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Tat-APE1/ref-1 protein inhibits TNF-α-induced endothelial cell activation

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Abstract

Apurinic/apyrimidinic endonuclease 1/redox factor-1 (APE1/ref-1) is a multifunctional protein involved both in DNA base excision repair and redox regulation. In this study we evaluated the protective role of Tat-mediated APE1/ref-1 transduction on the tumor necrosis factor (TNF)-α-activated endothelial activation in cultured human umbilical vein endothelial cells. To construct Tat-APE1/ref-1 fusion protein, human full length of APE1/ref-1 was fused with Tat-protein transduction domain. Purified Tat-APE1/ref-1 fusion protein efficiently transduced cultured endothelial cells in a dose-dependent manner and reached maximum expression at 1 h after incubation. Transduced Tat-APE1/ref-1 showed inhibitory activity on the TNF-α-induced monocyte adhesion and vascular cell adhesion molecule-1 expression in cultured endothelial cells. These results suggest Tat-APE1/ref-1 might be useful to reduce vascular endothelial activation or vascular inflammatory disorders.

Keywords: Apurinic/apyrimidinic endonuclease 1/redox factor-1 (APE1/ref-1); Tat-mediated transduction; Monocyte adhesion; Vascular cell adhesion molecule-1; Endothelial cells; Vascular endothelial activation

The direct intracellular delivery of proteins has been difficult to achieve primarily due to the bioavailability barrier of the plasma membrane, which effectively prevents the uptake of macromolecules by limiting their passive entry. One approach to circumvent these problems is the use of HIV-TAT-mediated protein transduction [1]. HIV-Tat protein contained 9–11 amino acids for its transduction function [2]. Protein transduction domains offer an exciting therapeutic opportunity for the treatment of many diseases, such as airway inflammation, stroke and diabetes [3–5].

Atherosclerosis is an inflammatory disease. Monocyte rolling and adhesion to the vascular endothelial lining and subsequent diapedesis are not only the first steps, but also seem to be crucial events in the pathological process [6]. As the phenotype of endothelial cells is subject to change by oxidative stress or inflammatory cytokine, such as tumor necrosis factor (TNF)-α, endothelial activation is implicated in the pathogenesis of cardiovascular disorders [7]. Apurinic/apyrimidinic endonuclease/redox factor-1 (APE1/ref-1) is an essential endonuclease in the base excision repair pathway of oxidatively damaged DNA, as well as having reducing properties that promote the binding of redox-sensitive transcription factors such as activator protein-1 to their cognate DNA sequences [8,9]. In addition to a nuclear role of APE1/ref-1, an extra-nuclear role of APE1/ref-1 in the regulation of endothelial oxidative stress has been uncovered. In particular, APE1/ref-1 suppresses oxidative stress through modulation of cytoplasmic rac1-regulated ROS generation [10,11] and increases nitric oxide bioavailability through phosphorylation of endothelial nitric oxide synthase [12].

Our aim was to evaluate the potential usefulness of a Tat-APE1/ref-1 fusion protein to inhibit vascular endothe-

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lial cell activation. In the present study, we investigated the transduction of full length human Tat-APE1/ref-1 fusion protein into endothelial cells and the resulting biological activity on monocyte adhesion and vascular cell adhesion molecule-1 expression in human umbilical vein endothelial cells.

Materials and methods

Cell culture and reagent. Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (Cambrex Bio Science, MD, USA) and were grown and maintained in endothelial growth medium. HUVECs were used between passages 3 and 6. Human monocyte cell line, U937 cells, was obtained from American type culture collection (Manassa, VA, USA). Anti-VCAM-1 (SC-8304), anti-APE1/ref-1 (SC-5572), anti-His (SC-804) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-labeled anti-rabbit antibody was from PIERCE Biotechnology (Rockford, IL, USA). Ni–nitrilotriacetic acid Sepharose was purchased from Qiagen (Valencia, CA, USA). Isopropyl-β-thiogalactoside (IPTG) was purchased from USB (Clevelend, OH, USA) and human TNF-α were purchased from Sigma (St. Louis, MO, USA).

