Antisense Oligodeoxynucleotide Against Tissue Factor Inhibits Human Umbilical Vein Endothelial Cells Injury Induced by Anoxia-Reoxygenation

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Key Words

Tissue factor • Antisense oligodeoxynucleotide • Human umbilical vein endothelial cells • Anoxia-reoxygenation

Abstract

Aims: The role of antisense oligodeoxynucleotide against tissue factor (aODN/TF) in cultured human umbilical vein endothelial cells (HUVECs) subjected to anoxia-reoxygenation (A/R) was investigated. **Methods:** HUVECs were divided randomly into control group, A/R group, aODN/TF+A/R group, sense oligodeoxynucleotide (sODN/TF) + A/R group and mismatched oligodeoxynucleotide (mODN/TF) + A/R group, in the latter 3 groups, HUVECs were transfected with aODN/TF, sODN/TF and mODN/TF respectively. HUVECs in all A/R groups underwent 3 hrs of anoxia and followed by 2 hrs of reoxygenation. In order to investigate the potential mechanisms of how increased TF may contribute to A/R injury in HUVECs, another set of HUVECs were incubated with human recombinant active site blocked factor VII (FVIIai) during A/R. **Results:** After A/R, TF expression

at both mRNA and protein level was increased, furthermore, cell viability and the concentrations of SOD, GSH-PX and NO were declined, while LDH, MDA and ET-1 were overproduced ($P < 0.05$ to 0.001 versus control group). In HUVECs of aODN/TF+A/R group, however, TF expression was inhibited, while the declined cell viability and the concentrations of SOD, GSH-PX, NO as well as the enhanced LDH, MDA and ET-1 levels occurred during A/R were ameliorated and reversed effectively ($P < 0.05$ to 0.01 versus those in other A/R groups). The results also showed that ROS was increased and PAR-1, PAR-2, p38 MAP kinase and p42/44 MAP kinase were all activated after A/R ($P < 0.001$ versus HUVECs under normoxia), while FVIIai inhibited the increment of ROS, PAR-1, PAR-2, p38 MAP kinase and p42/44 MAP kinase, and improved the changes of TF:C, MDA, SOD, GSH-PX, cell viability and LDH occurred during A/R ($P < 0.05$ to 0.001 versus HUVECs without FVIIai treatment). **Conclusion:** Tissue factor plays an important role in the development of HUVECs injury induced by anoxia-reoxygenation, inhibition of TF with antisense oligodeoxynucleotide is an effective approach to ameliorate the damage.

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Introduction

Vascular endothelium provides a barrier in our body and keeps vascular homeostasis under normal circumstances. Anoxia-reoxygenation or ischemia-reperfusion is a pathological process involving oxidative stress. It plays an important role in the pathogenesis of a wide variety of clinical conditions such as peripheral vascular disease, stroke and myocardial infarction, etc [1, 2]. As a crucial site, endothelial cells undergo cellular activation even death following anoxia-reoxygenation or ischemia-reperfusion injury. Data have shown that endothelial cells, under ischemic or hypoxic conditions, present dysfunction of energy metabolism, overproduction of oxygen free radicals and malondialdehyde (MDA), etc. which activate endothelial cells themselves and lead to the destruction of the integrity of the vascular endothelium [3].

Tissue factor (TF) is a 47 kDa transmembrane glycoprotein and a member of the cytokine receptor superfamily, which is constitutively expressed at extravascular sites. TF expression is usually induced on macrophages and endothelial cells during the inflammatory or immune response. As a high-affinity receptor and cofactor for plasma factor VII/activated factor VII (FVII/FVIIa), TF is the primary cellular initiator of blood coagulation, which is involved in thrombosis and inflammation associated with sepsis, atherosclerosis, angiogenesis, embryogenesis, cancer and metastasis [4]. Studies showed renal ischemia reperfusion injury was protected by TF deficiency [5]. However, the intimate nature of TF in the injury of endothelial cells induced by anoxia-reoxygenation remains unclear [6]. In the present study, we attempted to investigate the role of TF in cultured human umbilical vein endothelial cells subjected to anoxia-reoxygenation by using antisense oligodeoxynucleotide to specifically inhibit the expression of TF.

Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were acquired as follows [7]: Human umbilical veins were flushed with phosphate buffered saline (PBS), and filled with PBS containing collagenase (200 mg/ml) and incubated for 30 min at room temperature. Then the veins were washed with PBS, the collagenase solution and the wash were collected and centrifuged for 10 min at 200×g, cells were resuspended in RPMI 1640 complete medium (GIBCO) supplemented with 20% fetal bovine serum (FBS, HyClone), penicillin (100 U/ml) and

streptomycin (100 µg/ml), and then were seeded on glass slides ($\approx 1.8 \times 10^6$ cells per slide) coated with gelatin and incubated at 37°C in the presence of humidified 95% air and 5% CO₂. Upon reaching 75% confluence, cells were subcultured by the ratio of 1:3. When grew into monolayer like cobble, cells were characterized as HUVECs by morphological criteria (uniform polygonal shape) and by immunofluorescent demonstration of von Willebrand factor antigen.

Oligodeoxynucleotides synthesis

The published DNA sequence of human TF was used as a template for the synthesis of a phosphorothioate antisense oligodeoxynucleotide (aODN) of thirty bases. The sequence that we chose was shared by tissue factors from human and non-human sources, and contained the rare tripeptide motif, Trp-Lys-Ser (WKS), which had been predicted as a functional motif involved in the interaction with serine proteases. Two further phosphorothioate oligodeoxynucleotides were also synthesized for the purpose of providing negative controls: (a) TF sense oligodeoxynucleotide (sODN/TF)- of identical sequence to part of the human TF cDNA sequence, (b) TF mismatched oligodeoxynucleotide (mODN/TF)- of identical bases to the TF aODN but the sequence order was totally mismatched. Sequence homology of all three phosphorothioate ODNs with other known genes from Genbank computer search was found to be less than four bases. The nucleotide sequence comparisons of part of the human TF cDNA and the ODNs used in the experiments are shown below [8]:

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Human TF cDNA284 TAA GCA CTA AGT CAG GAG ATT GGA  
AAA GCA AAT GCT TTT ACA CA320  
aODN/TF 5'-AAA GCA TTT GCT TTT CCA ATC TCC TGA  
CTT-3'  
sODN/TF 5'-AAG TCA GGA GAT TGG AAA AGC AAA TGC  
TTT-3'  
mODN/TF 5'-TGT TTT AAC CAT ACC TCA TTG GAT CAC  
TCT-3'
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Cell transfection

Identified HUVECs were divided randomly into control group, anoxia-reoxygenation (A/R) group, antisense oligodeoxynucleotide/TF plus A/R (aODN/TF+A/R) group, sense oligodeoxynucleotide/TF plus A/R (sODN/TF+A/R) group and mismatched oligodeoxynucleotide/TF plus A/R (mODN/TF+A/R) group, with each group containing 6 dishes (approximately 6×10^5 viable cells per dish) of HUVECs in duplicate (in which one would be harvested before anoxia-reoxygenation, and the other would be harvested after anoxia-reoxygenation). HUVECs in aODN/TF+A/R group, sODN/TF+A/R group and mODN/TF+A/R group were washed in 4 ml PBS before oligodeoxynucleotide transfection.

