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Regulation of nitric oxide-induced apoptosis by sensitive to apoptosis gene protein

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Abstract
Sensitive to apoptosis gene (SAG) protein, a novel zinc RING finger protein that protects mammalian cells from apoptosis by redox reagents, is a metal chelator and a potential reactive oxygen species (ROS) scavenger, but its antioxidant properties have not been completely defined. Nitric oxide (NO), a radical species produced by many types of cells, is known to play a critical role in many regulatory processes, yet it may also participate in collateral reactions at higher concentrations, leading to cellular oxidative stress. In this report, we demonstrate that modulation of SAG expression in U937 cells regulates NO-induced apoptosis. When we examined the protective role of SAG against NO-induced apoptosis with U937 cells transfected with the cDNA for SAG, a clear inverse relationship was observed between the amount of SAG expressed in target cells and their susceptibility to apoptosis. We also observed the significant decrease in the endogenous production of ROS and oxidative DNA damage in SAG-overexpressed cells compared to control cells upon exposure to NO. These results suggest that SAG plays an important protective role in NO-induced apoptosis, presumably, through regulating the cellular redox status.

Keywords: *SAG, nitric oxide, U937, apoptosis, antioxidant protein*

Introduction

Nitric oxide (NO) is an endogenous short-lived free radical formed in a variety of mammalian cells including endothelium, neuronal cells, smooth muscle cells, macrophages, neutophiles, platelets, fibroblasts and type II pneumocytes by the enzymatic oxidation of L-arginine to citrulline and is implicated in diverse physiological processes including vasorelaxation, platelet inhibition, macrophage-mediated cytotoxicity and neurotransmission [1,2]. However, an excessive amount of NO causes nitrosative stress on surrounding tissues. It has been also suggested that relatively high concentrations of NO induce an oxidative stress [3–7] and apoptosis [8–10] in cells, presumably, through several different mechanisms, such as depletion of GSH, inhibition of antioxidant enzymes, inhibition of mitochondrial respiration and formation of peroxynitrite.

Sensitive to apoptosis gene (SAG) protein is a novel, evolutionally conserved, zinc RING finger protein that protects cells from apoptosis induced by redox reagents [11]. In human tissues, SAG is ubiquitously expressed at high levels in skeletal muscles, heart and testis. SAG is localized in both the cytoplasm and the nucleus of cells. SAG encodes a protein that consists of 113 amino acids including 12 cysteine residues with a molecular weight of 12.6 kDa [11]. Several biological roles of SAG have been suggested [11–14]. Besides being involved in the protection of cells from apoptosis induced by oxidative stress, other biological functions have been considered. For instance, it has been proposed that on accounts of its

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high cysteine content, SAG might play an antioxidant role in the cell through a metal chelator or reactive oxygen species (ROS) scavenger [11,12,15].

In the present study, the role of SAG in NO-induced oxidative stress and apoptosis was investigated using the U937 cell transfected with the SAG cDNA. The results suggest that SAG has an important protective role in NO-induced apoptosis, presumably, through acting as an antioxidant protein or a regulatory protein for the antioxidant defense mechanism.

Materials and methods

Materials

S-Nitroso-N-acetylpenicillamine (SNAP), propidium iodide (PI), 4′,6-diamidino-2-phenylindole (DAPI) and anti-rabbit IgG tetramethylrhodamine isothiocyanate (TRITC) conjugated secondary antibody were purchased from Sigma Chemical Company (St Louis, MO). 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and dihydrorhodamine (DHR) 123 dye were purchased from Molecular Probes (Eugene, OR). Antibodies against Bcl-2, Bax, lamin B, cleaved caspase-3, and cleaved poly(ADP-ribose) polymerase (PARP) were purchased from Santa Cruz (Santa Cruz, CA). Anti-SAG IgG was purchased from Abcam (Cambridge, MA).

Cell culture

Human premonocytic U937 cells (American Type Culture Collection, Rockville, MD) were transfected with the pcDNA-SAG by Lipofectamine 2000 (Invitrogen) as described by the manufacturer. The U937 cell line transfected with vector alone was used as a control. Cells were grown in RPMI 1640 culture medium supplemented with 10% (v/v) FBS, penicillin (50 units/ml) and 50 μg/ml streptomycin at 37°C in a 5% CO_2/95% air humidified incubator.

