

Human connective tissue growth factor gene transfection promoted the proliferation of human umbilical vein endothelial cells *via* Erk and *Akt* signal pathways

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Connective tissue growth factor (CTGF), a member of the CCN (Cyr61, Ctgf, Nov) family, is involved in many biological processes such as angiogenesis. In this study we analyzed the action of CTGF in transfected human umbilical vein endothelial cells (HUVECs) and explored the possible mechanism. The eukaryotic expression vector of CTGF gene was constructed in this study, and cDNA was amplified from HUVECs. The expression vector (pcDNA3.1 (-)/CTGF) which contains the entire coding region was verified by DNA sequence analysis and transferred successfully into HUVECs. Western blot analysis demonstrated that CTGF protein in HUVECs was over-expressed at 48h after transfection. To elucidate the putative mechanism of CTGF's transfection on HUVEC proliferation, the intracellular signaling pathways included mitogen-activated protein kinases (MAPK) and phosphoinositol 3-kinase (PI3K)/*Akt* were detected. The results demonstrated CTGF transfection promoted the proliferation of HUVECs; ERK1/2 and PI3K/*Akt* activities were increased when HUVECs transfected with CTGF; Special inhibitors (LY294002 and U0126) could partially prevent the pro-proliferative action of CTGF transfection. In conclusion, our data showed that CTGF transfection promoted the proliferation of HUVECs, and the ERK1/2 and *Akt* signal pathways involved in.

Keywords: Connective tissue growth factor, HUVECs, proliferation, ERK, *Akt*, angiogenesis

INTRODUCTION

Connective tissue growth factor (CTGF or CCN2) belongs to the CCN family of immediate early genes (Bork, 1993), which includes CTGF (also known as fisp-12), CYR61 (cysteine-rich 61/CEF10), Nov (nephroblastoma overexpressed) and the discovered WISP1/elm1, WISP2/rCop1, and WISP3 (Babic et al., 1999; Brigstock, 1999; Lau et al., 1999; Perbal, 2004). Although all members of the CCN family are highly conserved among species and display a similar gene structure, their functions remain largely unknown due to the fact that members of the family exhibit such diversity of functions

which have defied simple classification (Lin et al.). It has been well established that CTGF, which is originally identified in conditioned medium of human umbilical vein cells (HUVECs) (Bradham et al., 1991), plays an important role in various human diseases including systemic sclerosis (Igarashi et al., 1995), atherosclerosis (Cicha et al., 2008; Oemar et al., 1997), renal diseases (Ito et al., 1998), hepatic fibrosis in biliary atresia (Williams et al., 2000), and malignant melanoma as well (Walker et al., 2002). Currently, a lot of studies found CTGF/CCN2 was essential for the angiogenic process in microvascular endothelial cells, to mediate the S1P-induced endothelial cell migration and capillary-like formation, and related to a lot of proangiogenic factors such as VEGF, HGF, IL-15 as well (Hose et al., 2009; Markiewicz et al., 2011; Wang et al., 2010).

In vitro, depending on the cell types, CTGF exhibits diverse biological actions including mitogenesis, matrix production, cell proliferation and apoptosis (Grotendorst, 1997; Shimo et al., 1999) as well as an important pro-angiogenic activity during hypoxia (Kondo et al., 2002). There are accumulative evidences indicating that inhibition of the endogenous expression of CTGF by its antisense oligonucleotide and antisense RNA or its specific antibodies or could suppress the proliferation and migration of vascular endothelial cells including HUVECs (Aikawa et al., 2006; Shimo et al., 2006; Shimo et al., 1999); on the other hand, the fragment or modules of CCN2 protein could promote the cell proliferation and migration (Kubota et al., 2006). In our previous study, we successfully transfected HUVECs with the whole CTGF gene and found the cells got a high migration effect (Luo et al., 2006), but it still remains to be established in-depth the whole CTGF gene transfection whether has any direct actions on cell proliferation and explore the putative mechanism during the process.