5 µg aODN/TF, sODN/TF and mODN/TF dissolved in TE buffer (10mM Tris-Cl, 1mM EDTA, pH8.0) were mixed respectively with RPMI 1640 medium containing no serum, proteins, or antibiotics to a total volume of 90 µl, which was then mixed with 15 µl SuperFect Transfection Reagent (QIAGEN) and incubated for 10 min at room temperature to allow transfection-complex formation. 1 ml RPMI 1640 medium containing serum and antibiotics was added respectively into

the reaction tube containing the transfection complexes, and the total volume containing each oligodeoxynucleotide was mixed and transferred immediately to the corresponding washed HUVECs in 35-mm dish respectively and incubated for 3 hrs at 37°C and 5% CO₂. Then the RPMI 1640 medium containing the remaining complexes was gently removed and the cells were washed 4 times with 4 ml PBS. At last, fresh RPMI 1640 medium (containing serum and antibiotics) was added into the cells and incubated for 48 hrs.

After cell transfection, HUVECs in each group including control group and A/R group were passaged at 1:3 in 35-mm dishes and incubated to confluence, so each group contained 18 dishes (35-mm) of cells (approximately 6×10⁵ viable cells per dish) in duplicate in the experiments, the N number of HUVECs for each group was 18. There were totally 5 groups of HUVECs in this study, so the number of HUVECs for all experiments was 90 dishes of cells in duplicate.

Anoxia-reoxygenation of HUVECs

HUVECs were grown on gelatinized 35-mm dishes to confluence in endothelial growth medium (25 mmol/l HEPES, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, 20% heat-inactivated FBS in RPMI 1640 medium). To induce anoxic stress, culture medium was aspirated off cells and replaced with anoxic endothelial growth medium when introduced into an anoxia chamber (Forma Scientific). Cells in four A/R groups were maintained under anoxic stress at 37°C in a 0% O₂/5% CO₂/ $<12\%$ H₂/balance N₂ atmosphere within the anoxic chamber. The chamber environment was rendered anoxic by a palladium catalyst, and O₂ concentration was maintained at 0 PPM (Coy Laboratories products). The cells were removed from the anoxic chamber after 3 hrs, and the medium was replaced with warm fresh medium to begin a period of reoxygenation. The cells were then placed in a 95% air/5% CO₂ incubator at 37°C for 2 hrs [1]. The cells in control group were incubated in a 95% air/5% CO₂ incubator (normoxia) at 37°C for totally 5 hrs. Supernatant and HUVECs were harvested to be analyzed.

Nuclear transcription assay

Nuclear transcription assay is also called nuclear run-on assay here, it measures a specific gene being transcribed in intact nuclei at a specific time, and provides information on the synthesis of the gene that occurs as a function of cell state, as opposed to a change in mRNA degradation or transport from the nucleus to the cytoplasm. The principle involved is that, when cells are lysed and the intact nuclei are retrieved, the isolated nuclei contain the full transcription machinery for synthesis of mRNA and stall on the DNA template due to acute loss of ribonucleotide substrates. Therefore, it is regarded as the gold-standard measurement of overall transcriptional activity of a specific gene [9]. Transcription has thus been halted at the point in time where the nuclei are removed, but can be started up again *in vitro* with the addition of new ribonucleotides. After isolation, the nuclei are incubated in the presence of radiolabeled UTP allowing the *in vitro* transcription of a labeled total mRNA over a defined time. After the transcription reaction, the radiolabeled RNA is then isolated

and hybridized with denatured DNA, which has been immobilized on a filter membrane and usually includes cDNA sequences of the gene of interest and of a housekeeping gene that is stably expressed as a standard. This allows the detection of specific mRNA transcripts by comparing the thickness of the hybridized lines of radiolabeled RNA synthesized in one nuclei prep with another [10]. In this study, within a given experimental condition, TF gene transcription could be accurately assessed by using nuclear transcription assay.

Isolation of nuclei

HUVECs were washed in PBS, the cell pellets were harvested and lysed by lysis buffer (10 mM NaCl, 3 mM MgCl₂, 10 mM Tris-HCl, pH 7.4, 0.5% Nonidet P-40). The pellets were dispersed with repeated pipetting and spun for 3s in cold-room microcentrifuge to pellet the nuclei. The nuclear pellets were estimated in volume and resuspended in an equal volume of nuclei storage buffer (40% glycerol, 50 mM Tris-HCl, pH 8.5, 5 mM MgCl₂, 0.1 mM EDTA) and stored at -80°C.

cDNA slot blot preparation

10 µg linear fragments of human TF cDNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were denatured respectively by chilling on ice and neutralized with one volume of 2 M ammonium acetate after adding 30 µl of 10 M NaOH to each sample, 1 µg (150 µl) of the cDNA solution was filtered on to a nitrocellulose membrane on the top of one piece of 3 MM Whatman paper by using slot blot apparatus. The nitrocellulose membrane was cut into strips, with each containing one set of cDNA samples to be tested, and baked in a vacuum oven for 2 hrs at 80°C and stored at room temperature.

Transcription reaction

The nuclei was thawed on ice, 50 µl of nuclei (using wide-bore tip) was added to 50 µl of freshly prepared 2× reaction cocktail [50 µl ³²P-UTP (500µCi), 250 µl 4× reaction mix (100 mM HEPES, pH 7.5, 10 mM MgCl₂, 10 mM DTT, 300 mM KCl, 20% glycerol), 125 µl 8× tri-phosphate mix (14 µl 25 mM ATP, 14 µl 25 mM GTP, 14 µl 25 mM CTP, 0.4 µl 1 mM UTP and 83 µl ddH₂O, with the final concentrations of 2.8 mM ATP, GTP, CTP and 3.2 mM UTP), 75 µl ddH₂O] and incubated at room temperature for 20 min. The reaction was stopped by adding 2 µl of DNase I and incubating at 37°C for 10 min. 300 µl stop buffer (2% SDS, 7 M urea, 0.35 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), proteinase K with a final concentration of 1 mg/ml and 100 µg tRNA were added to each reaction, which was then homogenized by pipetting and incubated at 45°C for 2 hrs. After precipitation by adding ice cold trichloroacetic acid (TCA) to each reaction to a final concentration of 10%, incubation on ice for 20 min and spin for 15 min, the nucleic acids pellet was washed with cold absolute ethanol to remove any trace of TCA and was incubated at 65°C for 25 min to dissolve the RNA after air-drying and resuspending in 50 µl of TE containing 0.5% sodium dodecylsulphate (SDS).

