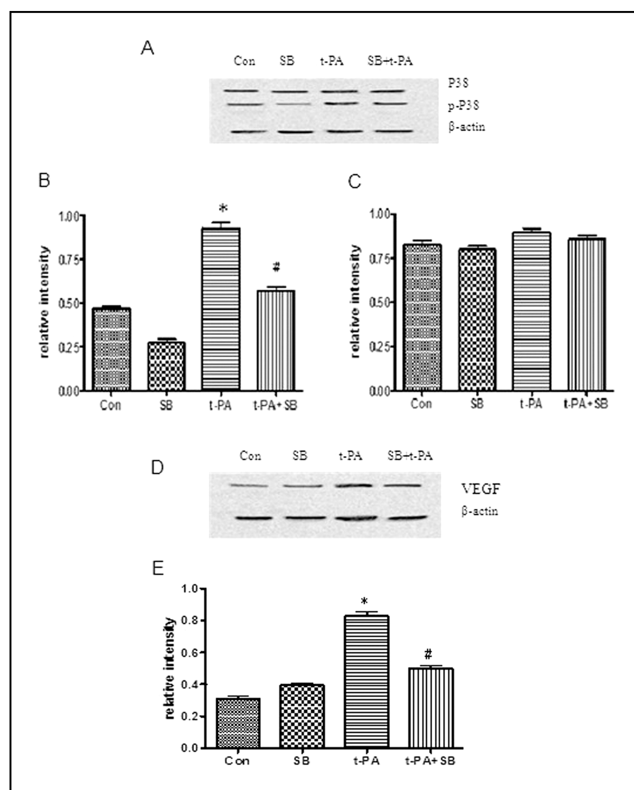

Fig. 7: Up-regulation of VEGF by t-PA overexpression was inhibited upon SB203580 treatment. ECV304 cells were infected with control adenovirus and recombinant t-PA adenovirus, or treated with SB203580, samples were collected, protein sample were prepared for p38 phosphorylation and VEGF protein level analysis by western blot. Representative figure (A, D) and statistics data (B, C, E) were shown from three independent experiments. $*p < 0.05$ *versus* 0 h sample, # $p < 0.05$ *versus* t-PA overexpressed group (Student's t-test).

3. Discussion

t-PA stimulates angiogenesis *in vitro* and *in vivo* (Form and Auerbach 1983; Spisni et al. 1992; Nancy and Barry 1994; Benezra 1978). t-PA concentration and expression are increased in a variety of thomb and vessel injuries (Rigas et al. 1993; Eberhart et al. 1994; Yang et al. 1998), thus implicating a role of t-PA in pathological angiogenesis (Gallo et al. 2001; Liu et al. 2000). However, the precise mechanism(s) by which t-PA stimulates angiogenesis is not explained. The vascular endothelial growth factor (VEGF) is known to induce angiogenesis by increasing endothelial cell proliferation, migration and microvascular hyperpermeability (Brown et al. 1992). Besides normal angiogenesis (e.g., during wound healing) (Jones et al. 1999; Brown et al. 1992), VEGF also stimulates pathological angiogenesis including cancer growth (Xu et al. 2013; Grothey and Ellis 2008; Hsu and Wakelee 2009). Although previous studies have reported that t-PA increases VEGF expression in other cells (osteoblasts, synovial fibroblasts and Muller cells)(Davis 1993; Chiang et al. 2012; Ohki et al. 2010), the effect of t-PA on the ultimate target cells for angiogenesis—endothelial cells—remain unknown. We report here that t-PA can induce VEGF expression in endothelial cells, and the underlying mechanism is stimulation of the ERK/p38 signaling pathways.
VEGF is regulated by extensive signaling pathways (Banerjee et al. 2007). Among them, p38 MAPK up-regulates VEGF expression by stabilizing the VEGF mRNA in response to cellular stress. During hypoxia or nutrient stress VEGF translation continues in a cap-independent manner *via* the internal ribosome entry site. This cap-independent manner is mediated by PI3K/AKT signal pathway, and transcription is driven by hypoxia inducible factor 1, which needs ERK activation. In this study, we found that t-PA overexpression stimulates VEGF expression, and this process is dependent on both p38 and ERK activation. This finding shed light on that there are some links between the p38 signaling and ERK signaling in the regulation of VEGF expression. More interestingly, using MAPK inhibitor, we found that during the process of t-PA stimulation of VEGF expression, ERK phosphorylation is above the signaling cascade of p38. This not only comfirmed the above hypothesis that there exists some link/between ERK and p38 signaling, but also revealed a totally novel signaling pathway from ERK to p38. However, the detailed signaling crosstalks between them need further exploration.
In this report we show that a significant up-regulation of VEGF in endothelial cells overexpressing t-PA, and t-PA stimulates VEGF in a ERK and p38 dependent manner. Interestingly, we also revealed that the ERK signaling is upstream the p38 signaling.
As VEGF plays an important role in the initiation and regulation of angiogenesis and is involved in wound healing and vessel growth, one potential implication of our results is that increased t-PA levels in thomb could facilitate vessel growth by stimulating VEGF synthesis and angiogenesis. *In vivo* studies will be necessary to characterize the physiological role of t-PA in angiogenesis and vascular maintenance.