MATERIALS AND METHODS

Cell culture

Human umbilical cords, obtained from the Department of Obstetrics, Beijing Gynecology and Obstetrics Hospital, were cleaned with isotonic PBS buffer and incubated for 20 minutes at 37°C with 0.2% collagenase (type I, Sigma, USA). Vein endothelial cells were collected and resuspended in Medium M200 (Cascade Biologics, Portland, Oreg., USA), containing low serum supplements (Cascade Biologics), penicillin (100U/ml) and streptomycin (100ug/ml). Cells were seeded and cultured in a humidified incubator equilibrated with 95% air/5% CO₂ at 37°C. After immunomagnetic separation (Dyna, ASA, Oslo, Norway) and immunochemistry identification (Factor VIII, purchased from (Santa Cruz Biotechnology, Santa Cruz, USA), cells with good conditions were selected for the following study. To avoid genetic mutation and low viability, no more than six passages of HUVECs were used.

Identification of endothelial cells by immunocytochemical staining with factor VIII

For identifying the cell's origin, we selected the factor VIII as a marker. Briefly, cells after immunomagnetic separation were grown on Superfrost-plus microscope slides (Menzel, Braunschweig, Germany). After fixation with buffered paraformaldehyde, cells were treated with Pronase for 30 min, followed by incubation with 0.3% H₂O₂ in methanol for 30 min to eliminate endogenous peroxidase activity and rinsed in PBS; then they were incubated with normal blocking serum for 20 min. Afterwards, cells were incubated with rabbit anti-factor VIII overnight at 4°C. Incubation and visualization with a ploy-HRP anti-rabbit IgG were performed according to the

manufactures protocol (DAB Kit, Promega, Madison, USA).

CTGF gene transfection in HUVECs

RNA isolation and RT-PCR

HUVECs were challenged with PD-ECGF (10pg/ml, Santa Cruz, CA, USA) for 2h after 24h starvation. Total RNA was then isolated from these cells according to the illustration of Trizol agent kit. According to human CTGF cDNA sequence, a pair of specific primers containing restriction sites of Xba I and Hind III were designed, respectively.

5'-GCTCTAGAGCAGTGCCAACCATGACC-3'

5'-CCCAAGCTTCTTCATGCCATGTCTCCGTA-3'

Total RNA was reverse transcribed according to the illustration of cDNA first chain construction agent kit (Invitrogen Co., Shanghai, China) with random primers Oligo dT. The logarithmic phase of PCR amplification was performed with specific primers in the following condition: 1min denaturation at 95°C, 1min annealing at 56°C and 2min extension at 72°C for 35 cycles. The final elongation time was 7min at 72 °C.

Ligation of CTGF and pcDNA3.1(-)

The CTGF cDNA fragments were ligated with expression plasmid in a total reaction volume of 10μL containing CTGF cDNA fragments 3μL, retrieved pcDNA3.1(-) (Invitrogen Co., Shanghai, China) fragments 2μL, T4DNA ligase 1μL, 10M Buffer 1μL and ddH₂O 3μL. The ligation was carried out overnight at 16°C and the reaction was stopped by heating at 65°C for 15 min..

Transfection in the Escherichia coli DH5α cells and identification of pcDNA3.1(-)/CTGF

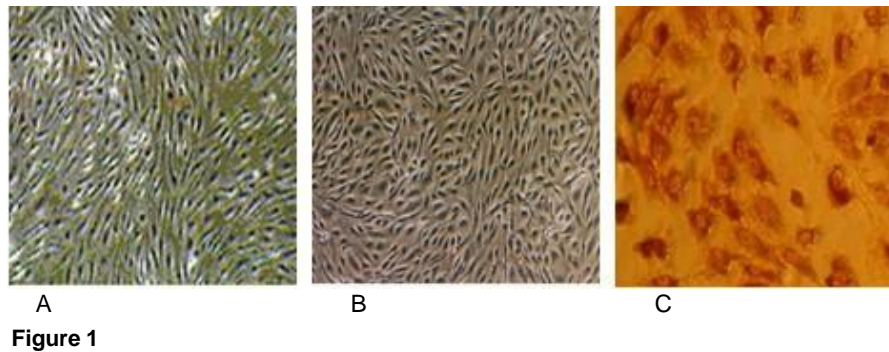
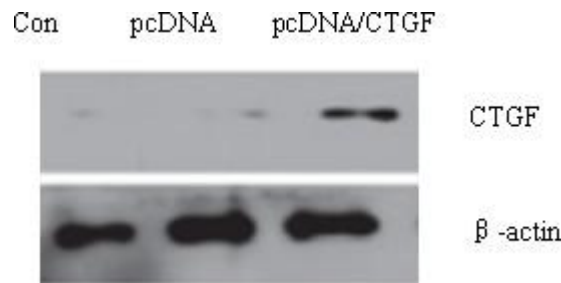
The DNA was transformed into *E. coli* DH5α cells by standard methods. The *E. coli* DH5α cells were then plated on LB plates containing ampicillin (100 μg/mL) and incubated at 37°C for 16 h. Single colonies were selected and grown in LB medium with ampicillin for 10 h. The plasmid DNA was then isolated and checked for the presence of the insert and for the correct orientation using *Hind*III plus *Xba*I restriction cleavage and DNA sequencing.