Hybridization reaction

Filter-bound cDNA was prehybridized in 2.5 ml hybridization buffer (50% formamide, 6× SSC, 10× Denhardt's

solution, 0.2% SDS) at 42°C for 8 hrs and hybridized at 42°C for 72 hrs after adding 50 µl ³²P-labeled RNA to the prehybridization buffer. Then the cDNA blots were washed once with 6× SSC and 0.2% SDS at room temperature for 10 min, twice with 2× SSC and 0.2% SDS, followed by two washes in 0.2× SSC and 0.2% SDS, all at 65°C for 20 min each wash. The cDNA blot strips were placed on an X-ray film as backing, covered with plastic wrap, and analyzed by autoradiography.

Tissue factor assay

TF procoagulant activity (TF:C) in HUVECs lysate was determined with one stage clotting assay [11]. Briefly, 100 µl of HUVECs lysate were added to 100 µl of normal pooled citrated plasma; after 150 seconds' incubation at 37°C, 100 µl of 0.025 mM CaCl₂ was added and clotting time was recorded. All samples were tested in duplicate. Consequently, clotting time was converted to arbitrary TF units (U)/2×10⁵ HUVECs by using logarithmic plots with clotting time versus dilution of a standard TF solution, which was obtained by means of commercial thromboplastin (Dade). Undiluted thromboplastin was assigned a value of 1000 TF units, corresponding to a clotting time of 14 seconds. This procoagulant activity was not seen with plasma deficient in factors VII, X, or V.

The enzyme linked immunosorbent assay (ELISA) was performed to measure antigen of TF (TF:Ag) in HUVECs lysate by using a commercial kit (Imubind Tissue Factor ELISA Kit, American Diagnostica Inc.). The assay recognized TF-apo, TF, and TF-FVII complexes and was designed such that there was no interference from other coagulation factors or inhibitors of procoagulant activity [11].

Assay of cell viability and lactate dehydrogenase activity

Cell viability was determined by trypan blue exclusion. HUVECs were seeded in 96-well plates at 1×10⁵ cells/well. After treated with anoxia-reoxygenation, subconfluent HUVECs were washed and harvested in PBS to prepare a single-cell suspension. 50 µl of the suspension was added to 0.95 ml 0.4% trypan blue solution. The total number of living and dead cells was counted with a haemocytometer [2].

The activity of lactate dehydrogenase (LDH) in cultured supernatant was assayed by a LDH assay kit (Jiancheng Biology Engineering Institute, Nanjing, China) according to the manufacturer's instruction.

Assay of malondialdehyde, superoxide dismutase and glutathione peroxidase

Lipid peroxidation was quantified by assaying the concentration of malondialdehyde (MDA) in HUVECs, and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) in HUVECs were determined by using nitroblue tetrazolium method and 5,5'-dithiobis-2-nitrobenzoic acid method. At the end of anoxia-reoxygenation, the HUVECs were washed with ice-cold PBS for three times. Then they were lysed in cold lysis buffer, collected with a rubber policeman, and sonicated in cold 20 mM HEPES buffer (EDTA 1 mM, 210 mM mannitol, 70 mM sucrose, pH 7.2) followed by centrifugation at 1500× g for 5 min at 4°C. The supernatant was evaluated for

MDA, SOD and GSH-PX with assay kits (Jiancheng Biology Engineering Institute, Nanjing, China) respectively according to the manufacturer's protocols, the absorbances of samples were read at 733nm, 550 nm and 412 nm respectively. Total cell protein was determined by the Lowry method.

Assay of nitric oxide and endothelin-1

After anoxia-reoxygenation, the culture medium was collected, and the amount of nitric oxide (NO) released by cells was determined using a NO assay kit (Jiancheng Biology Engineering Institute, Nanjing, China) according to the manufacturer's protocol. The method involved measuring the amount of NO metabolites (nitrite and nitrate), which were more stable than NO. The absorbance of samples was read at 550 nm.

The concentration of endothelin-1 (ET-1) was measured with a sandwich-enzyme immunoassay kit (R&D Systems, Inc.) according to the manufacturer's instruction. The absorbance of samples was read at 650 nm.

Investigation by means of active site blocked factor VII

In order to investigate the potential mechanisms of how increased TF may contribute to anoxia-reoxygenation injury in HUVECs, HUVECs were incubated with human recombinant active site blocked factor VII (FVIIai) during anoxia-reoxygenation. In this section of the experiments, HUVECs were divided randomly into 3 groups with each group containing 18 dishes (approximately 6×10⁵ viable cells per dish) in triplicate, in which one was used for investigating the generation of reactive oxygen species (ROS), one for measuring the expression of protease-activated receptor-1 (PAR-1), protease-activated receptor-2 (PAR-2), p38 mitogen-activated protein (MAP) kinase and p42/p44 MAP kinase respectively, and the other for determining TF:C, TF:Ag, the concentration of MDA, the activities of SOD and GSH-PX, cell viability, the activity of LDH as well as the concentrations of NO and ET-1. HUVECs in Group II and III underwent the same anoxia-reoxygenation mentioned above, but 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone (DEGRck) inactivated human FVIIa (FVIIai, from Novo-Nordisk, Bagsvaerd, Denmark) was added into the cell culture medium in Group III with the final concentration of 200 nM before anoxia-reoxygenation. FVIIai is identical to FVIIa but, because the active site is blocked, is devoid of enzymatic activity. The cells in Group I were incubated under normoxia at 37°C for totally 5 hrs.

