Articles

HIV-TAT mediated protein transduction of heme oxygenase-1 protects HaCaT cells from ionizing radiation

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ABSTRACT

الأهداف: تفسير تأثير بروتين الخلايا المشتقة القرنية البشرية وبروتين أكسجين الهيم المنصهر على الخلايا المشتقة القرنية البشرية الناتجة من الإشعاع.

الطريقة: أجريت هذه الدراسة خلال الفترة من مايو 2010م حتى فبراير 2013م في قسم طب الإشعاع والحماية، جامعة سوشو، سوتشو، الصين. صممت هذه الدراسة المخبرية لمعرفة الدور الوقائي TAT–HO في خلايا الجلد. انصهر جين HO البشري مع ترميز جين TAT PTD الجزئي لإنتاج TAT–HO–1. تم قياس توزيع TAT–HO–1 باختبار المقايسة المناعية. كما تم تحديد الأثر الإشعاعي TAT–HO–1 بمقايسة مولد النسائل. وتعديل تحليل موت الخلايا بواسطة التدفق الخلوي.

النتائج: أن بروتين TAT–HO–1 الصافي والظاهر لديه القدرة على الاتحاد مع الخلايا المشتقة القرنية البشرية. وبإمكاننا تقييم الدور الوقائي TAT–HO–1 ضد الإشعاع المؤين. كما قمنا بتقييم الدور الوقائي TAT–HO–1 ضد الإشعاع المؤين. يقلل TAT–HO–1 من تكاثر أصناف أنواع الأكسجين التفاعلية ويقلل TAT–HO–1من حساسية الإشعاع للتعرض للأشعة السينية. إضافة إلى ذلك، تمهد الخلايا المشتقة القرنية البشرية معالجة مع TAT–HO–1 وينتج موت أقل للخلايا بواسطة الإشعاع. إضافة إلى ذلك، قد ينفذ TAT–HO–1 جلد الفأر.

خاتمة: تشير النتائج أن TAT–HO–1 قد يحمي الخلايا المشتقة القرنية البشرية من الإشعاع الأيوني.

Objectives: To elucidate the effects of human keratinocyte-derived HaCaT cells (HIV-TAT) protein transduction domains (PTD) coupled heme oxygenase-1 (HO-1) fusion protein (TAT-HO-1) on radiation-induced human keratinocyte-derived HaCaT cells.

Methods: This study was conducted between May 2010 and February 2013 in the School of Radiation Medicine and Protection, Soochow University, Suzhou, China. This experimental study was designed to explore the protective role of TAT-HO-1 in skin cells. The human HO-1 gene was fused with a gene fragment encoding TAT PTD to produce in-frame TAT-HO-1. The distribution of TAT-HO-1 was measured by immunostaining and Western blot. The radioprotection for TAT-HO-1 was determined using clonogenic assay. Alterations in apoptosis were analyzed by flow cytometry.

Results: The expressed and purified TAT-HO-1 recombinant protein could be incorporated into human HaCaT cells. We then evaluated the protective role of TAT-HO-1 against ionizing radiation. The TAT-HO-1 attenuated the generation of reactive oxygen species and decreased HaCaT cell radiosensitivity to irradiation. Moreover, HaCaT cells pretreated with TAT-HO-1 resulted in less apoptosis by radiation. In addition, TAT-HO-1 could penetrate rat skin.

Conclusion: These results suggest that TAT-HO-1 can protect HaCaT cells from ionizing radiation.

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The currently used delivery systems for gene transfer including viral vectors, and non-viral vectors are confronted with many limitations. Low transfection efficiency and unstable levels of expression have greatly hindered their application.[1,2] Moreover, due to concerns regarding continued exogenous gene expression, new

approaches have been sought for gene transfer in humans. Protein transduction domains (PTDs) are able to facilitate the internalization of both homologous and heterogeneous cargos into cells.[3] Protein transduction domains, which possess a characteristic positive charge on the basis of their enrichment in arginine and lysine residues, have demonstrated high efficiency of transmembrane ability.[4] When PTDs are covalently linked to macromolecules, they are capable of facilitating internalization of the cargos. The first discovered PTD was human keratinocyte-derived HaCaT cells (HIV-TAT) in 1988.[5] Subsequently, an increasing number of PTDs have been reported, both natural PTDs such as the virus protein 22 (VP22) of Herpes simplex virus, the antennapedia homeoprotein of Drosophila, and the chemically synthesized ones such as 8 mers of polyarginine and polylysine.[6,7] The appearance of PTDs may hold promise in therapy for diseases.