Recombinant protein expression and purification. Tat-APE1/ref-1 was generated by insertion of APE1/ref-1 DNA into pTAT-2.1. The human APE1/ref-1 was isolated from the pDsRed/APE1/ref-1[12] by PCR using following two primers; sense primer was 5′-CGG AAT TCC ATG CCG AAG CGT GGG AAA AAG GGA G-3′ (containing an EcoRI restriction site), and the antisense primer was 5′-CGC AAG CTT TCA CAG TGC TAG GTA TAT GGT G-3′ (containing a HindIII restriction site). After digesting with EcoRI and Hind III, full length of APE1/ref-1 constructs were cloned into the pTAT bacterial expression vector (pTAT-2.1, kindly donated from Steven Dowdy) and pET-28b vector (Novagen), which contains a six-histidine tag, for easy purification. pTAT-APE1/ref-1 and pET28b-APE1/ref-1 plasmids were then transformed into the BL21(DE3) strain of *Escherichia coli*. Following 4 h of induction with IPTG, the cells were sonicated in buffer Z (8 M urea, 100 mM NaCl, 20 mM HEPES) and recombinant proteins were purified on a Ni–NTA agarose column (Qiagen). After washing, TAT-APE1/ref-1 and APE1/ref-1 were eluted from 250 mM imidazole containing Buffer Z followed by desalting on a PD-10 column (Amersham Pharmacia Biotech) into PBS, and frozen in 10% glycerol at −80 °C.

Transduction of Tat-APE1/ref-1 into cultured HUVEC. HUVEC cells were grown in EGM-2 at 37 °C in humidified 95% air/5% CO_2. HUVEC cells were grown to confluence on a 6-well plate, and then cells were treated with various concentration of Tat-APE1/ref-1 for indicated times. Cells or cell extracts were prepared for monocyte-endothelial cell adhesion assay and Western blot analysis.

Immunofluorescent staining. The transduction of Tat-APE1/ref-1 protein into the endothelial cells was measured with immunofluorescent staining. For immunofluorescent staining, HUVECs were grown on glass coverslips. Following exposure to Tat-APE1/ref-1 for 1 h, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100, and blocked with 2% bovine serum albumin and 5% fetal bovine serum for 1 h. Cells were stained using Ant-His Tag, Alexa Flour 488 (1:2000) antibody (Millipore, C#16–254) in 2% bovine serum albumin for overnight at 4 °C. Coverslips were mounted on microscope slides, and fluorescence for Alexa 488 (FITC wavelength) was visualized with an Olympus confocal microscope.

Monocyte-endothelial cell adhesion assay. U937 cells were fluorescently labeled with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester (BCECF-AM) for the quantitative adhesion assay. The U937 cells were fluorescently labeled by incubating the cells (1×10^7 cells/ml) with 1 μM BCECF-AM in RPMI-1640 medium for 30 min at 37 °C and 5% CO_2. HUVECs were seeded in 24-well plates to reach confluent monolayers and pretreated with Tat-APE1/ref-1 or APE1/ref-1 for 1 h in EGM-2 medium. Human recombinant TNF-α was added to appropriate wells (15 ng/ml) at 18 h before addition of labeled monocytes. Monocyte adhesion was quantified by measuring fluorescence with excitation (485 nm) and emission (535 nm). Wells containing HUVEC only without U937 cells were used as blanks.

Western blot analysis. For Western blot analysis, HUVECs were harvested with 100 μl of lysis buffer containing 20 mM Tris–Cl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM Na_3VO_3, 1 mM β-glycerophosphate, 4 mM Na pyrophosphate, 5 mM NaF, and 1% Triton X-100, and protease inhibitor cocktail. The lysate was centrifuged at 12,000 rpm for 20 min and the supernatant was collected. Protein (30 μg) was separated by 10% SDS–PAGE and was electrotransferred onto nitrocellulose membranes. After blocking with 5% skim milk for 2 h at room temperature, blots were incubated for overnight at 4 °C with specific primary antibody (1:1000) and subsequent detection with horseradish peroxidase (HRP)-conjugated secondary antibody was performed. Blots were developed for visualization using an Enhanced Chemiluminescence Detection kit (Pierce, USA).

Statistical analysis. Values are expressed as the mean ± SEM. Statistical evaluation was performed using Student-*t* test, with $p < 0.05$ considered significant.