Flow cytometry analysis

HUVECs employed to investigate the expression of ROS were loaded with 10 µmol/L dihydrochlorofluorescein diacetate (H₂DCF-DA) (Sigma-Aldrich, StLouis, MO, USA) in RPMI 1640 at 37°C for 30 min to establish a stable intracellular level of the probe and then washed twice with PBS before anoxia-reoxygenation, thus H₂DCF-DA was continuously present during anoxia-reoxygenation. H₂DCF-DA, which is a membrane permeable indicator, can penetrate the cells and is initially converted into DCFH by cellular esterase, then DCFH is in turn oxidized to dichlorofluorescein (DCF) in the presence of ROS, and upon excitation at 488 nm emits green fluorescence,

Fig. 1. Nuclear transcription assay for HUVECs. control: HUVECs from control group; A/R: HUVECs from A/R group; aODN/TF+A/R: HUVECs from aODN/TF+A/R group; sODN/TF+A/R: HUVECs from sODN/TF+A/R group; mODN/TF+A/R: HUVECs from mODN/TF+A/R group. Be: before anoxia-reoxygenation; Af: after anoxia-reoxygenation. The thickness of each line represents relative TF mRNA strength. Human GAPDH mRNA was served as inner control in each slot blotting.

proportional to the intracellular level of ROS. Thus the DCF fluorescence intensity of the cells is an index of intracellular levels of ROS. By quantifying fluorescence, the production of ROS could be quantified [12-16]. After anoxia-reoxygenation, the cells in 3 groups were washed and resuspended in PBS, and immediately detected on a Coulter Epics XL flow cytometer with the use of EXPO32 ADC software (Beckman Coulter, Inc., Miami, USA).

To investigate PAR-1 and PAR-2 expression, HUVECs undergone anoxia-reoxygenation were stained fresh for detection of surface expression of PARs and fixed in 4% paraformaldehyde and permeabilized in 0.1% saponin for detection of intracellular PARs. Cells were incubated directly with 1 in 20 dilution of PE-conjugated mouse anti-human PAR-1 monoclonal antibody and mouse anti-human PAR-2 monoclonal antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, USA) respectively for 30 min at 25°C, followed by two washes with staining buffer [PBS, 0.02% sodium azide, 0.1% bovine serum albumin (BSA), pH 7.4] [17-18]. Finally, cells were resuspended in PBS and labeled cells were analyzed by flow cytometry mentioned above.

Activated MAP kinases in HUVECs were demonstrated by flow cytometric analysis of intracellular phospho-p38 and phospho-p42/44 MAP kinase expression [19-21]. HUVECs suspensions were fixed using 4% paraformaldehyde for 15 minutes at 25°C. Following fixation, the cells were washed in PBS and pelleted by centrifugation and permeabilized in 0.1% saponin. The permeated HUVECs were subjected to staining of intracellular signal molecules by rabbit anti-human phospho-p38 and phospho-p42/44 MAP kinase monoclonal

antibodies (Cell Signaling Technology, Inc., Beverly, MA, USA) respectively for 30 min. This was followed by recognition with FITC-conjugated goat anti-rabbit Ig antibody (Invitrogen Corporation, Shanghai, China) for another 30 min staining. After washing twice with PBS, HUVECs were analyzed by using flow cytometry mentioned above.

Measurement of markers of HUVECs injury

In order to investigate the role of FVIIai on HUVECs subjected to anoxia-reoxygenation, TF, MDA, SOD, GSH-PX, cell viability, LDH, NO and ET-1 were measured by means of the same methods mentioned above.

Statistical analysis

Results were expressed as mean±SD. Inter-group comparisons were performed with 1-way ANOVA to test for difference. Statistical Package for the Social Sciences software (SPSS Inc.) was used for all analyses. Multiple group comparison corrections were made by using Dunnett's T3 and Dunnett's C methods when the variances among groups were not equal. Differences were considered to be significant at a value of $p < 0.05$.

Results

Statistical analysis showed that all data in this study followed normal distribution.

Fig. 2. TF:C and TF:Ag in HUVECs. A: TF:C in HUVECs (n=18); B: TF:Ag in HUVECs (n=18). Control: TF from HUVECs in control group; A/R: TF from HUVECs in A/R group; aODN/TF+A/R: TF from HUVECs in aODN/TF+A/R group; sODN/TF+A/R: TF from HUVECs in sODN/TF+A/R group; mODN/TF+A/R: TF from HUVECs in mODN/TF+A/R group. Be: before anoxia-reoxygenation; Af: after anoxia-reoxygenation. $\triangle$: $P < 0.001$ compared with control group; $\diamond$: $P < 0.01$ compared with A/R group; $\star$: $P < 0.01$ compared with aODN/TF+A/R group.

Nuclear transcription

Nuclear transcription assay measured TF gene transcribed in nuclei of HUVECs exposed to anoxia-reoxygenation. HUVECs were lysed and the intact nuclei were retrieved and incubated in the presence of radiolabeled UTP for *in vitro* transcription of labeled total mRNA. After the transcription reaction, the radiolabeled RNA was then isolated and hybridized with denatured TF cDNA, TF gene transcription was assessed by observing hybridized lines of TF mRNA transcripts. Before anoxia-reoxygenation, there was no difference of TF mRNA in HUVECs among groups. After anoxia-reoxygenation, HUVECs in A/R group, aODN/TF+A/R group, sODN/TF+A/R group and mODN/TF+A/R group displayed more increased TF mRNA than those in control group, principally reflecting enhanced TF gene transcription. While TF mRNA was less expressed in HUVECs of aODN/TF+A/R group than those of other 3 A/R groups (Fig. 1).

TF:C and TF:Ag in HUVECs

The expression of TF gene was assessed by determining TF:C with one stage clotting assay and TF:Ag with ELISA respectively. Before anoxia-reoxygenation, TF:C was (2.59 ± 0.53) U/ 2×10^5 cells, (2.47 ± 0.46) U/ 2×10^5 cells, (2.63 ± 0.57) U/ 2×10^5 cells, (2.56 ± 0.51) U/ 2×10^5 cells and (2.60 ± 0.62) U/ 2×10^5 cells in HUVECs from control group, A/R group, aODN/TF+A/R group, sODN/TF+A/R group and mODN/TF+A/R group respectively.

While after anoxia-reoxygenation, HUVECs expressed stronger TF procoagulant activity. TF:C was (2.52 ± 0.49) U/ 2×10^5 cells, (63.28 ± 5.76) U/ 2×10^5 cells, (23.18 ± 1.64) U/ 2×10^5 cells, (62.74 ± 5.21) U/ 2×10^5 cells and (65.58 ± 5.95) U/ 2×10^5 cells in HUVECs from each group in order respectively.

Before anoxia-reoxygenation, TF:Ag was (11.68 ± 1.95) pg/ 2×10^5 cells, (10.59 ± 1.72) pg/ 2×10^5 cells, (12.06 ± 2.01) pg/ 2×10^5 cells, (11.98 ± 1.99) pg/ 2×10^5 cells and (10.95 ± 1.88) pg/ 2×10^5 cells in HUVECs from control group, A/R group, aODN/TF+A/R group, sODN/TF+A/R group and mODN/TF+A/R group respectively. After anoxia-reoxygenation, however, it was (10.96 ± 1.87) pg/ 2×10^5 cells, (95.35 ± 5.65) pg/ 2×10^5 cells, (46.66 ± 3.24) pg/ 2×10^5 cells, (93.26 ± 5.19) pg/ 2×10^5 cells and (96.32 ± 6.03) pg/ 2×10^5 cells in HUVECs from control group, A/R group, aODN/TF+A/R group, sODN/TF+A/R group and mODN/TF+A/R group respectively.

