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Original Contribution

Regulation of heat shock-induced apoptosis by sensitive to apoptosis gene protein

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ABSTRACT

Heat shock may increase oxidative stress due to increased production of reactive oxygen species and/or the promotion of cellular oxidation events. 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 scavenger, but its antioxidant properties have not been completely defined. In this report, we demonstrate that modulation of SAG expression in U937 cells regulates heat shock-induced apoptosis. When we examined the protective role of SAG against heat shock-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 a significant decrease in the endogenous production of reactive oxygen species and oxidative DNA damage in SAG-overexpressed cells compared to control cells on exposure to heat shock. In addition, transfection of PC3 cells with SAG small interfering RNA markedly decreased the expression of SAG, enhancing the susceptibility of heat shock-induced apoptosis. Taken together, these results indicate that SAG may play an important role in regulating the apoptosis induced by heat shock presumably through maintaining the cellular redox status.

Introduction

Most living cells have an essential, highly conserved, and exquisitely regulated cellular response to sudden heat exposure. Moderately elevated temperature induces the selective synthesis of a small number of highly conserved proteins [1]. These are referred to as heat shock proteins (Hsps), and they appear to impart resistance toward elevated temperatures [2]. Hsps also can be induced by a variety of oxidizing agents including hydrogen peroxide and menadione, as well as by radiation, whose cytotoxicity is thought to be primarily due to the generated reactive oxygen species (ROS) [3–6]. Exposure to oxygen induces heat shock proteins in *Drosophila* [7], Chinese hamster ovary cells [8], and liver [9]. In contrast, heat shock induced superoxide dismutase (SOD) in mammalian cells [10] and manganese SOD (MnSOD) in *Escherichia coli* [11]. In addition, the fact that the deletion of the genes which encode the antioxidant enzymes results in sensitization of cells to heat shock stress and the overexpression of catalase and SOD genes caused an increase in thermotolerance [12] suggests that heat and oxidative stress have common cellular effects.

ROS such as superoxide anion ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) are generated in vivo from the incomplete reaction of oxygen during aerobic metabolism or from exposure to environmental agents such as radiation, redox cycling agents, or stimulated host phagocytes [3,13]. These oxygen species can cause widespread damage to biological macromolecules leading to lipid peroxidation, protein oxidation, and DNA base modifications and strand breaks [3], which may induce cell death including apoptosis. Since ROS appear to be mediators of the cellular damage induced by heat shock, antioxidant proteins that regulate the fate of such species may play a role in the protection of cells against heat shock-induced apoptosis.

Sensitive to apoptosis gene (SAG) protein is a novel, evolutionally conserved, zinc RING finger protein that protects cells from apoptosis induced by redox reagents [14]. 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 [14]. Several biological roles of SAG have been suggested [14–17]. 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 account of its high cysteine content, SAG might play an antioxidant role in the cell through a metal chelator or ROS scavenger [14,15,18].

Abbreviations: BSO, L-buthionine-(*S*,*R*)-sulfoximine; CMAC, t-butoxycarbonyl-Leu-Met-7-amino-4-chloromethylcoumarin; DAPI, 4,6-diamidino-2-phenylindole; DCF, 2′,7′-dichlorofluoroscein; DCFH-DA, 2′,7′-dichlorofluoroscein diacetate; DHR, dihydrorhodamine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FITC, fluorescein isothiocyanate; GSH, reduced glutathione; GSSG, oxidized glutathione; Hsp, heat shock protein; 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; IDPm, mitochondrial $NADP^+$-dependent isocitrate dehydrogenase; MnSOD, manganese SOD; MPT, membrane permeability transition; MB, methylene blue; NAC, *N*-acetyl-L-cysteine; NEM, *N*-ethylmaleimide, PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SAG, sensitive to apoptosis gene; siRNA, small interfering RNA; SOD, superoxide dismutase.

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In the present study the role of SAG in heat shock-induced oxidative stress and apoptosis was investigated using U937 cells transfected with the SAG cDNA and PC3 cells transfected with small interfering RNA (siRNA). The results suggest that SAG has an important protective role in heat shock-induced apoptosis, presumably, through acting as an antioxidant protein or a regulatory protein for the antioxidant defense mechanism.