Results and discussion

Construction, expression and purification of Tat-APE1/ref-1

To explore the protective effect of cell permeable APE1/ref-1 on endothelial activation, we constructed Tat-APE1/ref-1 and APE1/ref-1. Tat-APE1/ref-1 expression vector contained a consecutive cDNA sequence encoding human APE1/ref-1, the Tat-PTD (RKKRRQRRR), and six-histidine residues at the amino-terminus (Fig 1A). Purified Tat-APE1/ref-1 and APE1/ref-1 proteins were visualized by Coomassie brilliant blue staining after SDS–PAGE (Fig. 1B). Two kinds of proteins could be eluted by the treatment of 250 mM imidazole containing buffer Z. The purified Tat-APE1/ref-1 and APE1/ref-1 proteins were further confirmed by Western blotting using an anti-APE1/ref-1 and anti-histidine antibody, which showed a molecular weight of approximately 40.9 kDa (Fig. 1C).

Transduction of Tat-APE1/ref-1 into cultured endothelial cells

To evaluate the transduction ability in the cultured endothelial cells, purified Tat-APE1/ref-1 (100 nM) were added to cultured endothelial cells for various incubation times. After the incubation of Tat-APE1/ref-1, cells were harvested and the change of transduced APE1/ref-1 was analyzed by Western blotting with anti-APE1/ref-1 antibody. As shown in Fig. 2A, Tat-APE1/ref-1 fusion protein was detected in the cell lysates within 1 min and its transduction reached a maximum at 1 h. A significant level of transduced Tat-APE1/ref-1 was present in the cells at 18 h (Fig. 2A).

Cellular transduction of Tat-APE1/ref-1 was also investigated with immunofluorescent staining with anti-His-FITC as Tat-APE1/ref-1 was conjugated with 6×His. As shown in Fig 2B, in the un-stimulated cells, fluorescent signal of anti-His-FITC was not detected; however, fluorescent signals were detected in the only nucleus of endothelial cells by the incubation of Tat-APE1/ref-1