RT-PCR analysis of SAG

RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instruction. Total RNA (1 μg) were reverse transcribed into cDNA using a first strand cDNA synthesis kit (Invirogen) according to the manufacturer's protocol. The cDNA template was then amplified by quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using a Perkin–Elmer GemeAmp PCR System 2400 (Perkin–Elmer Cetus, Emeryville, CA) according to the manufacturer's protocol. The primers for SAG cDNA amplification were: forward primer, 5′-GTG ATG GAT GCC TGT CTT AGA T; and reverse primer, 5′-TCA TTT GCC GAT TCT TTG GAC C. The primers for β-actin cDNA amplification were: forward primer, 5′-TCT ACA ATG AGC TGC GTG TG; and reverse primer, 5′-ATC TCC TTC TGC ATC CTG TC. β-Actin gene was used as an internal control for relative mRNA amount and results were calculated after standardization on β-actin mRNA content. The single-stranded cDNA obtained from the reverse transcription reaction was then used as a template for PCR amplification. The amplified DNA products were resolved on a 1.5% nondenaturing agarose gel, which was stained with ethidium bromide.

Immunoblot analysis

Proteins were separated on 10–12.5% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and subsequently subjected to immunoblot analysis using appropriate antibodies. Immunoreactive antigen was then recognized by using horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

DAPI staining

DAPI staining was used for apoptotic nuclei determination. U937 cells were collected at 2000*g* for 5 min, washed once with cold PBS, fixed in ice-cold methanol/acetic acid (1:1, v/v) for 5 min, and stained with 0.8 μg/ml DAPI in the dark state [16]. The morphological changes of apoptotic cells were analyzed by the Zeiss Axiovert 200 microscope at fluorescence DAPI region (excitation, 351 nm; emission, 380 nm).

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To determine the portion of apoptotic cells, cells were analyzed with PI staining [17]. U937 cells were collected at 2000*g* for 5 min and washed once with cold PBS, fixed in 70% ethanol, decant ethanol by centrifuge and stained with 1 ml of solution containing 50 mg/ml PI, 1 mg/ml RNase A, 1.5% Triton X-100 for at least 1 h in the dark at 4°C. Labeled nuclei were subjected to flow cytometric analysis and then gated on light scatter to remove debris, and the percentage of nuclei with a sub-G_1 content was considered apoptotic cells.

Cellular redox status

Intracellular ROS production was measured using the oxidant-sensitive fluorescent probe DCFH-DA with confocal microscopy. Cells were grown at 2×10^6 cells/100 mm plate containing slide glass coated with poly-L-lysine and maintained in the growth medium for 24 h. Cells were treated with 10 μM DCFH-DA for 15 min and exposed to SNAP. Cells on

the slide glass were washed with PBS and a cover glass was put on the slide glass. DCF fluorescence (excitation, 488 nm; emission, 520 nm) was imaged on a laser confocal scanning microscope (DM/R-TCS, Leica) coupled to a microscope (Leitz DM REB) [18]. To evaluate the levels of mitochondrial ROS U937 cells in PBS were incubated for 20 min at 37°C with 5 μM DHR 123 and cells were washed, resuspended in complete growth media, and 0.2 mM SNAP was applied to the cells. The cells were then incubated for an additional 40 min. FACS was used for fluorescence intensity quantification.

Oxidative DNA damage

8-Hydroxy-2′-deoxyguanosine (8-OH-dG) levels of U937 cells were estimated by using a fluorescent binding assay as described by Struthers et al. [19]. After U937 cells were exposed to SNAP, cells were fixed and permeabilized with ice-cold methanol for 15 min. DNA damage was visualized with avidin-conjugated TRITC (1:200 dilution) for fluorescent microscope with 540 nm excitation and 588 nm emission.

Caspase activity assay

For colorimetric assay for caspase activity, cells were collected with a cell scraper, washed with PBS, mixed with lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT and 0.1 mM EDTA) for 10 min at 0°C, and centrifuged at 10,000*g* for 10 min at 4°C. Supernatant containing 100 μg protein was added to reaction buffer (100 mM HEPES, pH 7.4, 0.5 mM PMSF, 10 mM dithiothreitol, 1 mM EDTA and 10% glycerol) containing Ac-DEVD-pNA (Calbiochem, San Diego, CA) and incubated for 2 h at 37°C. The absorbance was then monitored at 405 nm to determine the caspase activity.

Quantitation of relative fluorescence

The averages of fluorescence intensity from fluorescence images were calculated as described [20].

Replicates

Unless otherwise indicated, each result described in the paper is representative of at least three separate experiments.