Radiotherapy has become a valuable alternative to primary surgical approaches for cancer patients.[8,9] However, the skin is the outer covering of the human body, which makes it the primary target during radiotherapy.[10,11] Radiation-induced skin damage remains a serious concern during radiotherapy, which may affect the duration and the dose of radiation.[12,13] Radiation exposure generates reactive oxygen species (ROS) and reactive nitrogen species such as oxygen ions, peroxides, and peroxynitrite. These detrimental free radicals are likely to attack nuclear DNA, proteins, lipids, and carbohydrates, resulting in skin damage.[14] Heme oxygenase-1 (HO-1), also known as heat shock protein 32, is reported to be a cytoprotective protein.[15,16] The HO-1 expression is strongly induced by various stimuli, including heat shock, cytokines, metals and oxidative stress, which directly or indirectly generate ROS.[17] The HO-1 catalyzes the heme ring into carbon monoxide (CO), free iron, and biliverdin.[18] The abundant and ubiquitous biliverdin reductase then converts biliverdin to bilirubin. The antioxidant and anti-inflammatory properties of CO and bilirubin have been well documented.[19,20] In skin cells, the expression of HO-1 is strongly induced by ultraviolet A radiation within hours.[21,22] The HO-1 is also involved in skin immunity, and HO-1 inhibition promotes cutaneous inflammation, which was abrogated by treatment with the HO-1 inducer cobaltic protoporphyrin.[23]

We have demonstrated that adenovirus mediated HO-1 overexpression can ameliorate radiation-induced skin injury.[24] However, clinical application of adenovirus-based gene therapy remains a concern.[25] Adenoviral vectors tend to elicit strong immune and inflammatory responses in vivo and usually mediate a short-term gene expression.[26] Protein transduction domains have provided a rapid and controlled system for therapeutic applications. Whether HO-1 mediated by PTDs is able to effectively protect skin cells from ionizing radiation is unclear. In the present study, human HO-1 was fused with HIV-TAT PTD. Purified TAT-HO-1 was analyzed for its transduction efficiency into human keratinocyte-derived HaCaT cells and evaluated for its protective role against ionizing radiation.

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Methods.

This study was conducted between May 2010 and February 2013 in the School of Radiation Medicine and Protection, Soochow University, Suzhou, China.

Experimental design. This experimental study was designed to explore the protective role of TAT-HO-1 in skin HaCaT cells. The HaCaT cells were assigned to control group and TAT-HO-1-treated group. The effect of TAT-HO-1 on the survival rates of HaCaT cells was determined using clonogenic assay. Alterations in apoptosis were analyzed by flow cytometry. Ethical approval for the study was obtained from the Medical School of Soochow University.

Construction of TAT-HO-1 expression vector. The coding sequence for HIV-TAT domain (YGRKKRRQRRR) was synthesized and subcloned into the BamHI and EcoRI sites of pET-28a (Novagen, Madison, WI, USA) to generate pET-28a-TAT. The human HO-1 cDNA was amplified by polymerase chain reaction using the following primers: forward 5'-CGGAATTCATGGA GCGTCCGCAACCCGAC-3' and reverse 5'-CCGCTCGAGTCACATGGCATAAAGCCCT ACAG-3'. The amplification product was then subcloned into the EcoRI and XhoI sites of pET-28a-TAT to generate pET-28a-TAT-HO1 (**Figure 1A**).

Purification of target fusion protein. The host *Escherichia coli* (*E.coli*) BL21 (DE3) was transformed with plasmids encoding the pET-28a-TAT-HO1, and then the transformants were selected on a lysogeny broth plate containing kanamycin. The BL21 (DE3) cells containing the expression plasmid were grown at 37°C to an optical density OD_{600} of 0.8. Isopropyl-beta-D-thiogalactoside (IPTG) was added to a final concentration of 1.0 mM, and the cells were then incubated for an additional 8 hours at 25°C. Cells were sonicated and the supernatants were recovered and applied to a column of Ni-nitrilotriacetic acid agarose (Qiagen, Valencia, CA, USA). Then, the mixture was maintained at 4°C with shaking at 50 x g. After the TAT-HO-1 had been absorbed by the column, the column was put into a plastic filter. After the Ni2+ column had subsided to the bottom, 4 ml washing buffer (10 mM imidazole, 0.5 M sodium chloride [NaCl], and 50 mM monosodium phosphate [NaH2PO4] at pH 8.0) was added to the filter. The initial filtrate was passed through the column a second time. Then, the fusion protein was eluted with an elution buffer (150 mM imidazole, 0.3 M NaCl, and 50 mM NaH2PO4 at pH 8.0). The fractions containing fusion proteins were combined. The expression and purification of enhanced green fluorescent protein (EGFP) and TAT-EGFP were described previously.[27,28]