Before anoxia-reoxygenation, no statistical differences of TF:C and TF:Ag in HUVECs were displayed among groups. While, there were significant differences in TF:C and TF:Ag between HUVECs from control group and other groups after anoxia-reoxygenation ($P < 0.001$ compared with control group). Both TF:C and TF:Ag were much lower in HUVECs from aODN/TF+A/R group than those from A/R, sODN/TF+A/R and mODN/TF+A/R group respectively after anoxia-reoxygenation ($P < 0.01$) (Fig. 2).

Cell viability and LDH activity in HUVECs

In this study, HUVECs injury was assessed by cell viability and LDH activity. Before anoxia-reoxygenation, there was no statistical difference of cell viability in HUVECs among groups. While after anoxia-reoxygenation, cell viability declined from (96.2±8.2)% to (75.3±5.8)% (A/R group), (94.9±7.1)% to (89.2±6.9)% (aODN/TF+A/R group), (95.3±7.3)% to (74.6±5.5)% (sODN/TF+A/R group) and (94.5±7.0)% to (76.4±5.3)% (mODN/TF+A/R group) respectively. There was a significant difference between HUVECs from control group [(95.7±7.6)% to (93.1±7.2)%] and A/R, sODN/TF+A/R and mODN/TF+A/R group after anoxia-reoxygenation ($P<0.05$ versus control group), however, there was no statistical difference between HUVECs from control group and aODN/TF+A/R group.

Meanwhile, no statistical difference of LDH activity in HUVECs among groups was shown before anoxia-reoxygenation. While after anoxia-reoxygenation, the activity of LDH in the supernatant increased from (28.9±1.2) U/L to (262.2±12.3) U/L (A/R group), (27.7±1.4) U/L to (65.6±8.1) U/L (aODN/TF+A/R group), (29.1±1.5) U/L to (273.9±14.6) U/L (sODN/TF+A/R group) and (28.3±1.4) U/L to (268.8±13.1) U/L (mODN/TF+A/R group) respectively. There was a significant difference between HUVECs from control group [(28.6±1.3) U/L to (30.5±1.4) U/L] and other groups after anoxia-reoxygenation ($P<0.05$ to 0.001 versus control group). While aODN against TF attenuated obviously the decline of HUVECs viability and the increase of LDH activity induced by anoxia-reoxygenation ($P<0.05$ to 0.01, compared with A/R group, sODN/TF+A/R group and mODN/TF+A/R group) (Fig. 3).

MDA concentration and the activities of SOD and GSH-PX

The concentration of MDA and the activities of SOD and GSH-PX revealed the level of lipid peroxidation. Our present results showed that there were no statistical differences of MDA concentration and the activities of SOD and GSH-PX in HUVECs among groups before anoxia-reoxygenation. While after anoxia-reoxygenation, MDA was overexpressed, and SOD and GSH-PX were less produced in HUVECs subjected to anoxia-reoxygenation ($P<0.05$ to 0.01, compared with control group). aODN against TF inhibited the overexpression of MDA and reductions of SOD and GSH-PX induced by anoxia-reoxygenation ($P<0.05$ to 0.01, compared with A/R group, sODN/TF+A/R group and mODN/TF+A/R group) (Tab. 1).

Fig. 3. Cell viability and LDH activity in HUVECs. A: cell viability (n=18); B: LDH activity (n=18). Control: HUVECs from control group; A/R: HUVECs from A/R group; aODN/TF+A/R: HUVECs from aODN/TF+A/R group; sODN/TF+A/R: HUVECs from sODN/TF+A/R group; mODN/TF+A/R: HUVECs from mODN/TF+A/R group. Be: before anoxia-reoxygenation; Af: after anoxia-reoxygenation. *: $P<0.05$ compared with control group; Δ : $P<0.001$ compared with control group; $\blacklozenge$: $P<0.05$ compared with A/R group; $\diamond$: $P<0.01$ compared with A/R group; $\star$: $P<0.05$ compared with aODN/TF+A/R group; $\star\star$: $P<0.01$ compared with aODN/TF+A/R group.

Concentrations of NO and ET-1

ET-1 and NO indicated HUVECs' function in vasoconstriction and vasodilation and the injury of HUVECs exposed to anoxia-reoxygenation. To investigate the release of NO and ET-1 from HUVECs subjected to anoxia-reoxygenation, NO and ET-1 release were measured simultaneously. Before anoxia-reoxygenation, there were no statistical differences of concentrations of NO and ET-1 in HUVECs among groups. After anoxia-reoxygenation, however, the

Groups	MDA concentration (nmol/mg Pro)		SOD activity (mIU/min./mg Pro)		GSH-PX activity (μ mol/min./mg Pro)	
	before A/R	after A/R	before A/R	after A/R	before A/R	after A/R
Control	13.68 $\pm$ 3.49	13.95 $\pm$ 3.53	20.87 $\pm$ 4.84	19.33 $\pm$ 4.28	55.29 $\pm$ 8.68	53.88 $\pm$ 8.15
A/R	13.55 $\pm$ 3.24	26.55 $\pm$ 6.35 [▲]	21.21 $\pm$ 5.12	8.86 $\pm$ 2.18 [▲]	54.94 $\pm$ 9.23	26.79 $\pm$ 3.77 [▲]
aODN/TF+A/R	14.06 $\pm$ 3.09	16.27 $\pm$ 4.86 [◇]	19.96 $\pm$ 4.15	14.22 $\pm$ 3.46 ^{*◇}	53.85 $\pm$ 7.81	46.38 $\pm$ 7.92 ^{*◇}
sODN/TF+A/R	13.96 $\pm$ 3.72	26.87 $\pm$ 5.93 ^{▲★}	20.63 $\pm$ 4.44	9.02 $\pm$ 2.54 ^{▲★}	54.36 $\pm$ 8.14	25.89 $\pm$ 7.86 ^{▲★}
mODN/TF+A/R	13.83 $\pm$ 3.09	27.02 $\pm$ 6.48 ^{▲★}	21.03 $\pm$ 4.98	8.99 $\pm$ 2.25 ^{▲★}	53.91 $\pm$ 8.74	26.33 $\pm$ 9.05 ^{▲★}

Table 1. Concentration of MDA, activities of SOD and GSH-PX (mean $\pm$ SD, n=18). *: P<0.05 compared with control group; ▲: P<0.01 compared with control group; ◇: P<0.05 compared with A/R group; ◇: P<0.01 compared with A/R group; ★: P<0.05 compared with aODN/TF+A/R group.