Gene transfer protocol

The pcDNA3.1(-)/CTGF expression plasmid was introduced into the cultured cells according to the modified method described by Hein (Hein et al., 1998). Briefly, 2μg of the pcDNA3.1 plasmid and 4μL lipofectin2000 were incubated separately in 100μL culture medium free of antibiotics and serum for 30 min at room temperature, followed by another 30 min after mixing of the two volumes. Then, 800μl of serum-free medium was added. The cells were transfected in a 1 ml total volume for 24 h at 37°C. 24 h later, the transfection solution was replaced with normal cell growth medium.

Proliferation Assays (MTT methods)

For the assessment of the proliferation rate of HUVECs, transfection and stimulation were performed in 96-well plates. HUVECs were

**Figure 1****Figure 2**

seeded into 96-well culture plates at a density of 15,000 cells/well and incubated in serum-free medium 200 for 24 hours. The cell viability was calculated by typan blue exclusion. The OD values were read by a scanning multiwell spectrophotometer by measuring the absorbance of the dye with a wavelength of 570 nm and a reference wavelength of 630 nm. Cell survival was calculated as a percentage of the control values.

The experiments were conducted in the absence or presence of pharmacological inhibitors of selected signaling pathway. Cells were treated with inhibitor for 30 minutes before the duration of the stimulation with CTGF. The inhibitor used was phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 and ERK inhibitor U0126 (Sigma, St. Louis, USA).

Western Blotting Assay

Cells were collected and lysed in the specific cell lysis buffer containing 10mM Tris-HCL (PH 8.0), 1%NP-40, 2mM EDTA (PH8.0), 100ug/mL DTT, 100ug/mL PMSF, and 1ug/mL aprotinin. Following centrifugation at 100,000 rpm for 10 min, the supernatant were collected and subpackaged. The protein concentration was determined by Bradford protein assay. Equal amounts of total protein was segregated by SDS-PAGE and blotted onto PVDF membrane. The protein blot was hybridized with the primary antibody of CTGF, *Akt*, *pAkt* ERK1/2 and p-ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, USA), and then with the corresponding secondary antibody, followed by detection with Western Blotting Luminol Reagent (ECL) kit (Amersham, New Jersey, USA).

Statistical Analysis

Statistical analysis was performed using the statistical program SPSS 10.0 for windows (SPSS Inc. Chicago, USA). All data are presented as mean \pm SEM and p values < 0.05 were considered as statistically significant

