Endothelin-1 promoted proliferation of vascular smooth muscle cell through pathway of extracellular signal-regulated kinase and cyclin D1

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ABSTRACT

AIM: To investigate whether endothelin-1 (ET-1) can promote human umbilical artery smooth muscle cell (HUASMC) proliferation through pathway of extracellular signal-regulated kinase (ERK) and cyclin D1. **METHODS:** The effects of ET-1 and PD98059 on HUASMC were evaluated by MTT assay. The content of DNA was defined by [^{3}H]TdR assay and cell cycle was analyzed by flow cytomerty. Western blot analysis was employed to detect the active phosphorylated state of ERK and the expression of cylin D1. **RESULTS:** Firstly, ET-1 (100 nmol/L) stimulated HUASMC proliferation compared with the group without ET-1 ($P<0.05$) and PD98059 group ($P<0.05$). PD98059 inhibited the HUASMC proliferation stimulated by ET-1 ($P<0.05$). Secondly, ET-1 stimulated DNA synthesis of HUASMC compared with the group without ET-1 ($P<0.05$). Thirdly, ET-1 promoted the cell cycle transition from G_0/G_1 phase to S phase. G_0/G_1 phase cell percentage was obviously decreased compared with the group without ET-1 ($P<0.05$). S phase cell percentage was increased compared with the group without ET-1 ($P<0.05$). Fourthly, ET-1 increased the phosphorylated level of ERK and the expression of cylin D1, an inhibitor of ERK blocked phosphorylated level of ERK and cyclin D1 expression. ERK phosphorylated level of ET-1 group was evidently increased compared with PD98059 group ($P<0.05$), Cyclin D1 protein expression also was increased compared with PD98059 group ($P<0.05$). While nonphosphorylated ERK expression remained unchanged. **CONCLUSION:** Endothelin-1 promoted vascular smooth muscle cell proliferation through pathway of ERK and cyclin D1.

INTRODUCTION

Vascular smooth muscle cell (VSMC) proliferation is a key feature in the development of arteriosclerosis[1], and can occur in response to many different humoral and mechanical stimuli. A number of vasoactive agents have proven abilities to modulate vascular tone, and have also been considered as possible promoters of VSMC growth[2]. One of them is endothelin-1 (ET-l), a 21-amino acid peptide, which is known the most potent natural vasoconstrictor[3]. The endothelium is the major source of ET-l, and the serum ET-1

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level of atherosclerosis patients was notably increased. *In vivo*, ET-1 has been shown to augment the neointimal formation response to balloon injury of the rat carotid artery[4]. ET has two receptor subtypes, ET_A and ET_B, which have been shown to be expressed on several different cell types[5]. The two kinds of receptors are transmembrane G protein-coupled receptors which mediate intracellular calcium rises via phospholipase C-mediated phospho-inositide hydrolysis[6].

Mitogen-activated protein (MAP) kinase-mediated signal transduction pathways contribute to cell growth and differentiation[7,8]. The MAP kinase isoforms of 42 and 44 kDa, so-called extracellular signal-regulated kinases (ERK1/2), are expressed in many mammalian cell types. ERK1 and 2 were initially identified as 2 protein kinases that became phosphorylated on tyrosine[9]. ERK-mediated signal pathway is a multistep phosphorylation cascade that transmits signals from the cell surface to cytosolie nuclear targets, which are responsible for the activation and phosphorylation of a number of other regulatory proteins, including p90rsk, cPLA2, and transcription factors needed for the expression of genes involved in cell proliferation. Therefore, MAP kinase-mediated signal pathways play a key role in initiating cell proliferation and differentiation.

The expression and activation of a series of serine/threonine kinases, called cyclin-dependent kinases, control the transition and progress of the cell cycle [10]. The activity of these kinases is regulated in part by a group of proteins called cyclins. The *D*-cyclins are G_1-phase cyclins, and serve as growth factor sensor[11]. Upstream growth factors stimulate this cyclin bind, activate their partner kinases CDK4/CDK6, and promote the phosphorylation of substrates such as the retinoblastoma protein (pRB). The change of cyclin D1/CDK complex, which could stop or promote cell proliferation, plays a critical role in cell decision. Whether the link of mitogen action and cell cycle could influence cell proliferation needs to be elucidated.