Groups	NO (nmol/ml)		ET-1 (pg/ml)	
	before A/R	after A/R	before A/R	after A/R
Control	67.18 $\pm$ 7.73	65.23 $\pm$ 6.95	25.41 $\pm$ 2.46	26.33 $\pm$ 3.02
A/R	69.22 $\pm$ 8.15	23.36 $\pm$ 4.31 [△]	26.84 $\pm$ 2.43	93.62 $\pm$ 5.75 [△]
aODN/TF+A/R	68.65 $\pm$ 7.20	53.67 $\pm$ 5.32 ^{▲◇}	27.22 $\pm$ 2.87	40.83 $\pm$ 4.89 ^{*◇}
sODN/TF+A/R	68.42 $\pm$ 7.11	24.25 $\pm$ 3.97 ^{△☆}	25.62 $\pm$ 2.71	90.88 $\pm$ 5.19 ^{△☆}
mODN/TF+A/R	66.84 $\pm$ 6.29	25.06 $\pm$ 4.47 ^{△☆}	26.96 $\pm$ 2.62	91.33 $\pm$ 5.06 ^{△☆}

Table 2. Concentrations of NO and ET-1 (mean $\pm$ SD, n=18). *: P<0.05 compared with control group; ▲: P<0.01 compared with control group; △: P<0.001 compared with control group; ◇: P<0.01 compared with A/R group; ☆: P<0.01 compared with aODN/TF+A/R group.

concentration of NO decreased and that of ET-1 increased significantly (P<0.05 to 0.001, compared with control group), while they were ameliorated by aODN against TF significantly (P<0.01, compared with A/R group, sODN/TF+A/R group and mODN/TF+A/R group) (Tab. 2).

Investigation by means of active site blocked factor VII

Flow cytometry analysis

There were background expressions of ROS, PAR-1, PAR-2, p38 MAP kinase and p42/44 MAP kinase in HUVECs from Group I after 5 hours of normoxia, the mean fluorescence intensity (MFI) of which was (3.6 $\pm$ 0.7) (DCF), (3.3 $\pm$ 0.6) (PAR-1), (3.8 $\pm$ 0.8) (PAR-2), (3.5 $\pm$ 0.7) (p38 MAP kinase) and (3.8 $\pm$ 0.8) (p42/44 MAP kinase) respectively. While after anoxia-reoxygenation, the results showed increased expressions of ROS, PAR-1, PAR-2, p38 MAP kinase and p42/44

MAP kinase in HUVECs from Group II, the MFI of which was (568.7 $\pm$ 32.8), (362.1 $\pm$ 33.3), (53.1 $\pm$ 9.5), (309.1 $\pm$ 26.5) and (255.5 $\pm$ 19.7) respectively. There were significant differences between HUVECs from Group I and Group II after anoxia-reoxygenation (P<0.001 versus Group I). In HUVECs from Group III, which FVIIai was added into the culture medium, the MFI of DCF, PAR-1, PAR-2, p38 MAP kinase and p42/44 MAP kinase was (16.6 $\pm$ 5.1), (3.6 $\pm$ 0.8), (27.8 $\pm$ 4.7), (10.2 $\pm$ 2.8) and (22.3 $\pm$ 4.4) respectively after anoxia-reoxygenation. FVIIai inhibited the activation of ROS, PAR-1, PAR-2, p38 MAP kinase and p42/44 MAP kinase induced by anoxia-reoxygenation (P<0.05 to 0.001, compared with Group II) (Fig. 4 A and 4B).

Markers of HUVECs injury

TF and biomarkers of peroxidation such as MDA, SOD and GSH-PX expressed at background levels in HUVECs from Group I after 5 hours of normoxia, which

Fig. 4. ROS, PAR-1, PAR-2, p38 MAP kinase and p42/44 MAP kinase in HUVECs subjected to anoxia-reoxygenation. A: flow cytometry plots; B: statistical column diagram from flow cytometry. DCF: dichlorofluorescein, which indicated the level of ROS. *: P<0.05 compared with Group I; ▲: P<0.01 compared with Group I; △: P<0.001 compared with Group I; ◆: P<0.05 compared with Group II; ※: P<0.001 compared with Group II.

Fig. 5. Markers of HUVECs injury in HUVECs treated with FVIIai during anoxia-reoxygenation. *: P<0.05 compared with Group I; ▲: P<0.01 compared with Group I; △: P<0.001 compared with Group I; ◆: P<0.05 compared with Group II; ※: P<0.001 compared with Group II.

Fig. 6. Cell viability, LDH, NO and ET-1 in HUVECs treated with FVIIai during anoxia-reoxygenation. *: P<0.05 compared with Group I; ▲: P<0.01 compared with Group I; △: P<0.001 compared with Group I; ◆: P<0.05 compared with Group II.

were (2.56±0.55) U/2×10⁵ cells (TF:C), (11.36±1.88) pg/2×10⁵ cells (TF:Ag), (11.51±4.08) nmol/mg Pro (MDA), (17.92±3.32) mIU/min./mg Pro (SOD) and (50.36±5.87) μmol/min./mg Pro (GSH-PX) respectively. While after anoxia-reoxygenation, the results showed increased expressions of TF and biomarkers of peroxidation in HUVECs from Group II (P<0.01 to 0.001, compared with Group I), which were (65.76±6.45) U/2×10⁵ cells (TF:C), (94.98±5.37) pg/2×10⁵ cells (TF:Ag), (23.32±5.84) nmol/mg Pro (MDA), (9.35±1.90) mIU/min./mg Pro (SOD) and (30.96±3.94) μmol/min./mg Pro (GSH-PX).

respectively. In HUVECs from Group III, which FVIIai was added into the culture medium, TF and biomarkers of peroxidation were (2.59 ± 0.54) U/ 2×10^5 cells (TF:C), (95.79 ± 5.84) pg/ 2×10^5 cells (TF:Ag), (18.15 ± 5.37) nmol/mg Pro (MDA), (11.29 ± 2.92) mIU/min./mg Pro (SOD) and (41.75 ± 5.77) μ mol/min./mg Pro (GSH-PX) respectively, there were statistical differences between Group III and Group II in TF:C, MDA, SOD and GSH-PX ($P < 0.05$ to 0.001 , compared with Group II) (Fig. 5).