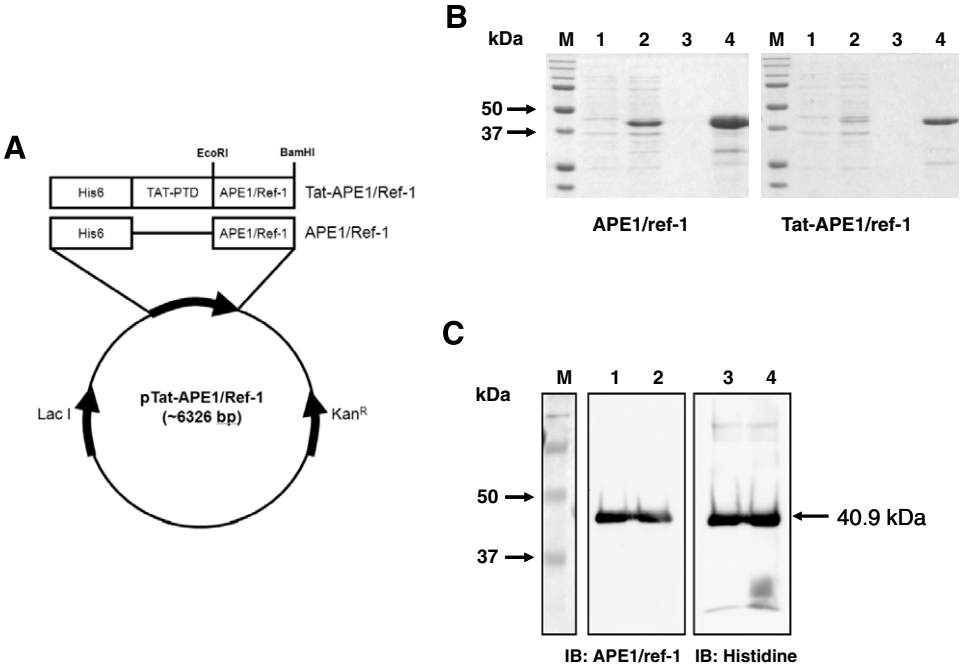

Fig. 1. Schematic diagrams, purification and Western blot analysis of Tat-APE1/ref-1 fusion protein. (A). The coding frame of human APE1/ref-1 is presented with six histidine and Tat PTD domain (RKKRRQRRR). Diagram of APE1/ref-1 fusion proteins were remarked on the vectors. (B). Coomassie blue staining of APE1/ref-1 protein (left) and Tat-APE1/ref-1 protein (right). Lane 1, *Escherichia coli* BL21(DE3) lysates without IPTG induction. Lane 2, *Escherichia coli* BL21(DE3) lysates after IPTG induction. Lane 3, eluate with 10 mM imidazole-containing buffer Z. Lane 4, eluate with 250 mM imidazole-containing buffer Z. (C). Western blot analysis of purified APE1/ref-1 (lanes 2 and 4) and Tat-APE1/ref-1 fusion protein (lanes 3 and 5) using anti-APE1/ref-1 and anti-histidine antibody. M shows molecular weights standard of protein as indicated on the left. APE1/ref-1 and Tat-APE1/ref-1 shows same molecular weight as approximately 40.9 kDa.

(100 nM) for 1 min or more, suggesting a reliable evidence for cellular transduction of Tat-APE1/ref-1 as an amino-terminus of APE1/ref-1 has putative nuclear localization signal [12].

This data shows the potential for Tat-APE1/ref-1 protein transduction to be applied to study in the endothelial activation or inflammation. Especially, Tat-PTD (RKKRRQRRR) efficiently helped to translocate APE1/ref-1 across plasma membrane [13]. Due to rapid transduction into the cells, it is applicable to vascular inflammation model and can avoid the need for genetic manipulation such as adenoviral DNA transfection.

Tat-APE1/ref-1 inhibited TNF-α-stimulated vascular cell adhesion molecule-1 (VCAM-1) expression

We next studied whether transduced Tat-APE1/ref-1 inhibits VCAM-1 expression in TNF-α-activated endothelial cells. Endothelial cells were incubated with Tat-APE1/ref-1 or APE1/ref-1 for 1 h before the treatment of TNF-α. After treatment with TNF-α for 12 h, cultured cells were harvested and transferred for Western blot analysis. As shown in Fig. 3, VCAM-1 expression was not detected in un-stimulated endothelial cells, but treatment with TNF-α resulted in a marked increase of VCAM-1 expression. Pretreatment of Tat-APE1/ref-1 at the range of 30–100 nM inhibited TNF-α-induced VCAM-1 expression in endothelial cells. In contrast, APE1/ref-1 protein did not inhibit VCAM-1 expression (Fig. 3), suggesting Tat-APE1/ref-1 has specific inhibitory action on VCAM-1 expression induced by TNF-α in endothelial cells. A previous report has shown that adenoviral overexpression of APE1/ref-1 suppressed TNF-α-induced VCAM-1 expression via inhibition of superoxide production and p38 MAPK activation in the endothelial cells [14]. In particular, cellular adhesion molecules are important components in atherosclerosis and the response to vascular injury [15]. Early atherosclerotic events and the initiation of lesion formation appear particularly dependent on VCAM-1 [16]. Furthermore, Dansky and colleagues' findings in $Vcam1^{D4D/D4D}Apoe^{-/-}$ mice [17] suggested a major role for VCAM-1 in the initiation of atherosclerotic process. This likely reflects an important function for VCAM-1 in recruitment of monocytes to the arterial intima. Therefore, application of Tat-APE1/ref-1 may be useful to reduce or inhibit the vascular activation in the response to inflammatory cytokine such as TNF-α.

Tat-APE1/ref-1 inhibited TNF-α-stimulated monocyte adhesion on the endothelial cells

Atherosclerosis is an inflammatory disease of the vessel wall characterized by monocyte infiltration in response to pro-atherogenic factors such as oxidized lipids or inflammatory cytokine. Firm adhesion of monocyte on the endo-