Materials and methods

Materials

β-$NADP^+$, 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), *N*-acetyl-L-cysteine (NAC), *N*-ethylmaleimide (NEM), L-buthionine-(*S*,*R*)-sulfoximine (BSO), KO_2, methylene blue (MB), 4,6-diamidino-2-phenylindole (DAPI), xylenol orange, propidium iodide (PI), mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (IDPm), avidin-conjugated TRITC, anti-rabbit IgG TRITC-conjugated secondary antibody, and anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated secondary antibody were obtained from Sigma. 2′,7′-Dichlorofluorescein diacetate (DCFHDA), t-butoxycarbonyl-Leu-Met-7-amino-4-chloromethylcoumarin (CMAC), dihydrorhodamine (DHR) 123, and rhodamine 123 were purchased from Molecular Probes (Eugene, OR). In order to prepare recombinant SAG protein, *E. coli* harboring pET14b-SAG were grown and lysed, and the recombinant protein was purified on Ni-NTA-agarose as described [19]. Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling (Beverly, MA). Anti-SAG IgG was purchased from Abcam (Cambridge, MA).

Oxidative damage to protein and DNA

IDPm was treated with 6 μM Cu/2 mM ascorbate, 5 mM H_2O_2, 10 mM KO_2, or 50 μM MB/light, which produces singlet oxygen, for 1 h. The remaining activity was determined as described [20]. DNA single-strand breaks were assayed by measuring the conversion of covalently closed circular double-stranded supercoiled DNA (form I) to open relaxed circular double-stranded DNA (form II) as described [19].

Cell culture

Human premonocytic U937 cells (American Type Culture Collection, Manassas, VA) 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. After incubation, cells were exposed to heat at 42°C for 1 h.

Knockdown of SAG by siRNA

SAG siRNA and control (scrambled) siRNA were purchased from Samchully Pharm (Seoul, Korea). The sequences of the double-stranded RNAs of SAG and control used in the current experiments are as follows. For SAG, sense and antisense siRNAs are 5′-GUCUUA GAUGUCAAGCUGA (dTdT)-3′ and 5′-UCAGCUUGACA UCUAAGAC(dTdT)-3′, respectively. For scrambled control, sense and antisense siRNAs are 5′-CUGAUGACCU-GAGUGAAUGdTdT-3′ and 5′-CAUUCACUCAGGUCAUCAG dTdT-3′, respectively. PC3 cells, derived from human prostate cancer, were transfected with 20 nM oligonucleotide by using Lipofectamine 2000 (Invitrogen) in serum-free conditions according to the manufacturer's protocol. After incubation for 5 h, the cells were washed and supplemented with fresh normal medium containing 10% FBS and 50 units/ml penicillin, 50 μg/ml streptomycin. After 2 days of incubation at 37°C in a humidified atmosphere of 5% CO_2, cells were exposed to heat at 42°C for 1 h.

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) 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 amplication 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 amplication 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.

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. The morphological changes of apoptotic cells were analyzed by the Zeiss Axiovert 200 microscope at the fluorescent DAPI region (excitation, 351 nm; emission, 380 nm).

FACS

To determine the portion of apoptotic cells, cells were analyzed with PI staining. 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. ApoAlert annexin V apoptosis detection system (Clontech) was used to measure the relative distribution of apoptotic and necrotic cells. The cell suspension was double-stained with annexin V-FITC and PI and analyzed by flow cytometry. Cell membrane permeability was determined by YO-PRO-1/PI staining and flow cytometry.

DNA fragmentation

To determine the degradation of chromosomal DNA into nucleosome-sized fragments, a 500-μl aliquot of the lysis buffer (100 mM Tris-HCl, pH 8.5, EDTA, 0.2 M NaCl, 0.2% SDS, and 0.2 mg/ml proteinase K) was added to the cell pellet (2×10^5 cells) and incubated at 37°C overnight. DNA was obtained by ethanol precipitation, separated in a 0.8% agarose gel, and visualized under UV light. DNA fragmentation was also determined using the diphenylamine assay as previously described [21].

Cellular redox status

Intracellular peroxide production was measured using the oxidant-sensitive fluorescent probe DCFH-DA with confocal microscopy. Cells were grown at 2×10^6 cells per 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 heat at 42°C for 1 h. 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