Transduction of TAT-HO-1 into cultured cells in vitro. To determine if the TAT-HO-1 was able to facilitate internalization into cells, human keratinocyte-derived HaCaT cells were used. They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin G, 100 units/mL streptomycin sulfate; Gibco, Grand Island, NY, USA). To analyze the transduction efficiency of fusion proteins, HaCaT cells were grown on a 6-well plate. Thereafter, these cells were treated with 200 nM EGFP, TAT-EGFP, or TAT-HO-1. Four hours after the addition, cells were washed 3 times with phosphate buffered saline (PBS) to remove free proteins. Images were collected with a Leica TCS SP2 confocal microscope (Leica, Wetzlar, Germany).

Immunostaining. The HaCaT cells or 3 μm thick skin frozen sections were fixed with 4% paraformaldehyde, washed with PBS, and permeabilized with 1% Triton X-100 in PBS. Cells were blocked with blocking buffer (PBS, 1% Triton X-100, and 5% bovine serum albumin) and incubated at 4°C with HO-1 antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight. The rhodamine-conjugated mouse anti-goat (1:100) was added for 30 minutes at room temperature. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO, USA).

Clonogenic assay. For standard clonogenic assays, cells were seeded into 6-well plates at 500-1,000 cells/well depending on the dose of radiation. Twenty-four hours after seeding, the cells were treated with TAT-HO-1 for 4 hours The cells were irradiated using a 6-MV x-ray linear accelerators (Clinac 2100EX; Varian Medical Systems Inc, Alto, CA, USA) at a dose rate of 2 Gy/min; a 1.5-cm bolus was used as a compensator. After radiation, the drug-containing medium was immediately replaced by fresh DMEM medium. The cells were then grown from 7-10 days to allow for colony formation and were subsequently fixed and stained using crystal violet. Colonies consisting of 50 or more cells were counted as a clone.

Reactive oxygen species assays (ROS). 2'7'-dichlorofluorescein diacetate (DCFH-DA) is permeable to the cellular membrane and once inside the cell is rapidly hydrolyzed by cellular enzymes to non-fluorescent 2'7'-dichlorofluorescein (DCFH). Oxidation of DCFH by ROS produces the fluorescent indicator 2'7'-dichlorofluorescein.[29] Cells were treated with TAT-HO-1 for 4 hours. The DCFH-DA (Beyotime, Nantong, China) was prepared at a concentration of one mM and added to the adherent HaCaT cells before irradiation. The incubation time was 15 minutes at 37°C in the dark. Cells were then irradiated, and fluorescence was measured at 24 hours after irradiation. The pcDNA3.1-HA-HO-1 was a kind gift from Prof. Wei-Qun Ding (The University of Oklahoma). Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Measurement of apoptosis. Cells were treated with 200 nmol/L TAT-HO-1 for 4 hours prior to treatment with 20 Gy irradiation. Apoptosis was measured using propidium iodide / annexin-V double-staining (Beyotime, Nantong, China). The cells were harvested at 48 hours after 20 Gy irradiation as reported previously.[30] Apoptotic fractions were measured using flow cytometry (Beckman, Brea, CA, USA).