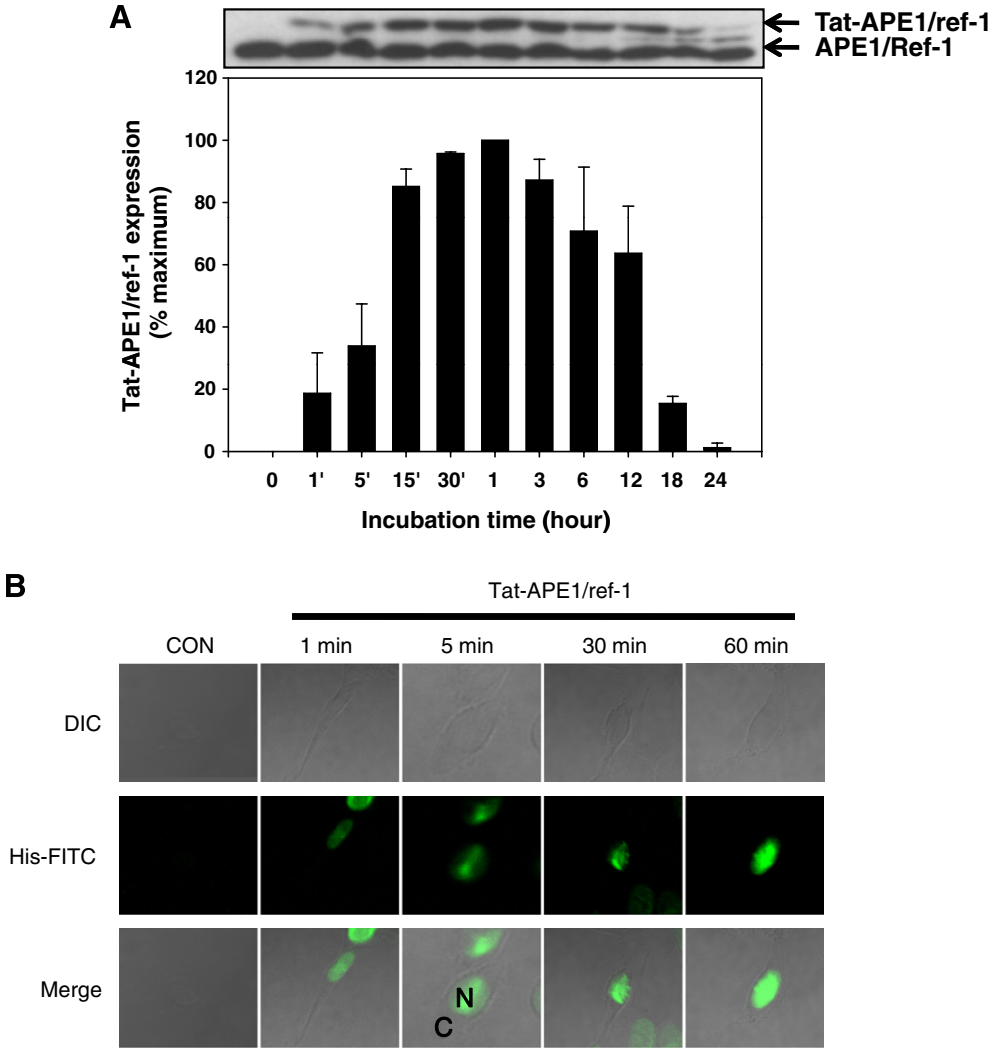

Fig. 2. (A) Transduction of Tat-APE1/ref-1 fusion proteins in cultured endothelial cells. Purified Tat-APE1/ref-1 (100 nM) was added in the cultured medium for indicated times, and then cell lysates were subjected with Western blot analysis for anti-APE1/ref-1 antibody. Densitometric analysis data was plotted in the bottom. Expression levels are expressed as % expression to maximum. Each bar shows mean ± SE ($n = 3$). (B) Immunofluorescent staining against Anti-His antibody in the cultured endothelial cells. Endothelial cells were treated with Tat-APE1/ref-1 (100 nM) for indicated time. Cells were stained using anti-His-Tag, Alexa Fluor 488 (Green). CON: untreated cells, N: nucleus, C: cytoplasm. (For interpretation of the references to colour in this figure legend the reader is referred to the web version of this paper).

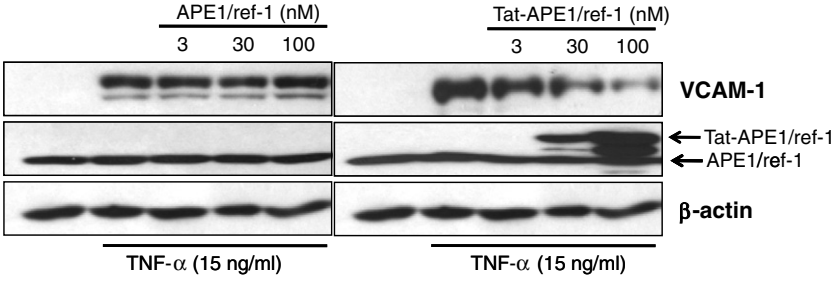

Fig. 3. Effect of Tat-APE1/ref-1 on the TNF-α-induced VCAM-1 expression in the endothelial cells. Endothelial cells were incubated with an indicated concentration of Tat-APE1/ref-1 or APE1/ref-1 for 1 h before the treatment of TNF-α. After treatment with TNF-α for 12 h, cultured cells were harvested and transferred for Western blot analysis as described in the Materials and method section. β-actin was used as a loading control. Left panel: effect of APE1/ref-1, Right panel: effect of Tat-APE1/ref-1.

thelial cells was mediated by the expression of adhesion molecules in the endothelial cells [14,15].