Results

To study the relationship between SAG expression and NO-induced apoptotic cell death, the two kinds of U937 transfectant cells were constructed. The U937 cells were transfected with the pcDNA containing either a SAG gene as a sense orientation, SAG(+), or the vector alone, control. Both SAG mRNA level measured by RT-PCR and SAG protein level measured by Western blotting were increased in SAG(+) cells compared to control cells (Figure 1).

Exposure of U937 cells to 0.2 mM SNAP for 18 h caused shrinkage of the cell and plasma membrane blebbing that was apparent by light microscopy (data not shown). To assess whether these changes were attributable to apoptotic changes, nuclear morphology was assessed by fluorescence microscopy using DAPI and flow cytometry using PI. As shown in Figure 2, nuclear condensation and fragmentation were apparent in control cells treated with SNAP when compared to SAG(+) cells. Figure 3 shows a typical cell cycle plot of U937 transfectant cells that were untreated or treated with 0.2 mM SNAP for 18 h. Apoptotic cells were estimated by calculating the number of subdiploid cells in the cell cycle histogram. When cells were exposed to SNAP, apoptotic cells were increased markedly in control cells (15.63%) as compared to SAG(+) cells (9.3%).

To investigate the role of SAG in cellular defense against NO-induced oxidative stress, we determined the cellular redox status in U937 transfectants unexposed or exposed to SNAP. The levels of intracellular ROS in U937 cells were evaluated with an oxidant-sensitive probe DCFH-DA. As shown in Figure 4a, an increase in DCF fluorescence was observed in U937 cells when they were exposed to SNAP. The increase in fluorescence was significantly reduced in SAG(+) cells. The levels of intracellular ROS in the mitochondria of U937 cells were evaluated by confocal microscopy with the oxidant-sensitive probe DHR 123. As shown in Figure 4b, the intensity of fluorescence was significantly lower in SAG(+) cells (six-fold) when compared to that in the mitochondria of control cells (3.2-fold) upon exposure to 0.2 mM SNAP. 8-OH-dG, the most abundant and most studied lesion in DNA generated by intracellular ROS, has been used as an indicator of oxidative DNA damage *in vivo* and *in vitro* [21]. Recently, it has been shown that 8-OH-dG level is specifically measured by a fluorescent binding assay using avidin-conjugated TRITC [19]. The fluorescent intensity which reflects the endogenous levels of 8-OH-dG in DNA was significantly increased in

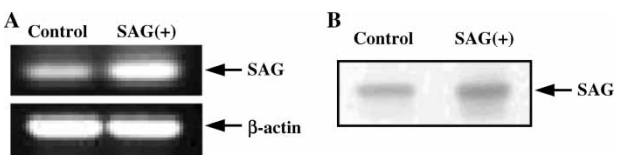

Figure 1. (a) RT-PCR analysis of SAG in U937 transfectant cells. Total RNA was isolated from U937 transfectant cells and SAG mRNA levels were determined by RT-PCR and analyzed on agarose gel electrophoresis. β-Actin from the same samples was amplified as control; (b) immunoblot analysis of SAG protein expressed in U937 transfectant cells. Cell extracts were subjected to 12.5% SDS-PAGE and immunoblotted with anti-SAG IgG.

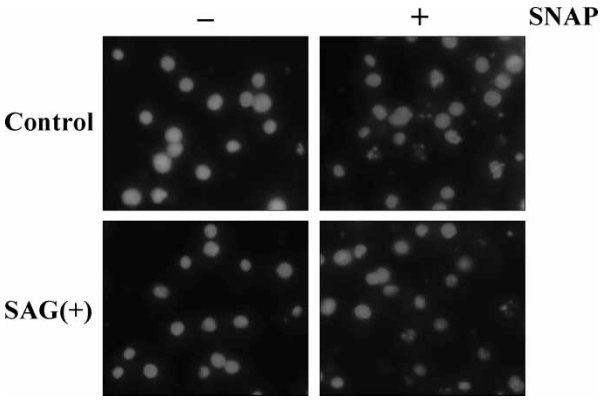

Figure 2. NO-induced nuclear condensation and fragmentation in SAG transfectant cells. SAG transfectant U937 cells were exposed to 0.2 mM SNAP for 18 h, and then harvested, fixed, permeabilized and loaded with 0.8 μg/ml DAPI for 5 min. The morphological changes of cells were analyzed by fluorescence microscopy (excitation, 351 nm; emission, 380 nm).