4. Experimental

4.1. Reagents

DMEM medium and heparin were obtained from GibcoBRL (Grand Island, NY). Antibiotic: antimycotic supplement and other tissue culture reagents

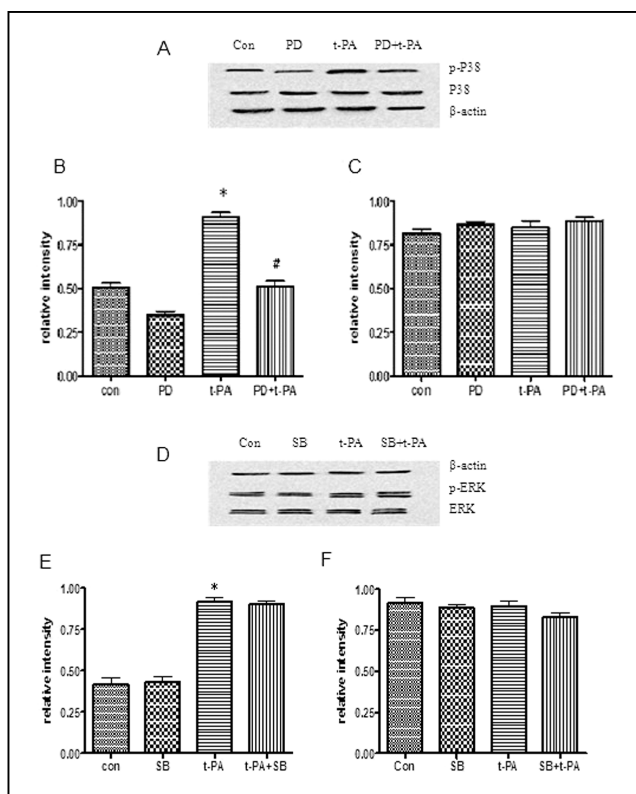

Fig. 8: t-PA induced ERK phosphorylation was at the upstream of p38 phosphorylation. ECV304 cells were infected with control adenovirus and recombinant t-PA adenovirus, or/and treated with PD98059/SB203580, samples were collected, protein sample were prepared for ERK and p38 phosphorylation analysis by western blot. Representative figure (A, D) and statistics data (B, C, E, F) were shown from three independent experiments. *$p < 0.05$ *versus* 0 h sample, # $p < 0.05$ *versus* t-PA overexpressed group (Student's t-test).

were obtained from Fisher Scientific. Fetal bovine serum (FBS) was obtained from Gibco (San Diego, CA). Rabbit polyclonal anti-ERK2, anti-VEGF, anti-JNK1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

4.2. Cell culture

ECV304 cells were routinely grown in endothelial–SFM medium supplemented with 20% FBS, 100 mg/ml heparin and antibiotics in an atmosphere of 5% CO_2 and 95% air at 37 °C in a humidified incubator. Subcultures were made from confluent stock cultures by trypsinization in PBS containing 0.5 mM EDTA and 0.25% trypsin.

4.3. Overexpression of t-PA in ECV304 cells

Adenovirus expressed full-length t-PA cDNA was transfected into ECV304 cells as previously described. The protein expression was detected by western blotting, These cells transfected with t-PA were routinely maintained in medium.

4.4. RNA isolation and RT-PCR

Total RNA was isolated using the TRIZOL reagent as previously described (Chomczynski and Sacchi 1987). Reverse transcription and polymerase chain reaction (RT/PCR) were performed using a TonekerBio RNA PCR kit and a DNA thermal cycler (Applied Biosystems, Foster City, CA) as previously described (Jones et al. 1999). Briefly, RT was performed at 42 °C for 15 min, at 99 °C for 5 min, and at 5 °C for 5 min. cDNA was amplified by using primers that recognize all four isoforms of VEGF mRNA. The nucleotide sequence of primers utilized were 5-CCTGGTGGACATCTTCCAGGAGTACC-3(sense) and 5-GAAGCTCATCTCTCCTATGTGCTGGC-3(antisense). The primers for b-actin were 5-TTGTAACCAACTGGGACGATATGG-3(sense) and 5-GATCTTGATCTTCATGGTGCTAGG-3 (antisense). The primers for b-actin were purchased from Clontech, Palo Alto, CA. The PCR amplification was performed for 28 cycles of 1 min at 94 °C for denaturing, 1 min at 55 °C for annealing and 2 min at 72 °C for extension. Ten microliter aliquots of the products were subjected to electrophoresis on a 1.25% agarose gel and DNA was visualized by ethidium bromide staining. Location of the products and their sizes were determined by using a 100-bp ladder (GIBCO, Gaithersburg, MD). The gel was photographed under ultraviolet illumination. For quantitative assessment of the PCR products, a video image analysis system (Image-1/FL, Universal Imaging Corp., Westchester, PA) was used. The image system can distinguish density on a scale of 0–255 units. Each measurement was standardized by subtracting the background intensity in average.

4.5. Western blot analysis

In brief, cells were washed with ice-cold PBS and lysed with a lysis buffer. The protein content of the clarified lysate was determined using a BCA protein assay kit (PierceChemical Co., Rockford, IL). Cell lysates containing equal amounts of proteins were subjected to SDS–PAGE and transferred onto a nitrocellulose membrane. Blots were stained with Ponceau Red to ensure equal loading and complete transfer of proteins. The blot was incubated with a blocking buffer, and probed with specific primary antibodies. Blots were washed and incubated with specific peroxidase-conjugated secondary antibodies. After washing, bound antibody was visualized by an ECL detection system (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions. The density of the protein bands was analyzed using a video image analysis system (Image-1/FL, Universal Imaging Corp., Westchester, PA).

4.6. Statistical analysis

All data are reported as mean ± SD. Statistical significance of differences between mean values was assessed by Student's t test for unpaired data. A P value less than 0.05 was considered statistically significant.

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