The aims of the present study were: (i) to investigate cell proliferative effects in terms of DNA synthesis, cell number, and cell cycle change; (ii) to further research whether ET-1 could influence cyclin D1 expression and ERK phosphorylation change; and (iii) to observe the relation, under the induction of ET-1, between the mitogenic pathways and cell cycle progression.

MATERIALS AND METHODS

Materials Mouse monoclonal phosphorylated antibodies ERK were purchased from Oncogene (USA). Cyclin D1 and actin monoclonal antibodies were from Santa Cruz Biotechemitry (USA), PD98059 from Sigma (MO, USA), and ET-1 from CalBioChem (USA). ERK polyclonal antibodies were from Gene Company Limited (Shanghai, China).

Cell culture Human umbilical cord was kindly provided by International Peaceful Hospital of Shanghai. Only sections of artery vessels were used for cell culture. The isolated vessel media was cut into 1 mm^3 pieces under sterile conditions, which were placed onto a sterile 100-mm plastic tissue culture dish. The explants were covered in growth medium and incubated at 37 °C in a humidified atmosphere of 5 % CO_2 in air. Cultured VSMC medium consisted of medium 199 (M199) supplemented with *L*-glutamine (3 mmol/L), benzylpenicillin (800 kU/L), streptomycin (0.1 mg/L) and 20 % (v/v) fetal calf serum (FCS). Cells were fed with fresh medium for about 13 d, and were allowed to grow out from the explants until reaching near-confluence, when they were removed by 0.25 % trypsin solution and seeded into larger flasks for propagation. Human umbilical artery SMC exhibited the characteristic hill and valley morphology at confluence under phase-contrast micro-scopy, by immunohistochemistry, stained positively for smooth muscle actin (Boshide Company). As general, human umbilical artery SMC were approximately at 70 % confluence, culture medium was replaced by serum-free medium and continued to be cultured for 48 h before incubation with ET-1. In this experiment, one is ET-1 group; another is PD98059 group. In PD98059 group, HUASMC was stimulated by PD98059 (10 μmol/L) for 30 min, subsequently with ET-1.

MTT assay for HUASMC proliferation induced by ET-1 The cells at a density of 1×10^3 cells per well were seeded into 96-well plates and incubated at 37 °C for 24 h. And then cells were exposed to ET-1 10, 100, and 1000 nmol/L for 1, 2, 3, 4, 5, and 6 d, respectively. The proliferative activities of ET-1 were evaluated by MTT assay.

Measurements of DNA synthesis DNA synthesis of HUASMC was measured by [^{3}H]thymidine incorporation. Sub-confluent HUASMC in 96-well plates (1×10^3 cells per well) were made quiescent by incubation for 48 h in M199 containing 0.5 % FCS (fetal

calf serum). At the start of the experiment, ET-1 was added in fresh M199 containing 0.5 % FCS (350 μL/well). Cells were incubated with ET-1 for a period from 12,24,36 and 48 h to 60 h. [^{3}H]Thymidine was added to each well at 18 h to a final concentration of 3.7×10^7 Bq/L. At the end of the incubation period, the incorporated radioactivity (Bq) was measured by fixation and solubilisation of cells.

Cell cycle analysis HUASMC were arrested to growth by M199 containing 0.5 % fetal calf serum for about 48 h. HUASMC were exposed to 0.5 % M199 with ET-1 at the final concentration of 100 nmol/L from 12,24, 36, and 48 h, the cells were digested by a mixture of 0.25 % trypsin and centrifuged at 1500×*g* for 6 min, the pellets were washed in PBS solution twice, and then cells were dyed in a mixture of PI (50 mg/L) and RNase (3000 U/L) for 30 min, and dyed with FITC at the final concentration of 0.05 mg/L. The cell cycle was determined by flow cytometry.