In addition, the results showed background expressions of cell viability, LDH, NO and ET-1 in HUVECs from Group I after 5 hours of normoxia, which were (96.2 ± 8.6) % (cell viability), (31.8 ± 1.2) U/L (LDH), (64.46 ± 7.27) nmol/ml (NO) and (26.32 ± 2.85) pg/ml (ET-1) respectively. While after anoxia-reoxygenation, both cell viability and concentration of NO decreased in HUVECs from Group II ($P < 0.01$, compared with Group I), which were (70.9 ± 5.3) % and (26.44 ± 3.98) nmol/ml respectively. LDH and the concentration of ET-1, however, increased obviously after anoxia-reoxygenation in Group II ($P < 0.001$, compared with Group I), which were (245.9 ± 12.5) U/L and (90.29 ± 4.94) pg/ml (ET-1) respectively. In FVIIai-treated HUVECs from Group III, the changes of cell viability and LDH were inhibited in a different degree ($P < 0.05$, compared with Group II), which were (80.3 ± 6.5) % (cell viability) and (210.5 ± 10.9) U/L (LDH) respectively. There were no statistical differences between Group II and Group III in the concentrations of NO and ET-1 after anoxia-reoxygenation, which were (28.38 ± 3.92) nmol/ml and (87.48 ± 4.49) pg/ml respectively in HUVECs from Group III (Fig. 6).

Discussion

Anoxia-reoxygenation injury is a serious clinical problem, which involves oxidative stress and leads to tissue injury. Endothelial cells are the tissue's first line of defense, oxidative stress activates endothelial cells and leads to injury even death of the cells [22]. In this study, we found that TF expression at the mRNA and protein level was both increased in HUVECs subjected to anoxia-reoxygenation. In fact, not only anoxia-reoxygenation induced the overexpression of TF, studies *in vivo* and *in vitro* showed that ischemia and hypoxia also produced soluble factors that either directly or indirectly stimulated TF expression [5, 23]. HUVECs subjected to anoxia-reoxygenation induces upregulation of TF mRNA by stimulating TF promoter via activating transcription factors such as activator protein-1 (AP-1), nuclear factor- κ B

(NF- κ B) and early growth response protein-1 (EGR-1), etc [24, 25]. Especially transcription factor NF- κ B plays an essential role in TF gene expression, it translocates from the cytoplasm to the nucleus, and binds to the putative κ B site in the promoter of TF gene [24], ultimately resulting in TF overexpression.

How does the increased TF act during anoxia-reoxygenation? Previous data showed that TF binds to FVIIa, which is the receptor of TF, and forms TF-FVIIa complex. TF-FVIIa complex not only irritates blood coagulation, but also triggers cell signaling [26]. In the present study, in order to investigate whether TF acts by binding with FVIIa or not, we used FVIIai to block the binding of TF to FVIIa. Flow cytometry showed that ROS increased and PAR-1, PAR-2, p38 MAP kinase and p42/44 MAP kinase were activated after anoxia-reoxygenation, while FVIIai inhibited the increment of ROS, PAR-1, PAR-2, p38 MAP kinase and p42/44 MAP kinase during anoxia-reoxygenation. PAR-1, PAR-2, p38 MAP kinase and p42/44 MAP kinase are important signal transduction molecules, the results indicated that during anoxia-reoxygenation, overexpressed TF binds to trace FVIIa in FBS contained in cell culture medium and forms TF-FVIIa complex, which triggers a set of cell signaling.

The increased TF may contribute to anoxia-reoxygenation injury of endothelial cells by inducing peroxidative injury via ROS generation and inflammation via proinflammatory mediators, strictly say, the role of ROS belongs also to inflammation [27].

On one hand, TF may contribute to anoxia-reoxygenation injury via ROS generation and subsequent lipid peroxidation injury. TF binds to trace FVIIa existed in FBS contained in the culture medium of HUVECs and forms TF-FVIIa complex, which activates p38 MAP kinase [28], a crucial mediator in ROS generation. p38 MAP kinase has been shown to be able to mediate phosphorylation and activation of xanthine dehydrogenase/xanthine oxidase (XDH/XO) in hypoxic vascular endothelial cells. XDH/XO is crucial enzymes responsible for generation of ROS [29]. XO normally occurs as an NAD⁺-dependent dehydrogenase [xanthine dehydrogenase (XDH)] incapable of ROS production. XDH activity converts by sulfhydryl oxidation or limited proteolysis (conditions that exist during reoxygenation) to XO. It was confirmed that p38 MAP kinase mediated phosphorylation of XO was an essential step for producing ROS including O₂⁻, H₂O₂ and $\cdot$ OH during reoxygenation [30]. Inhibition of p38 MAP kinase improves survival of cardiac myocytes with hypoxia via reducing oxidative

stress [31]. Therefore, p38MAP kinase mediates oxidative stress by upregulating XDH/XO directly in anoxic HUVECs, which is essential for producing ROS. In addition, there may be some other pathways in TF-induced ROS generation. TF binds to trace FVIIa and forms TF-FVIIa complex, which irritates phospholipase C-dependent calcium transients and subsequent generation of ROS, the presence of the cytoplasmic tail of TF is essential for TF-FVIIa complex driven phospholipase C-dependent generation of ROS [32]. Moreover, TF also contributes to ROS generation by activation of PAR-2 [33].

Under physiological conditions, the amount of ROS, e.g. $O_2^{\cdot-}$, H_2O_2 and $\cdot OH$ stays at a relatively stable level by regulating the balance between ROS-forming enzyme such as XO and ROS-scavenging enzymes such as SOD and GSH-PX. Under pathological conditions such as anoxia and reoxygenation, however, overexpression of TF induces ROS production. Accumulation of ROS thereby brings about lipid peroxidation, which results in overproduction of MDA, a biomarker of oxidative stress level, and consumptive decrease of SOD and GSH-PX activities. SOD and GSH-PX play a key role in detoxification of reactive oxygen metabolites (peroxides) and reactive electrophilic compounds and are commonly considered as anti-peroxidative biomarkers [34, 35]. Therefore, we detected the concentrations of SOD, GSH-PX and MDA to reveal HUVECs' ability in scavenging ROS and lipid peroxidation injury to HUVECs subjected to anoxia-reoxygenation in this study.

On the other hand, overexpressed TF may bind to trace FVIIa existed in the culture medium of HUVECs and form TF-FVIIa complex, which cleaves subsequently the N-terminal domain of G-protein-coupled PAR and exposes a new N-terminal domain, results in PAR signaling activation [36, 37]. The PAR family contains four members, PAR-1, PAR-2, PAR-3 and PAR-4 [38]. From existing literature, we know that deficiency of TF or lack of PAR signaling improves reperfusion injury by reduced inflammation [5]. TF-FVIIa complex induces HUVECs to express various inflammatory mediators such as adhesion molecules [intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), selectins, etc.], interleukins, growth factors [vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), etc.], cytokines [tissue necrosis factor (TNF)] and chemokines [monocyte chemoattractant protein (MCP-1)] by activation of PAR-1 and PAR-2 expressed on endothelial cells during anoxia-reoxygenation, and

subsequently elicits inflammation and thereby leads to the injury of the cells [39].