RESULTS

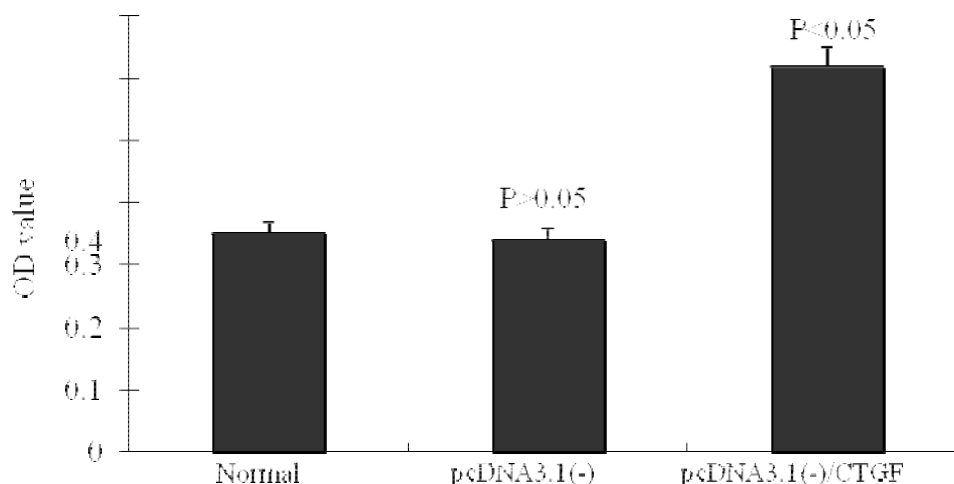
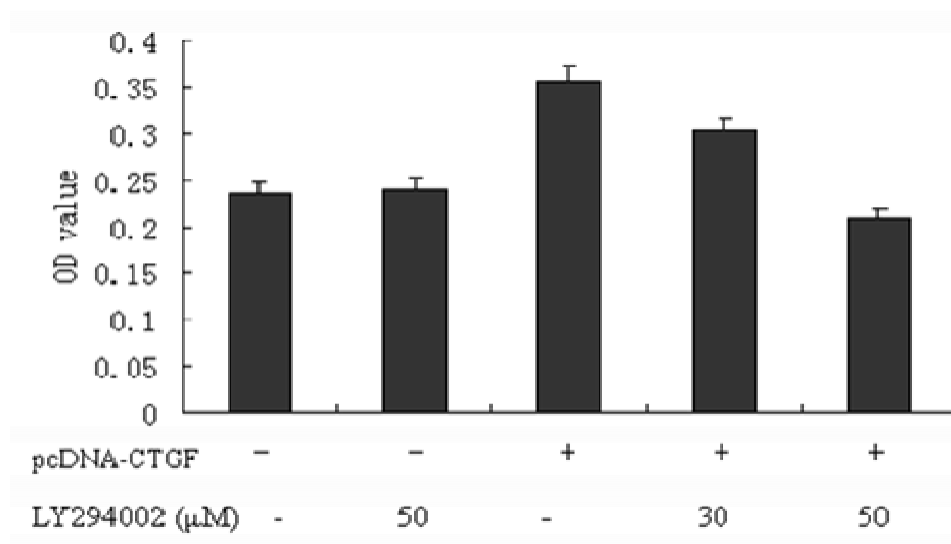
Under microscopy, the HUVECs formed a typical 'cobblestone' morphology (Figure 1A and Figure 1B) and the cells were identified by the presence of \square factor related antigen using the immunohistochemistry technique (Figure 1b).

Figure 1, A, Confluent human umbilical vein endothelial cells (HUVECs) showed the typical "cobblestone" appearance after immunomagnetic separation. B, HUVECs after CTGF gene transfection got higher proliferation; C: the cytochemical identification of HUVECs by the factor VIII (X400).

After 24h, CTGF protein expression is obvious in the transfected HUVECs, but very slight in the vehicle and normal controls (Figure 2), which suggested that CTGF was overexpressed in the cells transfected with pcDNA3.1(-)/CTGF.

Figure 2, the CTGF protein expression in HUVECs: Lane 1, normal control; Lane 2, pcDNA3.1(-) vehicle control; Lane 3, pcDNA3.1(-)/CTGF

Cell proliferation assay can identify proliferating cells

**Figure 3.****Figure 4 A**

under different conditions. Transfected cells were cultured for 24 hours. The results demonstrated that the transfected HUVECs got a higher proliferation effect than normal and vehicle control (Figure 3, $P < 0.05$).

Figure 3, Cell growth evaluated under different conditions by the MTT method. Mean values were obtained from three independent experiments. Compared to controls (normal group and pcDNA3.1 (-) group), the results showed that transfected CTGF induced HUVECs proliferation ($P < 0.05$), while no statistical difference between the normal and pcDNA3.1 (-) groups ($p > 0.05$).

Cell proliferation usually implies the activation of some major intracellular signaling pathways including mitogen-activated protein kinases (MAPK) and

phosphoinositol 3-kinase (PI3K)/Akt pathways (Kapoor et al., 2003). To determine whether these pathways were involved in CTGF-induced HUVECs proliferation in our experiments, cells were treated with the specific inhibitors LY294002 (30 μM and 50 μM) and U0126 (10 μM) during CTGF challenging. The results showed both inhibitors could decrease HUVEC proliferation ability when transfection with CTGF gene (Figure 4A and 4B), while no effect on the normal or vehicle controls. In our experiments, 50 μM LY294002 and 10 μM U0126 nearly completely depleted the pro-proliferative effect induced by CTGF gene transfection (vs controls, $P > 0.05$).

