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A protective role of 27-kDa heat shock protein in glucocorticoid-evoked apoptotic cell death of hippocampal progenitor cells

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Abstract

Hippocampus is one of the most vulnerable tissues to glucocorticoid (GC). In the present study, we demonstrate that dexamethasone (DEX), a synthetic GC, induces apoptotic cell death in hippocampal progenitor HiB5 cells without any additional insult. Interestingly, expression of 27-kDa heat shock protein (HSP27) was markedly induced by DEX in time- and dose-dependent manners. This induction was dependent on the production of reactive oxygen species (ROS), suggesting that DEX-evoked oxidative damage to HiB5 cells is responsible for the HSP27 induction. To evaluate a possible role of HSP27, we generated two mutant HiB5 cell lines, in which expression of HSP27 was inhibited or enhanced by the over-expression of HSP27 cDNA with antisense or sense orientation (AS-HSP27 and S-HSP27, respectively). DEX-induced apoptotic cell population was significantly increased in AS-HSP27 HiB5 cells and evidently decreased in S-HSP27 cells. These results indicate that HSP27 protects hippocampal progenitor cells from GC-induced apoptotic cell death.

Keywords: HSP27; Glucocorticoid; Apoptosis; Hippocampal progenitor; HiB5

Glucocorticoid (GC) acts as a final effector molecule of the stress-responsive hypothalamus–pituitary–adrenal (HPA) axis. The action of a GC on its target tissues is mediated by interaction with either a low affinity glucocorticoid receptor (GR) or a high affinity mineralocorticoid receptor (MR) [1]. Although GC plays a pivotal role in the physiological and behavioral adaptive responses to stress [2], excessive or prolonged GC secretion can result in damaging effects on the viability and structural integrity of neurons in several brain regions including the hippocampus, which abundantly contains both types of receptors [3,4].

It is noteworthy that both GR and MR mRNA can be found in the developing rat hippocampal region as early as embryonic day 15.5 when the hippocampus begins to form [5,6]. However, there is little evidence that GC has any potential damaging influence on the developing hippocampus, in spite of extensive studies on GC-evoked neuronal loss in the adult. Actually, maternal GC or administration of synthetic GC during the prenatal or neonatal periods is a potent programming factor for the developing hippocampus. Excessive maternal GC can permanently modify the expression of hippocampal corticosteroid receptors, followed by an altered HPA reactivity to stress in adulthood [7]. Administration of DEX on neonatal rats caused alterations in the subcellular distribution of several synaptic molecules in the hippocampus and resulting learning deficits in adulthood [8]. In addition to this information from animals, we recently demonstrated that GC could strongly inhibit growth factor-induced neuronal differentiation of HiB5 cells, a conditionally immortalized rat hippocampal progenitor cell line in vitro [9]. Thus, it is worthwhile to

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explore whether GC damages proliferating hippocampal progenitor cells.

Heat shock proteins (HSPs) are a family of proteins originally identified as being rapidly up-regulated in response to elevated temperature. Since a wide variety of physical, chemical, and biological stimuli, including oxidative stress, heavy metal, osmotic stress, and ionizing radiation, are also capable of inducing HSPs, their induction is now considered as an index for a variety of cellular insults [10,11]. The main inducible HSPs in the central nervous system are 70 and 27-kDa HSPs (HSP70 and 27, respectively), and they are shown to elicit neuroprotective functions [12–14]. Recently, we demonstrated that HSP27 was rapidly induced in HiB5 cells in response to heat shock and exhibited a potent thermo-protective function in these cells by both transcriptional induction and post-translational regulation [15]. HSP27 belongs to a small HSP family, which includes HSP27, HSPB3, HSPB8, HSPB9, α-crystalline, and related members, sharing a conserved C-terminal α-crystalline domain [10,16]. In addition to thermo-tolerance, a large body of evidence has accumulated, showing that small HSPs also act as negative regulators of apoptosis in various cell types [17–20].

At first, the present study examined whether GC has a direct cytotoxic effect on hippocampal progenitor cells in vitro. The extent of apoptotic cell death and expression of HSP27 were investigated for demonstrating GC-evoked cellular damage in HiB5 cells. In the later part of the study, we addressed the molecular mechanism leading to the altered expression of HSP27 and its functional relevance.

Materials and methods

Materials. Dexamethasone (DEX) was obtained from Sigma (St. Louis, MO). Materials for cell culture were obtained from Invitrogen (Carlsbad, CA) and other chemicals, if not mentioned, were from Sigma.

Cell culture. HiB5 cells (kindly provided by Dr. R.D. McKay, National Institutes of Health) were maintained in Dulbecco's modified Eagle's medium with 4 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin/streptomycin, and 10% fetal bovine serum under a humidifying atmosphere containing 5% CO_2 at 32 °C for proliferation [9,15]. The medium was changed every 2 days. DEX at an indicated concentration or vehicle (0.1% ethanol) was applied when cells were grown to 40–60% confluence.