After the inhibitory action of Tat-APE1/ref-1 in the TNF-α-induced VCAM-1 expression was confirmed, we

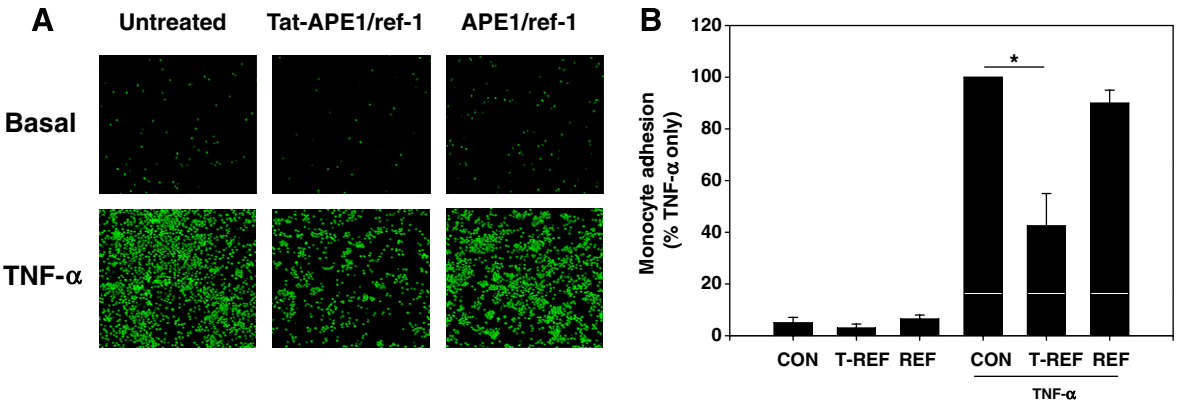

Fig. 4. Effect of Tat-APE1/ref-1 and APE1/ref-1 on the TNF-α-induced monocyte adhesion on the endothelial cells. Tat-APE1/ref-1 (100 nM, T-REF) or APE1/ref-1 (100 nM, REF) was pretreated for 1 h before TNF-α treatment. Note that transduced Tat-APE1/ref-1 suppressed TNF-α-induced monocyte adhesion, compared with APE1/ref-1. Monocyte adhesion was assessed by monocyte-endothelial cell adhesion assay as described in the Material and methods section. (A). Typical monocyte adhesion assay, (B). Densitometric data. Expression levels are represented as % expression to TNF-α only. Each bar shows mean ± SE ($n = 6$).). $^{*}p < 0.05$ (Tat-APE1/ref-1 *vs.* control [CON]).

evaluated the possible role of Tat-APE1/ref-1 on TNF-α-induced monocyte adhesion to endothelial cells. Cells were pretreated with Tat-APE1/ref-1 or APE1/ref-1 for 1 h before the treatment with TNF-α. After incubation with TNF-α for 18 h, monocyte adhesion assay was performed as described in Material and methods. As shown in Fig. 4, monocyte adhesion was minimal in un-stimulated endothelial cells, but treatment with TNF-α resulted in a marked increase of monocyte adhesion to endothelial cells. Pretreatment with Tat-APE1/ref-1 (100 nM) inhibited TNF-α-induced monocyte adhesion to endothelial cells. However, APE1/ref-1 protein did not inhibit monocyte adhesion (Fig 4B), suggesting that Tat-APE1/ref-1 has specific inhibitory action on monocyte adhesion induced by TNF-α to endothelial cells.

In conclusion, these data show that Tat-APE1/ref-1 suppresses vascular cell adhesion molecule-1 expression and monocyte adhesion to the endothelium in response to TNF-α, suggesting it may be useful in the suppression of vascular activation or vascular inflammatory disorders.

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