Figure 4, Both special inhibitors of PI3K/AKT LY294002 and ERK1/2 phosphorylation U0126 prevented the

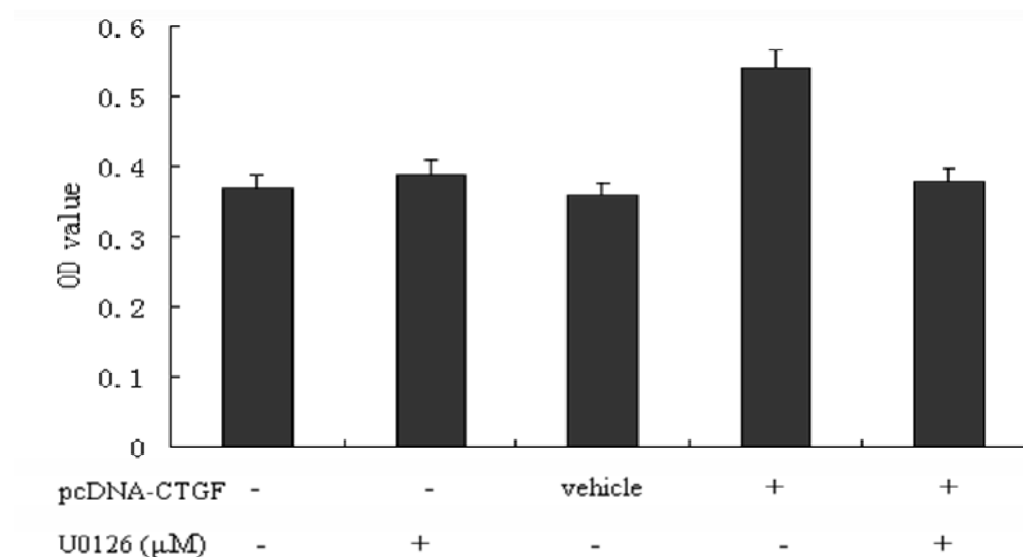


Figure 4 B

pro-proliferative effect on HUVECs transfected with CTGF, whereas no obvious effect on the normal control and vehicle control. A, LY294002 (30μM and 50μM) depleted the pro-proliferation effect in HUVECs after transfected with CTGF gene ($p < 0.05$, LY294002 30/50 μM/pcDNA-CTGF (+) group vs. pcDNA-CTGF (+) group), but no obvious effect on the proliferation in the controls ($p > 0.05$), and 50 μM LY294002 nearly completely depleted the pro-proliferative effect induced by CTGF gene transfection (vs. the normal control and vehicle control, $p > 0.05$); B, showed U0126 (10μM) also inhibited the proliferation effect induced by CTGF transfection ($p < 0.05$, U0126(+)/pcDNA-CTGF (+) group vs. the pcDNA-CTGF (+) group) and nearly completely depleted the pro-proliferative effect induced by CTGF gene transfection (the normal control and vehicle control, $p > 0.05$).

To further define whether these most important intracellular pathways (ERK1/2 and PI3K/Akt) were involved in the pro-proliferative effect of CTGF on HUVECs, CTGF-transfected cell lysates were probed to determine these protein levels and their phosphorylation status. Our previous study showed the expression of constitutive pERK and pAkt was very low, but markedly increasing in this study when transfecting with CTGF gene transfection, but this action was almost completely blocked by the inhibitors of PI3K/Akt and ERK1/2 (Fig. 5A and 5B). These results suggested CTGF overexpression promoted the proliferation of HUVECs probably by the activation of ERK and Akt signal pathways.

Figure 5, Western blot analysis showed that the activities of phosphorylated ERK1/2 and phosphorylated Akt in HUVECs with or without CTGF transfection after

24h. After transfection, the pERK1/2 and pAkt activity showed a marked increase ($p < 0.05$, vs. the normal control and vector control), and both inhibitors U0126 (10μM) and LY294002 (50μM) nearly completely prevented the activation induced by CTGF transfection ($p < 0.05$, vs. transfection group). All data were obtained from three representative experiments. A, the pAkt and tAkt expression in HUVECs and the action of LY294002; the results demonstrated pAkt activity markedly increased after transfection with CTGF ($p < 0.05$ vs. controls), and was obviously blocked by using LY294002 ($p < 0.05$ vs. CTGF transfection group). B, showed the pERK and tERK activity in HUVECs and the action of U0126; the results showed pERK activity increased obviously in transfection cells ($p < 0.05$ vs. controls), and was nearly completely inhibited when U0126 use ($p < 0.05$ vs. transfection group).