Western blot HUASMC were grown to near-confluence in 100-mm plates, and made quiescent by incubation for 48 h in M199 containing 0.5 % FCS. Afterwards, fresh medium was added containing ET-1 (100 nmol/L), incubated with the cells for different periods. After the incubation period, cells were rinsed twice with ice-cold PBS and lysed in 150 μL lysis buffer consisting of 10 % SDS, PMSF 10 μg/L, and 25 μg/L leupeptin. After running through a syringe needle 6 times, the lysates were centrifuged at 12 000× *g* for 5 min. Equal amounts (5-30 μg) of cell extracts were resolved by 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 1.5 h, and transferred onto a PVDF membrane for 1.5 h (Amersham, Little Chalfont, UK). The membrane was blocked for 6 h at room temperature in PBS containing 0.05 % Tween 20 and 5 % milk powder, followed by 1.5 h with mouse monoclonal anti-cyclin D1 antibody (Santa Cruz Biotechnology, USA) and mouse monoclonal phosphorylated antibodies ERK (Oncogene) at a 1:750 dilution. The membrane was washed several times with blocking solution, and incubated for a further 1.5 h with a horseradish peroxidase-conjugated recombinant anti-mouse secondary antibody (Dingguo, China). After washing the membrane, the immunoreactive protein bands were detected by enhanced chemiluminescence using the ECL reagent (Amersham, UK). Half-quantitative analysis of immunoreactivity was measured by the TOPTAL image analysis system in terms of calculating absorbency and area of each blot. To test crosslinking between ERK and cell cycle, HUASMC were prepared as described, but PD98059 10 μmol/L was added at 30 min before stimulation with ET-1 100 nmol/L.

Statistical analysis Data were analyzed with one-way-ANOVA and Independent samples *t*-test. The different protein concentration groups were analyzed with nonparametric correlation. In all cases, differences were considered significant if $P<0.05$.

RESULTS

Effects of ET-1 on growth of HUASMC HUASMC proliferation was gradually enhanced along with ET-1 concentration increase ($P=0.042$, $P<0.05$), but there was no statistically significant difference between ET-1 100 nmol/L and 1000 nmol/L group ($P=0.418$, P>0.05, Fig 1). ET-1 100 nmol/L promoted obviously the proliferation of HUASMC compared with the group without ET-1, with the maximum effect observed at d 6 ($P=0.002$, $P<0.05$). HUASMC proliferation was inhibited by PD 98059 compared with ET-1 group ($P=0.024$, $P<0.05$, Fig 2).

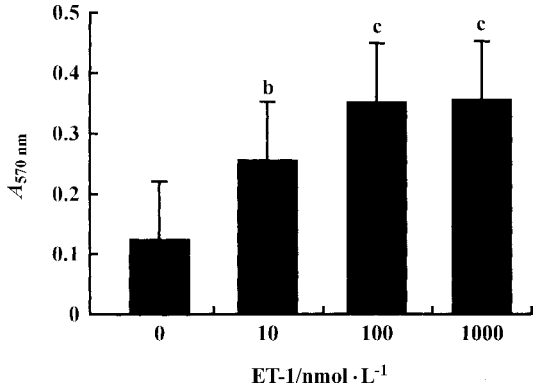

Fig 1. Effect of different concentrations of endothelin-1 (ET-1) on HUASMC proliferation after 48-h incubation. *n*=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control (ET-1 0 nmol/L).

ET-1 stimulated human umbilical artery SMC DNA synthesis DNA synthesis was decreased in the group without ET-1, but significantly increased in ET-1 group. The difference was significant ($P=0.011$, $P<0.05$, Fig 3).

Effects of ET-1 on cell cycle of HUASMC Flow cytometry analysis indicated that ET-1 urged HUASMC to S phase. G_0/G_1 and S phase cell ratios were 69.49 % and 29.93 %; 56.25 % and 33.97 %; 52.74 % and 47.08 %; 38.45 % and 58.32 % at 12, 24, 36, and 48 h, respectively. Whereas in the group without ET-1,

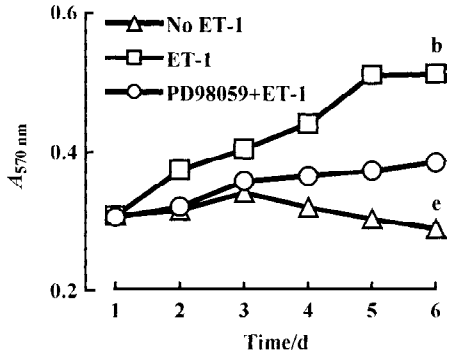

Fig 2. Time course curve of HUASMC proliferation induced by ET-1 (100 nmol/L) and the effect of PD98059 (10 μmol/L) on HUASMC proliferation. *n*=3. Mean±SD. $^{b}P<0.05$ *vs* no ET-1 group. $^{e}P<0.05$ *vs* ET-1 group.

