Free Radical Biology & Medicine 48 (2010) 969–977

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

# Transduced Tat–SAG fusion protein protects against oxidative stress and brain ischemic insult

Dae Won Kim [a], Sun Hwa Lee [a], Min Seop Jeong [a], Eun Jeong Sohn [a], Mi Jin Kim [a], Hoon Jae Jeong [a], Jae Jin An [a], Sang Ho Jang [a], Moo Ho Won [b], In Koo Hwang [c], Sung-Woo Cho [d], Tae-Cheon Kang [b], Kil Soo Lee [a], Jinseu Park [a], Ki-Yeon Yoo [b,*], Won Sik Eum [a,*], Soo Young Choi [a,*]

[a] Department of Biomedical Science, Research Institute for Bioscience and Biotechnology, and Medical & Bio-material Research Center, Hallym University, Chunchon 200-702, Korea
[b] Department of Anatomy & Neurobiology, College of Medicine, Hallym University, Chunchon 200-702, Korea
[c] Department of Anatomy and Cell Biology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea
[d] Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, Seoul 138-736, Korea

ARTICLE INFO

*Article history:*
Received 14 September 2009
Revised 9 January 2010
Accepted 14 January 2010
Available online 25 January 2010

*Keywords:*
Cell viability
Ischemic damage
Oxidative stress
Protein transduction
Sensitive to apoptosis gene
SAG
Free radicals

ABSTRACT

Reactive oxygen species (ROS) have been implicated in the pathogenesis of ischemic brain injury. Sensitive to apoptosis gene (SAG) is a RING-finger protein that exhibits antioxidant activity against a variety of redox reagents. However, the protective effect of SAG in brain ischemic injury is unclear. Here, we investigated the protective effects of a Tat–SAG fusion protein against cell death and ischemic insult. When Tat–SAG fusion protein was added to the culture medium of astrocytes, it rapidly entered the cells and protected them against oxidative stress-induced cell death. Immunohistochemical analysis revealed that, when Tat–SAG fusion protein was intraperitoneally injected into gerbils, wild-type Tat–SAG prevented neuronal cell death in the CA1 region of the hippocampus in response to transient forebrain ischemia. In addition, wild-type Tat–SAG fusion protein decreased lipid peroxidation in the brain compared with mutant Tat–SAG- or vehicle-treated animals. Our results demonstrate that Tat–SAG fusion protein is a tool for the treatment of ischemic insult and it can be used in protein therapy for various disorders related to ROS, including stroke.



Reactive oxygen species (ROS) are formed as inevitable by-products of various, normal cellular processes involving interactions with oxygen. Constant exposure to the harmful actions of ROS damages macromolecules and ultimately, ROS contribute significantly to the pathological processes of various human diseases [1,2]. ROS participate in a wide variety of cellular functions, and in pathological conditions they can thus induce cell death. A significant induction of ROS or a depletion of cellular antioxidants also induces cell death [3–6]. The interruption and reperfusion of blood initially induces an enormous increase in ROS in the hippocampal CA1 region and finally induces neuronal cell death [7–9].

Sensitive to apoptosis gene (SAG) is a zinc RING-finger protein that has been shown to be involved in protection against apoptotic cell death induced by oxidative stress in cells [10–12]. SAG is localized in both the cytoplasm and the nucleus of cells and consists of 113 amino acids including 12 cysteine residues, with a molecular mass of 12.6 kDa [11]. SAG is ubiquitously expressed in many organs and tissues, but predominantly in the heart, skeletal muscle, brain, and gonads [13,14]. Though several studies have shown that SAG plays an antioxidant role in cells as a metal chelator and ROS scavenger [10,11,14–16], the exact role of SAG in brain ischemic injury remains unclear.