Western blot. Cells were treated with 200 nmol/L TAT-HO-1 fusion protein for 4 hours. Cells were then washed twice with ice-cold PBS and then directly lysed in 200 μL of cell lysis buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 1% Triton X-100, 5 mM EDTA, 10 mM NaF, 2 μM leupeptin, one mM phenylmethylsulfonyl fluoride, one mM dithiothreitol, and 2 μM pepstatin A) in each well. The lysates were boiled, centrifuged at 15,000 x g, and then loaded onto a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE) gel. The samples were electrophoresed for 2 hours and then transferred onto transfer membranes. After blocking with 5% non-fat milk in PBS-Tween-20 for one hour at room temperature, the membranes were blotted with the anti-6 × histidine-tag or glyceraldehyde 3-phosphate dehydrogenase primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:1,000-1:2,000 dilution. The membranes were then incubated with the appropriate horseradish peroxidase-coupled secondary antibody at a 1:2000 dilution for one hour at room temperature. After washing with tris-buffered saline and tween, the blots were incubated in detection reagent (ECL Advance Western Blotting Detection Kit, Amersham Bioscience, Freiburg, Germany) and exposed to a Hyperfilm ECL film (Pierce, Rockford, IL, USA).

Statistical analysis. All analyses represent experiments that were performed at least in triplicate. The results were evaluated by one- or two-way ANOVA using the Statistical Package for Social Sciences version 17.0 software (SPSS Inc., Chicago, IL, USA) to determine significance. The radiation sensitivity enhancement ratio (SER) was measured according to the multi-target single hit model. Differences were considered statistically significant when $p<0.05$.

Results. *Purification of target fusion proteins.* To investigate the protective role of TAT-HO-1 fusion protein, we constructed a pET-28a-TAT-HO1 expression vector containing the cDNA sequences encoding the HO-1 and HIV-TAT basic domain (**Figure 1A**). As shown in **Figure 1B**, the fusion proteins were expressed at a very high level and can further be induced by IPTG. The fusion protein was present more in the supernatant of whole cell lysate (**Figure 1C**), indicating it was soluble in the *E.coli* host cells. Then, the TAT-HO-1 fusion protein was purified by affinity chromatography (**Figure 1D**). In addition, 2 control recombinant proteins, EGFP and TAT-EGFP were also expressed and purified.

Transduction of TAT-HO-1 into cultured HaCaT cells. To determine whether the HIV-TAT was able to facilitate internalization into human HaCaT cells, cells were mock treated or treated with 200 nmol/L EGFP or TAT-EGFP. Four hours after the administration, cells were washed 3 times with PBS to remove free proteins. Under a fluorescent microscope, green fluorescence can be observed from cells treated with TAT-EGFP, but not in the mock treated HaCaT cells (**Figure 2**). Cells treated with EGFP showed very weak fluorescence, which is possibly due to the EGFP protein attached to the cell surface (**Figure 2**). This result indicated that

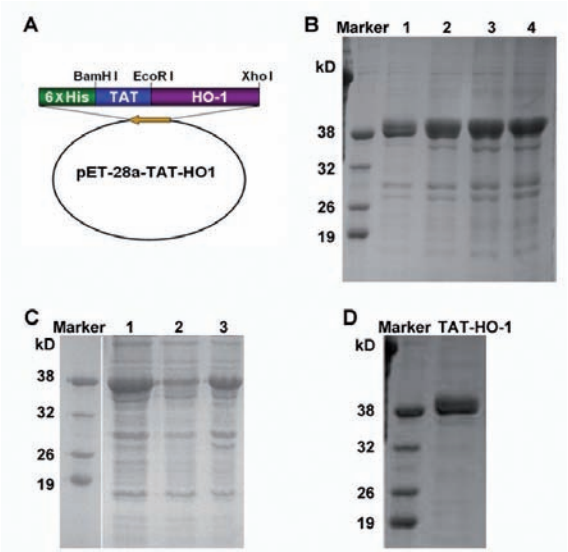

Figure 1 - Expression and purification of TAT-heme oxygenase-1 (HO-1) fusion protein in *Escherichia coli* (*E. coli*) showing: **A**) Diagram of TAT-HO-1 expression vector. Human heme oxygenase-1 (HO-1) gene was cloned into expression vector pET-28a. The coding frame of TAT-HO-1 is represented by HO-1 (purple box) along with 6 x histidine (His) (green box) and TAT (blue box). The resulting vector was named pET-28a-TAT-HO1. **B**) The *E. coli* BL21 (DE3) transformed with pET-28a-TAT-HO1 was induced with 1 mM isopropyl-beta-D-thiogalactoside (IPTG) for 8 hours at 30°C. Lane 1-4: induction of IPTG for 0, 2, 4 or 8 hours. **C**) The presence of TAT-HO-1 recombinant protein in lysed *E. coli* supernatant. Lane 1-3: whole cell lysate, cell pellet, and cell supernatant. **D**) Purified TAT-HO-1 by affinity chromatography on a nickel Sepharose column. Protein extracts of cells and purified fusion proteins were analyzed by 10% SDS-PAGE gel electrophoresis and stained with Coomassie Brilliant blue. His - histidine, SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