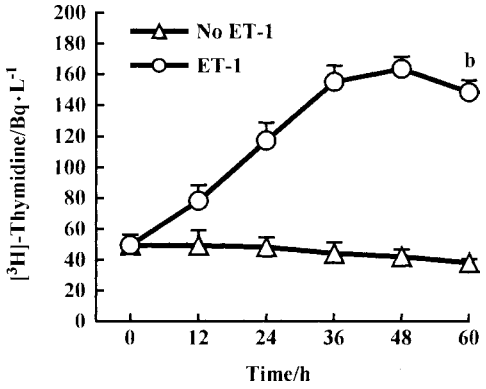

Fig 3. Effects of ET-1 100 nmol/L on DNA synthesis at different time points in human artery SMC. *n*=3. Mean±SD. $^{b}P<0.05$ *vs* no ET-1.

G_0/G_1 and S phase cell ratios were 67.69 % and 25.53 %; 74.11 % and 17.41 %; 75.24 % and 18.28 %; 77.31 % and 16.59 % at 12, 24, 36, and 48 h. (Fig 4). G_0/G_1 and S phase cell ratios of ET-1 group were different from those in the group without ET-1 (G_0/G_1 phase: P=0.030, P<0.05; S phase: P=0.033, P<0.05).

ET-1 induced expression of cyclin D1 and ERK phosphorylate level The effect of ET-1 on cell cycle progression was examined by comparing the levels of cyclin D1, a cell cycle-related protein, in quiescent cells treated with ET-1 for different periods. The results showed a marked increase in cyclin D1 protein levels at different time points, its maximum expression was at 32 h, and little fall at 40 h (β=0.816, t=2.818, P=0.048, P<0.05, Fig 5A). ET-1 induced increase in ERK phosphorylation at different time points; its maximum expression was at 15 min, and little fall at 30 min (β=0.897, t=3.518, P=0.039, P<0.05, Fig 6A). ERK nonphosphorylation expression had no obvious change (β=

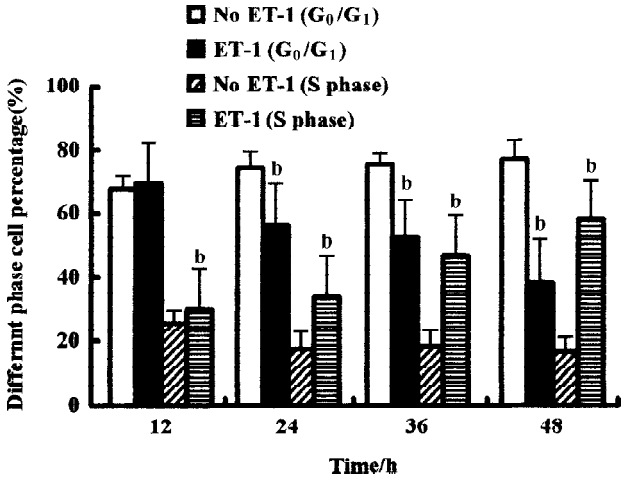

Fig 4. Effects of ET-1 (100 nmol/L) on cell cycle in HUASM. *n*=3. Mean±SD. $^{b}P<0.05$ *vs* no ET-1.