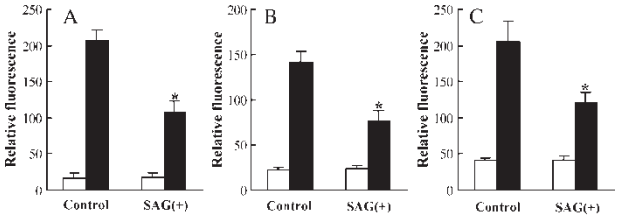

Figure 4. (a) Measurement of *in vivo* molecular oxidation. DCF fluorescence was measured in U937 transfectant cells exposed to SNAP. Fluorescence images were obtained under laser confocal microscopy; (b) effect of SAG protein expression on mitochondrial ROS generation. DHR 123 was employed to detect mitochondrial ROS. Fluorescence images were obtained under microscopy; (c) 8-OH-dG levels in U937 cells. The cells were fixed and permeabilized immediately after exposure to SNAP. 8-OH-dG levels reflected by the binding of avidin-TRITC were visualized by a fluorescence microscope. The averages of fluorescence intensity were calculated as described [20]. The results shown are the means ± SD of five separate experiments. Open and shaded bars represent U937 cells unexposed and exposed to SNAP, respectively. $\star p < 0.01$ compared to control cells exposed to SNAP.

control cells upon exposure to SNAP. In contrast, the overall DNA appeared to be markedly protected in SAG(+) cells even after exposure to the same dose of SNAP. These results indicate that SAG appears to protect cells from oxidative DNA damage caused by NO.

We evaluated changes in the apoptotic marker proteins as a result of treatment with SNAP and the influence of SAG expression levels. Caspase-3 activation in U937 cells was assessed by caspase colorimetric assay and by immunoblot analysis of lysates from cells that had been exposed to 0.2 mM SNAP. Caspase-3 activity increased significantly higher in control cells than SAG(+) cells upon exposure to SNAP (Figure 5a). As shown in Figure 5b, SNAP induced cleavage of caspase-3, however, the cleavage was significantly higher in control cells. Induction of the formation of fragments which represents proteolytic cleavage of PARP and lamin B, indicates an oncoming apoptotic process. The cleaved products of PARP and lamin B increased markedly in control cells compared to SAG(+) cells upon exposure to SNAP. Taken together, NO-induced cleavage of procaspase-3 into the active form of caspase-3 and caspase-3 induces degradation of PARP or lamin B. The results also indicate that SAG exhibits a protective effect on the NO-induced apoptosis. The role of mitochondrial pathway of apoptosis in the NO-induced death of U937 cells were examined by immunoblot analysis of the abundance of Bcl-2, an antiapoptotic protein, and of Bax, an proapoptotic protein. As shown in Figure 5b, the abundance of

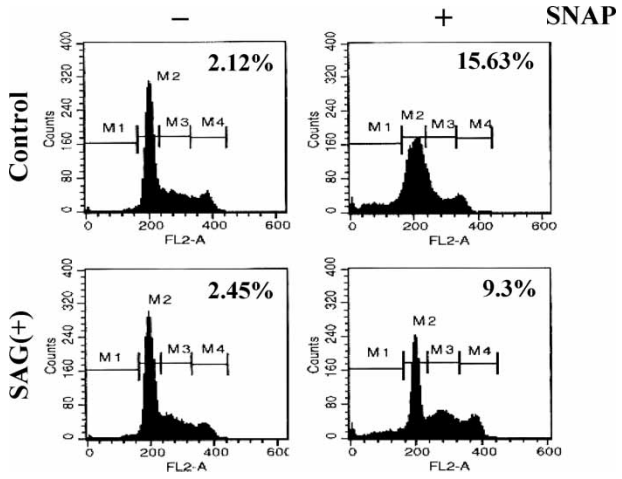

Figure 3. NO-induced apoptosis in SAG transfectant cells. Cell cycle analysis with cellular DNA content was examined by flow cytometry. The sub-G_1 region (presented as 'M1') includes cells undergoing apoptosis. The number of each panel refers to the percentage of apoptotic cells.