DISCUSSION

It is well known that the CCN families including CTGF were originally identified as the important regulators in many pathophysiological processes especially involved in renal fibrosis (Ito et al., 1998). But nowadays more and more compelling evidences have been found to support the close relationship between CTGF and tumor angiogenesis (Aikawa et al., 2006; Chang et al., 2006; Chien et al., 2006; Kondo et al., 2002; Pan et al., 2002; Shimo et al., 1999). In glioblastoma, CTGF expression was elevated in the proliferating endothelial cells and tumor cells, and was preferentially expressed in the vasculature at the juvenile pilocytic astrocytoma (Pan et al., 2002). Recent observation demonstrated that purified

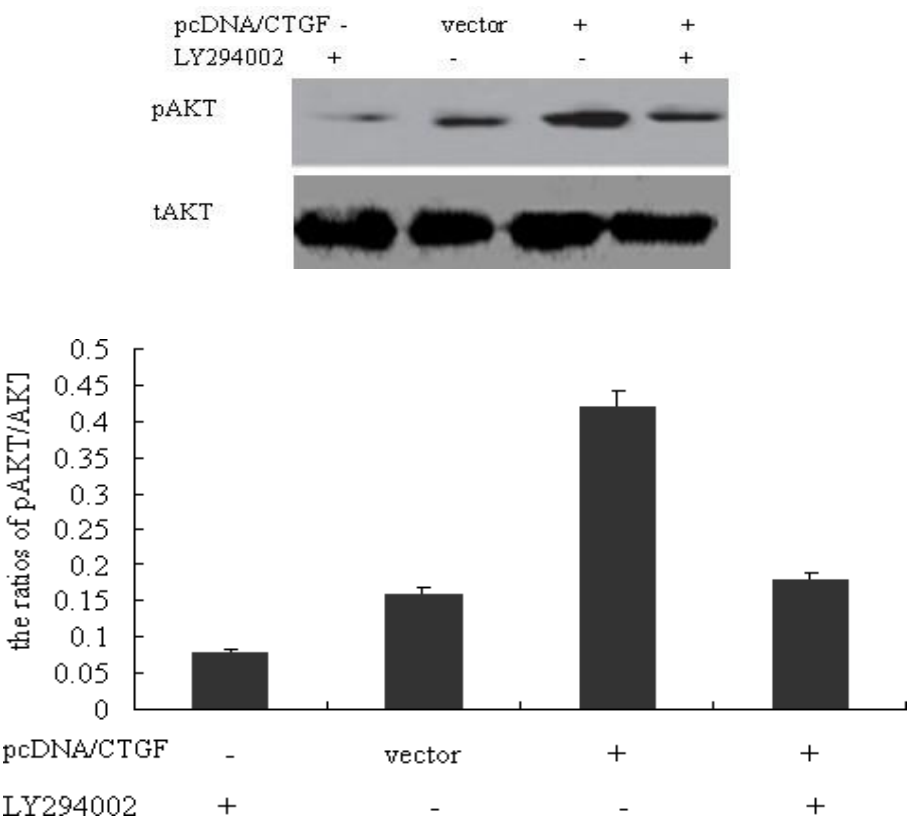


Figure A

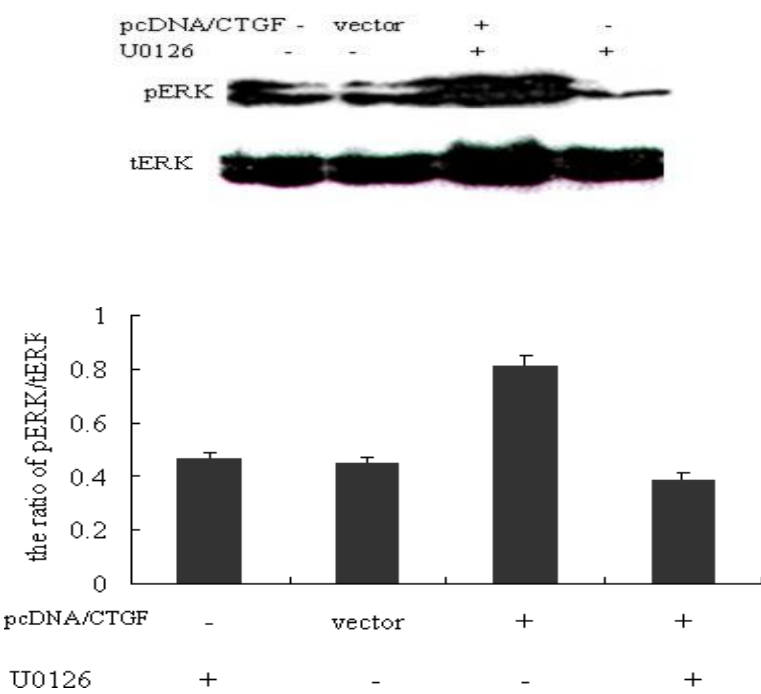


Figure B

recombinant CTGF promoted the proliferation and migration of bovine aorta endothelial cells(Shimo et al., 1999), and could provoke the expression of a number of metalloproteinases that played important roles in the invasive processes for neovascularization(Kondo et al., 2002). The matrix metalloproteinases-1, 2, 3 and 9 (MMP-1, 2,3,and 9), and MT1-MMP genes were up-regulated in response to CTGF treatment, which suggested that CTGF critically tilted the balance of the key proteinases and their respective endogenous inhibitors towards increasing proteolysis in the ECM, and eventually promoted vascular endothelial cell migration (Kondo et al., 2002). In our previous study we also confirmed that CTGF promoted the migration of HUVECs by scratch tests(Luo et al., 2006), and in this study we demonstrated its markedly pro-proliferative effect in transfected HUVECs. All suggested CTGF played an important role in physiological and pathological angiogenesis.

Normal endothelial cells (EC), which line the inner vessel wall and play an important role in regulating vascular function, are in a highly quiescent state in normal conditions. They switch into an active proliferative state under stimulations, then grow, migrate and form new blood vessels that supply oxygen and nutrients. Stimulation of EC proliferation could effectively promote angiogenesis(Risau, 1997). In this study, the transfection markedly promoted the proliferation of HUVECs, and this effect was certified basing on morphological observations and also on the basis of possible molecular biological exploration.