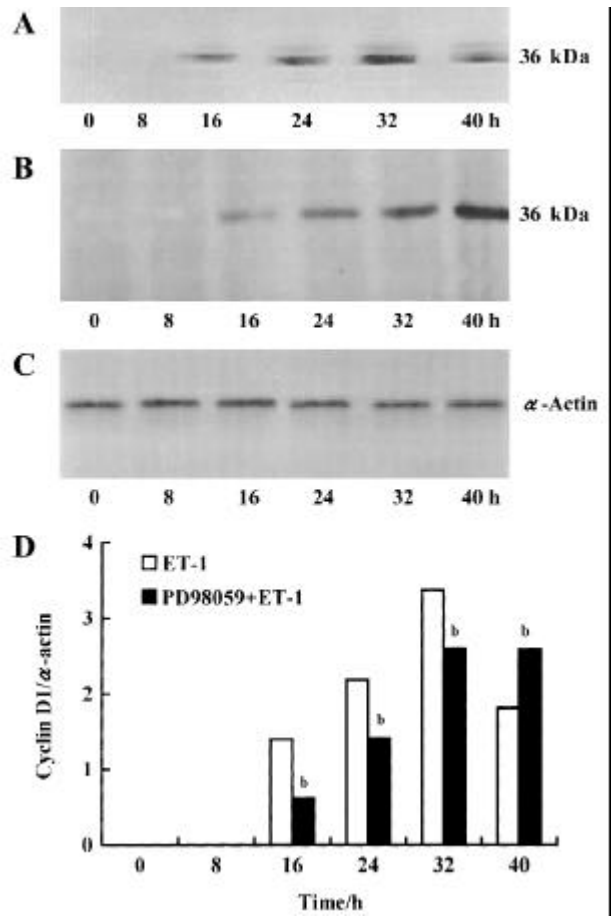

Fig 5. Influence of ET-1 and PD98059 on the expression of cyclin D1 in HUASMC. A: Treated with ET-1 (100 nmol/L); B: Pretreated with PD98059 (10 μmol/L); C: Standard a-actin; D: Half-quantitative analysis result. *n*=3. Mean±SD. $^{b}P<0.05$ *vs* ET-1.

0.810, t=2.393, P=0.097, P>0.05 Fig 6B). We can see

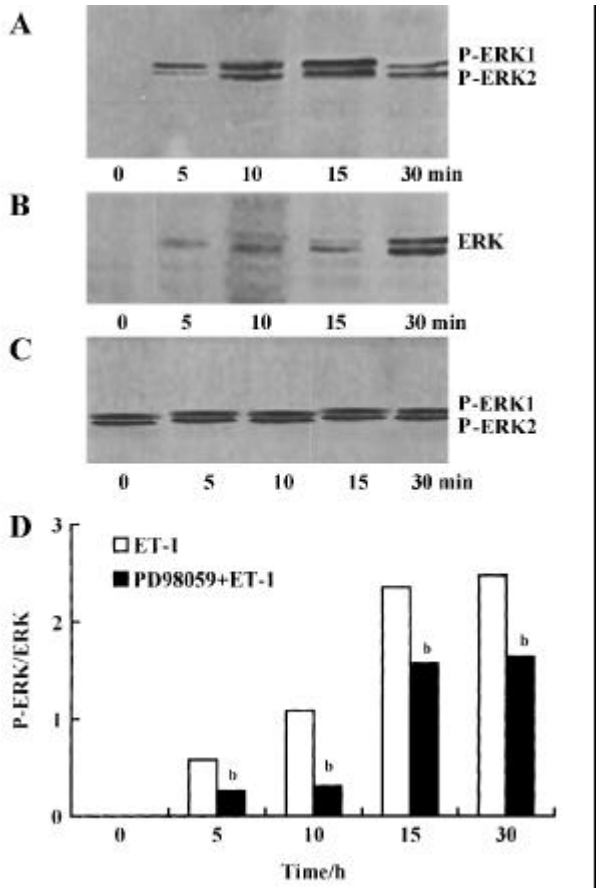

Fig 6. Influence of ET-1 and PD98059 on the level change of ERK phosphorylation at different time. A: Treated with ET-1 (100 nmol/L); B: ERK nonphosphorylation expression induced by ET-1 (100 nmol/L); C: Pretreated with PD98059 (10 μmol/L); D: Half-quantitative analysis result. *n*=3. Mean±SD. $^{b}P<0.05$ *vs* ET-1.

cyclin D1 protein half-quantitative analysis result (Fig 5D).