To date, many researchers have demonstrated the successful delivery of full-length Tat fusion proteins by protein transduction technology. Several small regions of proteins, called protein transduction domains (PTDs), have been developed to allow the delivery of exogenous protein into living cells [17]. In a previous study, we successfully transduced Tat–SOD directly into various cell lines, including pancreatic islet cells, and found that transduced Tat–SOD increased radical scavenger activity [18]. In addition, we reported that various transduced fusion proteins in vitro and in vivo efficiently protected against cell death [19–26].

In this study, we designed a Tat–SAG fusion protein by genetic in-frame cloning, for direct transduction in vitro and in vivo. Our results demonstrate that Tat–SAG fusion protein efficiently transduced and protected against cell death in vitro and in vivo. Therefore, we suggest that Tat–SAG fusion protein could be useful as a potential therapeutic agent for transient forebrain ischemia.

* Corresponding authors. Fax: +82 33 241 1463.
*E-mail addresses:* kyyoo@hallym.ac.kr (K.-Y. Yoo), wseum@hallym.ac.kr (W.S. Eum), sychoi@hallym.ac.kr (S.Y. Choi).

0891-5849/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2010.01.023

## Materials and methods

### Materials

Restriction endonuclease and T4 DNA ligase were purchased from Promega (Madison, WI, USA). Oligonucleotides were synthesized from Gibco BRL custom primers (Grand Island, NY, USA). $Ni^{2+}$-nitrilotriacetic acid Sepharose Superflow was purchased from Qiagen (Valencia, CA, USA). Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Duchefa Co. (Haarlem, Netherlands). Plasmid pET-15b and *Escherichia coli* strain BL21 (DE3) were obtained from Novagen (Hilden, Germany). A human SAG cDNA fragment was isolated using the polymerase chain reaction (PCR) technique. Primary antibodies against histidine (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were obtained commercially. All other chemicals and reagents were of the highest analytical grade available.

### Expression and purification of Tat–SAG fusion proteins

An HIV-1 Tat expression vector was prepared in our laboratory as described previously [27]. A synthesized oligonucleotide was ligated into a *Nde*I–*Xho*I-digested pET-15b vector. Next, on the basis of the cDNA sequence of human SAG, two primers were synthesized. The sense primer was 5′-CTCGAGATGGCCGACGTGGAAGACGGA-GAG-3′, containing an *Xho*I restriction site, and the antisense primer, 5′-GGATCCTCATTTGCCGATTCTTTGGACCAC-3′, contained a *Bam*HI restriction site. PCR was performed and the PCR products were purified by preparative agarose gel electrophoresis. The purified products were ligated into a TA cloning vector and then transformed into a competent cell. The plasmids of selected colonies were purified using a Plasmid Mini Kit (Qiagen). The purified TA vector containing human SAG cDNA was digested with *Xho*I and *Bam*HI. The SAG was then ligated into the expression vector, Tat, in-frame with six histidine open-reading frames to generate the expression vector, and cloned into *E. coli* DH5α cells.

To produce the Tat–SAG fusion protein, the plasmid was transformed into *E. coli* BL21 (DE3) cells. The transformed bacterial cells were grown in 100 ml of LB medium at 37°C to a $D_{600}$ value of 0.5–1.0 and induced with 0.5 mM IPTG at 37°C for 4 h. Harvested cells were lysed by sonication at 4°C in a binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9) containing 6 M urea. After centrifugation, supernatants containing Tat–SAG were immediately loaded onto a 2.0-ml $Ni^{2+}$-nitrilotriacetic acid Sepharose column (Qiagen). After the column was washed with 10 volumes of binding buffer and 6 volumes of washing buffer (35 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9), the fusion protein was eluted with an elution buffer (0.5 M imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9). The salts in the purified fractions were removed by PD10 column chromatography (Amersham, Braunschweig, Germany). The protein concentrations were estimated by the Bradford procedure using bovine serum albumin as a standard [28].