Cell proliferation usually implies activation of several important intracellular pathways, especially the mitogen-activated protein kinases (MAPK) and phosphoinositol 3-kinase (PI3K)/*Akt* pathways, and these signaling pathways were reported to play important roles in endothelial cells growth, survival and migration(Koyama et al., 1998; Lawlor et al., 2001; Shiojima et al., 2002). Both signaling pathways are activated by a variety of stimuli in endothelial cells and regulate multiple critical steps in angiogenesis, and also can regulate cardiovascular homeostasis and vessel integrity at least in part by controlling NO synthesis(Dimmeler et al., 1999). And in endothelial cells activation of PI3K - AKT signaling was inversely related to CCN2 expression (Samarin et al., 2009), so targeting these signaling axes could be an important therapeutic strategy for many diseases. In this study, to find out if the inhibition of these intracellular pathways could have any antiproliferative effect on HUVECs, we used inhibitors of ERK1/2 and PI3K pathways: U0126 and LY294002, respectively. We found out that both inhibitors in such concentrations decreased the cell growth only in the presence of CTGF transfection. Western blot analysis showed an increase of both phosphorylated molecules whereas no alteration of the total protein expression occurred, thus suggesting CTGF transfection promoted

the activation of the ERK1/2 and *Akt* in HUVECs; U0126 and LY294002 used in this study partially prevented the activation induced by CTGF transfection and subsequently inhibited HUVECs proliferation. The results demonstrated that the ERK1/2 and PI3K/*Akt* pathways were involved in the HUVECs proliferation process induced by CTGF transfection.

CONCLUSIONS

In summary, we demonstrated that CTGF transfection in HUVECs exerted an obvious proliferative effect, and that ERK1/2 and PI3K/AKT pathways were involved in this process. This study laid a foundation for further study on the role of CTGF gene in angiogenesis.

ACKNOWLEDGEMENT

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