PD98059 inhibited expression of cyclin D1 and ERK phosphorylation level Pretreatment of HUASMC with PD98059, an inhibitor of ERK kinase, largely reduced expression of cyclin D1, its maximum expression was at 40 h (β=0.963, t=7.109, P=0.02, P<0.05, Fig 5B). Meantime, it obviously blocked activation of ERK phosphorylation by ET-1, its maximum expression was at 30 min (β=0.879, t=3.197, P=0.049, P< 0.05, Fig 6C). We can see p-ERK protein half-quantitative analyzes result (Fig 6D). The difference of cyclin D1 protein expression was significant compared ET-1 group with PD98059 group at 16, 24, 32, and 40 h (at 16 h: t=2.9119, v=10, P<0.02, at 24 h: t=4.8001, v=10, P<0.001, at 32 h: t=5.8642, v=10, P<0.001, at 40 h: t=5.9721, v=10, P<0.001, Fig 5D). The difference of ERK phosphorylation was significant between ET-1 group and PD98059 group in 10, 15, and 30 min (at 10 min: t=3.3252, v=8, P<0.02, at 15 min: t=2.7922, v=8, P<0.05, at 30 min: t=4.82972, v=8, P<0.002), but not at 5 min (t=0.0017, v=8, P>0.05, Fig 6D).

DISCUSSION

Present study demonstrated that ET-1 stimulated HUASMC proliferation. ET-1 induced DNA synthesis, increased S phase cell number, as well as increased cyclin D1 protein expression and activated ERK phosphorylation, respectively. The cyclin D1 expression and ERK phosphorylation could be blocked by PD98059. Therefore, these findings demonstrated that ET-1 induced chronic growth-related changes, and this effect takes priority of those effects on vascular tone.

As a vasoconstrictor, the role of ET-1 has been well established, and also been demonstrated *in vivo* in the human circulation[12]. Nevertheless, as mitogenicity, many potential biological functions of the peptide need to be investigated in the human vasculature. For ability of inducing coronary artery smooth muscle cell prolifera-tion, ET-1 may play a significant role in human coronary artery disease, such as atherosclerosis. Increased ET plasma levels have been detected in several cardiovascular diseases, for example atherosclerosis, heart failure and myocardial infarction[13]. In HUASMC, ET-1 stimulated DNA synthesis and increased S phase cell number, which reflected cell progression from G_0/G_1 to S phase and new DNA synthesis prior to division. Cell cycle progression relies on a number of specific proteins, each of which controls specific stages of the cycle. Cyclin D l is an important cyclin, whose protein expression change is associated with the G_0/G_1 to S phase transition in the cell cycle[14]. In the present study, that HUASMC had been exposed to ET-1 from 0 to 40 h, we detected a maximum cyclin D1 level at 32 h, and a little decreased level at 40 h. In PD98059 group, cyclin D1 expression was blocked, and maximum expression was detected at 40 h. This observation parallels the mitogenic response. The hypothesis that ET-1 serves as a direct mitogen is further confirmed by our findings.

Activating ERK kinase cascades is one of the major pathways for the regulation of proliferation and cell growth in various cultured cells[15,16]. Reversible protein phosphorylation is the established mechanism of regulation of ERK kinase[17]. In cultured cells, a transient kinase activation or attenuation has been seen after chronic stimulation. The present studies demon-

strated that ERK phosphorylation was sharply elevated at 15 min in HUASMC, and a little decreased at 30 min. ERK phosphorylation was blocked by PD98059, and maximum expression at 30 min. Nonphosphorylation ERK expression has no obvious change. Hyperexpression and activation of these kinases may play a main role in regulation of cell proliferation in the pathogenesis of arteriosclerosis.

The present study demonstrated that ET-1 could promote vascular smooth muscle cell proliferation through pathway of ERK and cyclin D1. Our study provides new insights for the molecular mechanism for controlling the smooth muscle proliferation. The therapeutic effect of ERK antagonist or inhibitor on atherosclerosis should be addressed in future studies. Furthermore, understanding of cross-linking between ERK pathway and cell cycle regulation could lead to new strategies for the prevention or therapy of atherosclerosis.

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