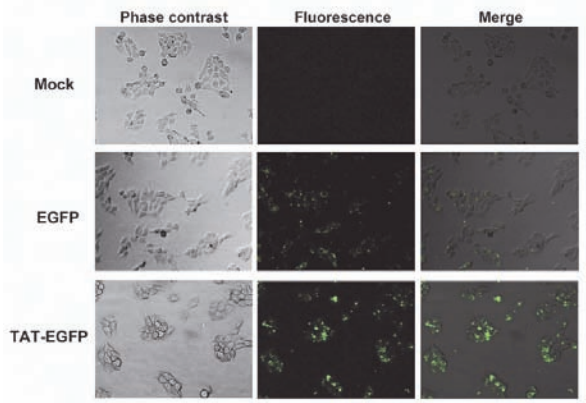

Figure 2 - Transduction of proteins into cultured HaCaT cells. The HaCaT cells were grown on a 24-well plate. Thereafter, these cells were mock treated or treated with 200 nmol/L enhanced green fluorescent protein (EGFP) or TAT-EGFP. Four hours after addition, cells were washed 3 times with phosphate buffered saline to remove free proteins. The distribution of fluorescence within cells was analyzed by a confocal microscope. Fluorescence signals were obtained under the same conditions.

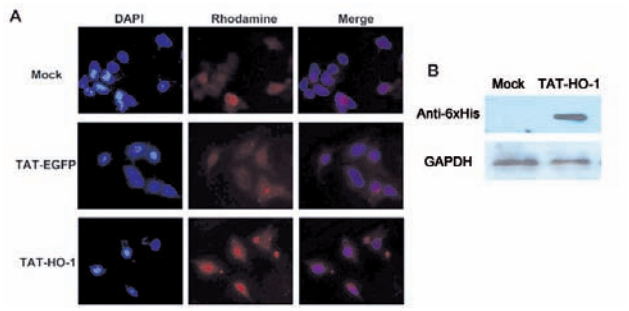

Figure 3 - Fluorescent microscopic analysis of HaCaT cells transduced with TAT-heme oxygenase-1 (HO-1). **A)** Transduction of the TAT-HO-1 protein into HaCaT cells. Four hours after addition, cells were washed 3 times with phosphate buffered saline to remove free proteins. Fluorescence signals were obtained under the same conditions by immunostaining. **B)** Cells were treated with 200 nmol/L TAT-HO-1 for 4 hours. The TAT-HO-1 and internal standard glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein levels were detected by Western blot using anti-6 × histidine (His) or anti-GAPDH primary antibodies. EGFP - enhanced green fluorescent protein. DAPI - 4,6-diamidino-2-phenylindole