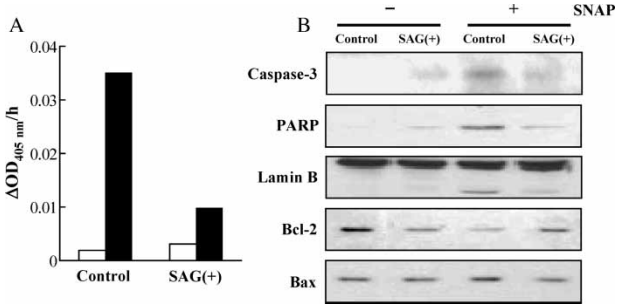

Figure 5. (a) Activation of caspase-3 in U937 transfectant cells exposed to SNAP. Cells were lysed and centrifuged. The supernatant was then added to Ac-DEVD-pNA and subjected to caspase colorimetric activity. Protease activity of caspase-3 was calculated by monitoring the absorbance at 405 nm. Open and shaded bars represent U937 cells unexposed and exposed to SNAP, respectively; (b) immunoblot analysis of various apoptosis-related proteins in U937 transfectant cells untreated or treated with 0.2 mM SNAP for 18 h. Cell extracts were subjected to 10–12.5% SDS-PAGE and immunoblotted with antibodies against cleaved caspase-3, cleaved PARP, lamin B, Bcl-2 and Bax.

Bcl-2 in U937 cells was significantly decreased in control cells as compared to that of SAG(+) cells when exposed to 0.2 mM SNAP. The increase of Bax was more pronounced in control cells as compared to that of SAG(+) cells upon exposure to SNAP.

Discussion

SAG is a cysteine-rich, metal-binding protein that is found in a wide range of organisms from yeast to human [8]. SAG is evolutionally conserved, with a 55 or 70% identity, respectively, between human and yeast or human and *Caenorhabditis elegans* sequences. Although, SAG is thought to serve as an important line of defense against oxidative stress-induced damage and redox-induced apoptosis, the biological roles of SAG have not been completely elucidated. SAG could be a growth-essential gene by functioning as an antioxidant molecule to protect cells against ROS-induced death or by promoting cell growth through other mechanisms [11]. Because of its peculiar structure, characterized by a large content of thiol groups, SAG is endowed with strong antioxidant and nucleophilic properties. Based on the prevention of LDL oxidation by copper ion and 2,2-azo-bis-2-amidinopropane hydrochloride (AAPH), a free radical generator, it has been suggested that SAG plays a role as a metal, especially Cu, chelator or free radical scavenger through use of its sulfhydryl groups [11,12]. SAG contains neither heme nor a flavin prosthetic group and it does not possess any activity of known antioxidant enzymes including catalase, superoxide dismutase and glutathione peroxidase. It has also been proposed that sulfhydryl cysteines in SAG could function as strong nucleophiles to destroy reactive nitrogen species such as peroxynitrite [22]. It remains to be studied and thus determined whether or not cysteines of SAG are involved in the protection against oxidative stress as a non-specific way or if any specific cysteines are involved in the catalysis. We reported that SAG exhibits thiol-linked peroxidase activity [15]. Therefore, it is possible that SAG acts in a way similar to peroxiredoxins. Alternatively, SAG indirectly affects the cellular redox status by regulating antioxidant defenses by other, yet unknown, mechanisms. The *in vivo* mechanism of SAG to maintain the cellular redox status should be studied further.

In the present study, we examined the apoptotic pathway initiated by NO in U937 transfectant cells. A temporal pattern of events was observed, starting from perturbation of redox status reflected by the modulation of intracellular ROS generation, followed by caspase-3 activation, cleavage of caspase target proteins, and finally DNA fragmentation. The SAG expression significantly improved redox status and inhibited the whole apoptotic pathway.

To demonstrate the NO-induced intracellular oxidation, we evaluated intracellular oxidants after exposure to SNAP. We noted that SNAP caused an increase in intracellular oxidants in U937 cells. However, the increase in intracellular oxidants was significantly higher in control cells than in SAG(+) cells. These data suggest that SAG protects cells from the cytotoxic actions of NO by decreasing the steady-state level of intracellular oxidants. Intracellular oxidants may induce the damage to DNA, which results in cellular damage. Cells have the lower level of SAG show a significant increase in oxidative damage to DNA, emphasizing the essential protective role of SAG in mitigating such damage.

The susceptibility to NO-induced apoptosis in U937 cells was significantly decreased in SAG(+) cells compared to that of control cells. Cleavage of caspase-3 and its target proteins such as PARP and lamin B, a signature event of apoptosis, was induced by SNAP. In the meantime, the Bcl-2 protein is a suppressor of apoptosis that homodimerizes with itself and forms heterodimers with a homologous protein Bax, a promoter of cell death [23]. Overexpression of SAG suppressed programmed cell death by decreasing apoptotic features including caspase activation, increasing anti-apoptotic molecules (Bcl-2), and decreasing pro-apoptotic molecules (Bax), presumably via modulation of redox status.

In conclusion, the present study demonstrates that SAG abrogates the NO-induced early production of ROS, leading to protection against apoptotic cell death.

Acknowledgements

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