### Construction, expression, and purification of Tat–SAG mutant fusion proteins

The construction, expression, and purification of SAG mutant protein were carried out as described previously [16]. A two-step PCR was performed to generate a single point mutation in wild-type SAG cDNA cloned with the pTat-SAG vector as the template. The primers used for generation of the SAG mutant were as follows: T7 promoter sense, 5′-TAATACGACTCACTATAGGG-3′; SAG mutant antisense, 5′-CCTGCTGGCTGAGAGGGC-3′; T7 terminator antisense, 5′-GCTAGTTATTGCTCAGCGG-3′; SAG mutant sense, 5′-GCCCTCTCAG-CCAGCAGG-3′. In the first step, two separate PCRs were performed using each of the sense and antisense primers. The PCR products containing a desired point mutation were mixed and served as templates for the second-step PCR. For the second step of the PCR, we used T7 promoter and T7 terminator primers. The PCR processes were performed with *Pfu* DNA polymerase premix according to the manufacturer's instructions (Intron Biotechnology, Korea). The PCR products were excised with *Xho*I and *Bam*HI restriction enzymes, extracted, and ligated into a pTat vector. The SAG mutant generated was verified by DNA sequencing. The SAG mutant contains two cysteine-to-serine mutations at codons 99 and 102 that abolish the second RING motif. SAG mutant protein was expressed and purified as detailed earlier.

### Transduction of Tat–SAG fusion protein into astrocyte cells

The astrocytes were cultured in Dulbecco's modified Eagle's medium containing 20 mM Hepes/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% fetal bovine serum, and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37°C under humidified conditions of 95% air and 5% $CO_2$. For the transduction of wild-type and mutant Tat–SAG, astrocytes were grown to confluence on a six-well plate. Then the culture medium was replaced with 1 ml of fresh solution. After the cells were treated with various concentrations of wild-type and mutant Tat–SAG for 1 h, the cells were treated with trypsin–EDTA (Gibco) and washed with phosphate-buffered saline (PBS). The cells were harvested for the preparation of cell extracts to perform Western blot analysis.

### Fluorescence analysis

For direct detection of fluorescein-labeled protein, purified wild-type and mutant Tat–SAG was labeled according to the manufacturer's instructions using an EZ-Label fluorescein isothiocyanate (FITC) protein labeling kit (Pierce, Rockford, IL, USA). Cultured cells were grown on glass coverslips and treated with 3 μM wild-type or mutant Tat-SAG fusion protein. After incubation for 1 h at 37°C, the cells were washed twice with PBS and trypsin–EDTA. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature. The distribution of fluorescence was analyzed using a confocal laser-scanning system (Bio-Rad MRC-1024ES).

### MTT assay

The biological activity of the transduced wild-type and mutant Tat–SAG fusion protein was assessed by measuring the cell viability of astrocytes treated with hydrogen peroxide. The cells were seeded into six-well plates at 70% confluence. The cells were pretreated with 3 μM wild-type or mutant Tat–SAG for 1 h, then hydrogen peroxide (0.7 mM) was added to the culture medium for 12 h. Cell viability was estimated by a colorimetric assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

### Measurement of intracellular ROS levels

Intracellular ROS levels were determined using DCF-DA, which is converted by ROS into fluorescent DCF. To investigate the effects of wild-type and mutant Tat–SAG on ROS production, astrocytes were incubated in the absence or presence of wild-type or mutant Tat–SAG for 1 h and then treated with hydrogen peroxide (100 μM) for 30 min. The cells were washed twice with PBS and incubated with DCF-DA (10 μM) for 30 min. The cellular fluorescence images were obtained using a Zeiss Axiovert S100 microscope with a confocal laser-scanning system (Bio-Rad MRC-1024ES) using laser excitation at 494 nm as described in [29,30]. Under the same experimental conditions, the fluorescence intensity quantification was measured at 485 nm excitation and 538 nm emission by a Fluoroskan ELISA plate reader (Labsystems Oy).