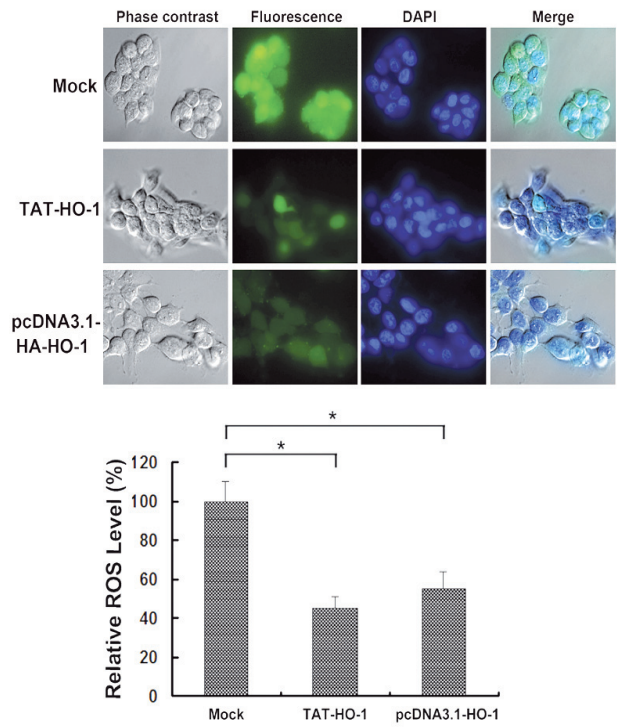

Figure 4 - The effect of TAT-heme oxygenase-1 (HO-1) on reactive oxygen species (ROS) generation. Cells were pretreated with TAT-HO-1 or transfected with pcDNA3.1-HA-HO-1. The ROS production after irradiation was determined by 2'7'-dichlorofluorescein diacetate (DCFH-DA) fluorescence 24 hours after x-ray irradiation. Fluorescence signals were obtained under the same conditions. Relative ROS level in indicated group of cells as calculated by ImageJ image analysis software (Bethesda, MD, USA). DAPI - 4,6-diamidino-2-phenylindole

the TAT domain can be used as a delivery vehicle for protein transfer.

We next investigated the internalization of HO-1 coupled with TAT PTD; HaCaT cells were treated with 200 nmol/L TAT-HO-1 or equivalent TAT-EGFP. As shown in **Figure 3A**, 4 hours after administration of TAT-HO-1 in the medium, it was distributed in both the cytoplasm and nucleus of the HaCaT cells. Western blot further confirmed that there was obviously increased histidine-tagged protein in the TAT-HO-1 treated cells (**Figure 3B**). These results indicated that the HIV-TAT could mediate internalization of HO-1 fusion protein into the HaCaT cells.

The TAT-HO-1 reduces intracellular ROS concentration. An increase in ROS is produced by irradiation. We therefore, tested if TAT-HO-1 could decrease ROS in HaCaT cells. The ROS production was measured by DCFH-DA fluorescence 24 hours after 20 Gy X-ray irradiation. As shown in **Figure 4**, compared with mock treated cells, pretreatment with TAT-HO-1 can significantly decrease ROS generation (p=0.016), which was comparable to transfection of an HO-1 expression vector (pcDNA3.1-HA-HO-1). This result indicates that TAT-HO-1 can decrease radiation-induced ROS generation in cultured HaCaT cells.

The effect of TAT-HO-1 on the radiosensitivity of HaCaT cells. To investigate the effect of TAT-HO-1 on the radiosensitivity of HaCaT cells, we performed an in vitro clonogenic cell survival assay using TAT-HO-1 treatment plus radiation. The HaCaT cells treated with 100 or 200 nmol/L TAT-HO-1 plus x-ray irradiation exhibited significantly higher clonogenic survival rates than cells treated with radiation alone. Compared with mock treated cells, using the multi-target model, the radiation SER was 0.86 for cells treated with 100 nmol/L TAT-HO-1, and 0.77 for cells treated with 200 nmol/L TAT-HO-1 (**Figure 5A**). The data were further analyzed using 2-way ANOVA to test the interaction effect between TAT-HO-1 and radiation. Our results indicated that the interaction effect between 200 nmol/L TAT-HO-1 and radiation was statistically significant (p=0.0034) for HaCaT cells, suggesting that TAT-HO-1 treatment could protect cells from x-ray irradiation. Taken together, these results demonstrated that treatment with TAT-HO-1 could attenuate the radiosensitivity of human HaCaT cells.

The inhibition of apoptosis by TAT-HO-1. We next investigated whether the increased clonogenic survival by treatment of TAT-HO-1 was associated with decreased apoptosis. Annexin-V staining-based flow cytometric analysis of apoptosis was performed.