Previous study shows that TF-FVIIa complex induces phospholipase C-dependent calcium transients and subsequent activation of p38 MAP kinase, p42/p44 MAP kinase, phosphatidylinositol 3-kinase (PI3-K) and C-jun N-terminal kinase (JNK) [28, 40, 41]. As a set of key signaling components, MAP kinases are essential for mRNA production of proinflammatory factors as well as for protein production. For example, p38 MAP kinase and p42/p44 MAP kinase have been shown to be involved in the TF-driven gene transcription of inflammatory factors such as interleukin-8 (IL-8), interleukin-1 β (IL-1 β) and leukemia inhibitory factor (LIF) [42]. Inhibition of p38 MAP kinase with SB203580, a specific p38 MAP kinase inhibitor, improves survival of cardiac myocytes with hypoxia possibly via reducing the production of cytokines and oxidative stress, providing strong evidence that TF-induced inflammation may cause anoxia-reoxygenation injury [43, 44].

Both lipid peroxidation and inflammation subsequently result in endothelial cells injury even death, which leads to ET-1 release. ET-1, a 21-amino acid peptide produced primarily in endothelial cells, is the most powerful endogenous vasoconstrictive agent and has been identified as a key player of endothelial dysfunction results from endothelial cells activation even injury [45]. NO is an unstable substance produced in endothelial cells and the sensitive biomarker of endothelial cells injury, which is involved in the regulation of blood pressure, inhibition of the adhesion of leukocytes to endothelium, decreased platelet and blood vessel wall interaction, inhibition of apoptosis induced by various apoptotic stimuli and proliferation and migration of vascular smooth muscle cells. Because of the very unstable chemical nature of NO, endothelium-derived NO can be rapidly inactivated by oxygen-derived free radicals due to the formation of the complex between NO and $O_2^{\cdot-}$, and thus, an enhanced production of ROS appears to be involved in the accelerated breakdown of NO. Excess vascular oxidative stress leads to the impairment of NO production, resulting in reduced NO in endothelial cells [46]. Therefore, we detected the concentrations of ET-1 and NO to assess HUVECs' function in regulating vasoconstriction and vasodilation and injury of HUVECs.

Impaired functions of endothelial cells in scavenging ROS and regulating vasodilation and vasoconstriction lead to a decline of cell viability and increase of LDH activity, which indicated directly the degree of cells injury. Therefore, we measured the cell viability and LDH activity

to evaluate the injury of HUVECs induced by anoxia-reoxygenation in the present study.

The results showed that after anoxia-reoxygenation, HUVECs subjected to anoxia-reoxygenation displayed lower activities of SOD and GSH-PX as well as higher concentration of MDA, which revealed the HUVECs' ability in scavenging ROS was damaged and HUVECs were injured by lipid peroxidation. While HUVECs in A/R groups presented higher concentration of ET-1 and lower concentration of NO after anoxia-reoxygenation, which demonstrated dysfunction of HUVECs in regulating vasoconstriction and vasodilation and injury of HUVECs. In addition, HUVECs viability declined and LDH activity increased to a certain degree in A/R groups, which indicated HUVECs were injured by anoxia-reoxygenation. This suggested the experimental model was reliable and successful [2].

TF appears to be responsible for anoxia-reoxygenation induced injury of HUVECs. On account of this, inhibition of TF expression should reduce anoxia-reoxygenation injury in endothelial cells through blocking TF-induced ROS production and inflammation. Inhibition of TF expression reduces anoxia-reoxygenation injury or ischemia-reperfusion injury has also been proved by previous publications. For example, pretreatment with lovastatin, which eliminates excessive expression of TF in mural endothelial cell, reduced hypoxia-reoxygenation injury in endothelial cells [6]. Anti-TF antibody reduces infarct size and improves the microcirculation in experimental occlusion/reperfusion injuries of heart in rabbit model [47]. Antisense oligodeoxynucleotide against TF prevents renal ischemia-reperfusion injury and inhibits hepatic ischemia-reperfusion injury respectively in the rat [48, 49]. As the results showed, FVIIai interrupted the procoagulant activity of TF and thereby improved the changes of MDA, SOD, GSH-PX, cell viability and LDH occurred during anoxia-reoxygenation. For FVIIai just binds to TF and blocks the procoagulant activity of TF, and has no way to impact the expression of TF in HUVECs during anoxia-reoxygenation, therefore, FVIIai does not inhibit TF:Ag. While antisense oligodeoxynucleotide against TF inhibits TF expression at both mRNA and protein level, on account of this, we chose the antisense method for the inhibition of TF expression in the present study. The results showed that HUVECs in aODN/TF+A/R group demonstrated weak TF transcription as well as lower TF:C and TF:Ag compared with the cells in the other 3 A/R groups. This indicated that synthetic phosphorothioate aODN/TF blocked specifically the expression of targeted TF at both

mRNA and protein level, so we considered that the characteristic of aODN/TF used in this study was suitable for the prevention of TF expression in HUVECs. Our results showed that aODN/TF inhibited TF expression and subsequently reversed more effectively the changes of SOD, GSH-PX, NO, MDA and ET-1, ameliorated the decline of HUVECs viability and the enhancement of LDH activity that occurred during anoxia-reoxygenation than sODN/TF and mODN/TF. This indicated aODN against TF reduced obviously anoxia-reoxygenation injury in HUVECs and ultimately improved endothelial cells survival by interrupting the two TF-dependent signal pathways albeit the time frame of the experiments was relatively short.

The aODN against TF used in this study contained the rare tripeptide motif, Trp-Lys-Ser (WKS), which had been predicted as a functional motif involved in the interaction with serine proteases. Actually, there are many antisense oligodeoxynucleotides against different sections of TF used in previous publications [48, 49, 50], which are also effective in inhibiting TF, and no studies showed which one was more effective than others in inhibiting TF up to now. Therefore it was not the aODN against the WKS section of TF that was more effective than that against any other section of TF.

Consequently, the present study showed that TF plays an important role in the development of anoxia-reoxygenation injury of the HUVECs via inducing ROS generation and inflammation, antisense ODN against TF approach is an effective method to inhibit TF expression and subsequently ameliorates lipid peroxidation and inflammation, ultimately protects HUVECs against the injury induced by anoxia-reoxygenation. However, anoxia-reoxygenation is a pathological condition and a complex process, which is the consequence of a lot of diseases, it is also a complicated subject to understand because several signaling pathways are involved. Moreover, injured endothelial cells present abnormalities in both functions and structure, whereas, most of the underlying mechanisms in detail of TF contributing to the injury induced by anoxia-reoxygenation still remain unclear and need to be further investigated.

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