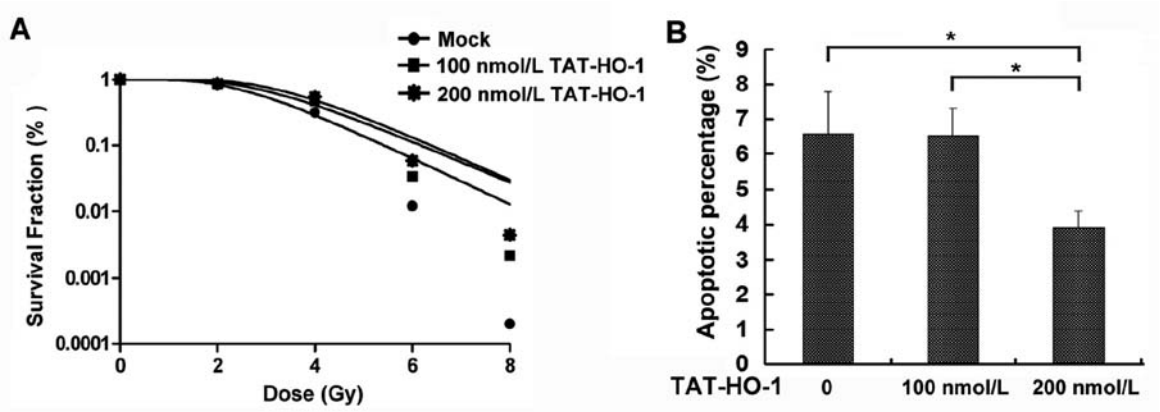

Figure 5 - The effect of TAT-heme oxygenase-1 (HO-1) on radiosensitivity and apoptosis in HaCaT cells. **A)** The HaCaT cells were pretreated with the indicated concentrations of TAT-HO-1 for 4 hours and then were exposed to 2, 4, 6, or 8 Gy irradiation. The survival data were normalized to that of the unirradiated control group. The radiation sensitivity enhancement ratio was calculated for HaCaT cells that were treated with 100 or 200 nmol/L TAT-HO-1 prior to x-ray irradiation. **B)** The effect of TAT-HO-1 on apoptosis in HaCaT cells. Cells were treated with 100 or 200 nmol/L TAT-HO-1 for 4 hours and then exposed to 20 Gy irradiation. Statistical analysis between the groups were determined by ANOVA, $^{*}p<0.05$.

The proportion of apoptotic cells is shown in **Figure 5B**, TAT-HO-1 treatment further reduced the apoptosis of HaCaT cells after 20 Gy of irradiation (6.57% versus 3.91%, p=0.003). Taken together, these results demonstrate that TAT-HO-1 reduces apoptotic cell death of HaCaT cells in response to irradiation.

Penetration of fusion proteins through rat skin. We then evaluated the ability of the HO-1 fusion proteins to transduce into rat skin. The fusion proteins were sprayed on rat skin, and the degree of skin penetration of these fusion proteins was analyzed by immunostaining. Because TAT-HO-1 could be incorporated into skin cells after 4 hours of incubation, we measured its transdermal ability after 4 hours. As shown in **Figure 6**, the fluorescence signal in skin treated with TAT-HO-1 fusion proteins was significantly detected in the epidermis as well as the dermis of the subcutaneous layer in rat skin tissue. These results demonstrate that the HO-1 fusion protein with HIV-TAT basic domain residues cannot only be transduced into cultured mammalian cells, but also penetrate the rat skin.

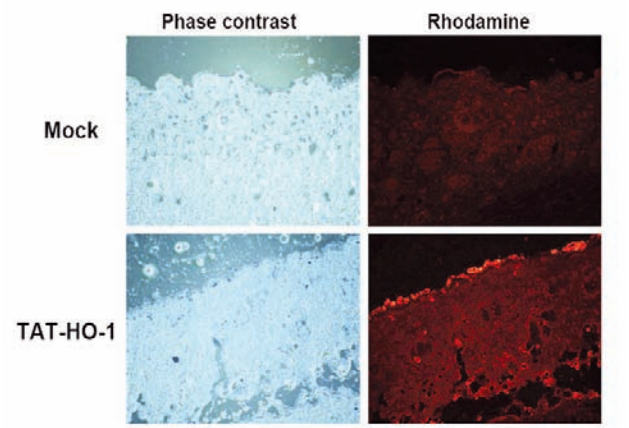

Figure 6 - Immunostaining of rat skin transduced with TAT-heme oxygenase-1 (HO-1) fusion proteins. Fifty microgram of TAT-HO-1 fusion proteins were topically applied onto the shaved area of the rat skin for 4 hours. Frozen sections of skin tissues were immunostained with goat anti-HO-1 immunoglobulin G (IgG) followed by staining with rhodamine linked mouse anti-goat IgG, as described in the methods section. Fluorescence signals were obtained under the same conditions by immunostaining. HO-1 - hemo oxygenase-1

Discussion. Protein transduction domains are strings of amino acids rich in arginine and lysine residues. They can convey molecules across the barrier of the cytoplasmic membrane and are promising devices for improving the delivery of large molecules. Since the discovery of the first PTD HIV-TAT, over 30 kinds of natural and synthetic PTDs have been reported.[31] These PTDs have been widely used powerful tools for protein delivery. The HIV-TAT PTD does not enhance the immunogenicity of a full-length recombinant protein.[32] Here, using the classical HIV-TAT PTD, we expressed and purified TAT-HO-1 recombinant protein. This fusion protein mediated the incorporation of HO-1 into HaCaT cells. The TAT-HO-1 decreased cell radiosensitivity to irradiation. Moreover, HaCaT cells pretreated with TAT-HO-1 resulted in less apoptosis by radiation. In addition, TAT-HO-1 can penetrate rat skin. These results suggest that TAT-HO-1 has shown its potential as protein therapy for radiation-induced skin injury.

The HO-1 has been assumed to be important in the cellular response against oxidative stress through modification of the pro-oxidant heme into less toxic catabolites that behave as antioxidants.[15,17] Our results indicate that TAT-HO-1 can decrease radiation-induced ROS in the cultured HaCaT cell, which is consistent with a previous report that HO-1 has been demonstrated to attenuate cellular ROS accumulation.[29] The protective role of HO-1 has been well documented for a variety of oxidative tissue injuries, such as hepatic fibrosis,[33] renal ischemia-reperfusion injury,[34] allograft and xenograft survival,[35] and type 1 diabetes.[36] Previously, we have demonstrated that adenovirus mediated HO-1 overexpression can ameliorate radiation-induced skin injury.[24] Overexpression of HO-1 in rat skin decreased lipid peroxidation and inhibited the induction of ROS scavenging proteins.[24] Moreover, HO-1 exerted an anti-apoptotic effect by suppressing apoptosis stimulating fragment (FAS) and FAS-ligand expression.[24] The currently used delivery systems for HO-1 gene transfer including viral vectors and plasmids, have their own limitations, including immune response and unstable levels of expression.[37] The possible advantage for TAT-HO-1 is that PTD can mediate protein internalization into cells indiscriminately and its required dose can be exactly controlled therefore resulting in decreased side effects. Moreover, TAT-HO-1 has exhibited a protective role after 4 hours pretreatment. This indicated that PTD-based delivery provided a reliable therapeutic approach, which was much faster than most gene transfer-based therapy. Consistent with adenovirus-based HO-1 overexpression, administration of TAT-HO-1 decreased apoptosis for HaCaT cells. The PTD-based approach has provided a promising alternative for therapy of radiation-induced injury.

Besides its transmembrane ability for cells, HIV-TAT has also shown its ability for skin penetration.[38,39] Here, we showed that 4 hours after topical use of TAT-HO-1, obvious increased HO-1 expression could be detected in both the epidermis and dermis of rat skin. The TAT-HO-1 fusion protein can be detected 3 days after it has been administrated (data not shown). The human epidermis is composed of 4 or 5 layers depending on the region of skin. The stratum corneum is the outermost layer of the epidermis, which accounts for most of the barrier functions of the epidermis. The stratum corneum blocks the penetration of extraneous molecules as a self-protection mechanism. Exploration of a skin penetration PTD is promising because it directly contributes to the protein therapy of skin-related diseases. Administration of drugs through the skin is hampered mainly due to the limitation of molecule weight. Proteins, whose molecule weights are usually above 500 Da can hardly be transdermally delivered.[40,41] Our results indicated that TAT can mediate the internalization of heterogeneous HO-1. We postulate that the TAT PTD is also able to facilitate other proteins through the hurdle of stratum corneum.

Study limitations. A limitation of this study is that the mechanism of skin cell and tissue penetration of TAT-HO1 remains elusive. Once we have illustrated its mechanism, we can improve the efficacy of PTDs. Moreover, animal models with radiation-induced skin injuries are warranted to confirm the protective role of TAT-HO1 in vivo.

In summary, we expressed and purified TAT-HO-1 recombinant protein, which can be incorporated into human keratinocyte-derived HaCaT cells. The TAT-HO-1 decreased cell radiosensitivity to irradiation. Moreover, the HaCaT cells pretreated with TAT-HO-1 resulted in less ROS and apoptosis by radiation. In addition, TAT-HO-1 can penetrate rat skin. These results suggest that TAT-HO-1 can protect HaCaT cells from ionizing radiation.

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