Propidium iodide staining and analysis of DNA contents. Quantitative analysis of apoptotic cells was conducted as described previously with minor modifications [15]. After drug treatment, both floating and adherent live cells were collected by trypsinization and centrifugation at 800*g* for 5 min. These cells were then fixed with 70% ethanol at 4 °C for 16 h. The fixed cells were washed with phosphate-buffered saline (PBS, pH 7.4) and then resuspended in PBS containing 500 μg/ml RNase A and 50 μg/ml propidium iodide. After 30 min of incubation at 37 °C, samples were analyzed for DNA contents on a Becton–Dickinson FACScan using LYSYSII software (BD Immunocytometry Systems, San Jose, CA). Haploid cells were considered as apoptotic.

DNA fragmentation assay. DNA fragmentation was analyzed essentially as described previously [15]. Briefly, cells were lysed for 20 min at 4 °C in a lysis buffer containing 5 mM Tris, pH 7.4, 0.5% Triton X-100, and 20 mM EDTA. After centrifugation at 20,000*g* for 15 min, the supernatants were extracted with a phenol–chloroform mixture (24:1), and nucleic acids were precipitated in ethanol before being analyzed by gel electrophoresis (1.5% agarose gel in Tris-acetate–EDTA buffer). Thereafter, the gel was incubated for 3 h at 37 °C in the presence of 20 μg/ml RNase A and visualized by staining with ethidium bromide.

Electrophoretic mobility shift assay (EMSA). After various treatments, nuclear extracts were prepared by a slight modification of the rapid nuclear extraction protocol described previously [21]. The sequences of p53-responsive element (p53RE) and heat shock element (HSE) oligonucleotides used are as follows (for simplicity, sequences of only one strand are shown): ctagTCGAGCCGGGCATGTCCGGGCATGTCCGGGCATGTC [22] and ctagAGAATCTTCCAGAAGTTTCC [23], respectively. Small characters indicate restriction enzyme-compatible ends for radiolabeling purpose, for which, 4 pmol of annealed oligo nucleotides was labeled with 20 μCi [α-^{32}P]dCTP (Amersham, Uppsala, Sweden) using the Klenow fragment (Promega, Madison, WI). Forty femtomolar of a labeled probe was incubated with 5–10 μg HiB5 nuclear extracts in 1× gel shift assay buffer (20 mM Hepes, pH 7.6, 0.1 mM KCl, 10% glycerol, 0.1% Nonidet P-40, and 1 mM DTT) containing 2.5 μg poly(dI–dC) in a 20 μl reaction mixture. Binding reactions were performed on ice for 20 min and then at room temperature for further 30 min. Protein–DNA complexes were resolved on a 5% native polyacrylamide (29:1) gel. For competition experiments, a 100-fold molar excess of unlabeled double-stranded competitor DNA was added with the ^{32}P-labeled probes.

Immunoblot analysis. Cell extracts were resolved on sodium dodecyl sulfate (SDS)–polyacrylamide gels, which were transferred to PVDF membranes (Millipore, Bedford, MA) using Towbin's buffer [25 mM Tris, pH 8.3, 192 mM glycine, and 20% (vol/vol) methanol]. The membranes were blocked in Tris-buffered saline (TBS: 150 mM NaCl, 10 mM Tris, pH 7.6, and 2 mM $MgCl_2$) containing 0.5% Tween 20 and 3% BSA, and then incubated with anti-HSP27 (Stressgen, San Diego, CA), anti-caspase-3 (Cell Signaling Technology, Beverly, MA), anti-PARP (Cell Signaling Technology) or anti-actin antibody (Sigma) at room temperature for 1 h. Blots were washed four times with TBS/0.5% Tween 20. Primary antibody binding was subsequently detected by incubation with secondary antibodies linked to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA). Then, the blots were washed four times as described above. Immunoreactive bands were visualized by Amersham ECL reagents following the manufacturer's instructions (Arlington Heights, IL).

Northern blot hybridization. Isolation of total RNA and Northern blotting were performed as described previously [15]. Briefly, total RNA was isolated by a single-step acid–guanidinium–phenol–chloroform method with minor modifications. Twenty micrograms of total RNAs was denatured, resolved on a 1.2% formaldehyde gel, and transferred to a Nylon membrane (Schleicher & Schuell, Dachen, Germany). The membrane was prehybridized at 42 °C for 2 h and then hybridized for 16 h in the presence of ^{32}P-labeled rat HSP27 cDNA probe. After autoradiography, membranes were stripped and rehybridized with a ^{32}P-labeled β-actin cDNA probe to ensure the equal amounts of loaded RNA.

Immunocytochemistry (ICC). Cells were grown on coverslips to 70% confluence, washed twice with PBS, and fixed in 4% paraformaldehyde, and then treated with 0.3% Triton X-100 in 3% fetal calf serum for 30 min. After washing with PBS, the cells were incubated with primary antibody (anti-HSP27; Stressgen) for 1 h, rinsed with PBS four times, and treated with FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) for 1 h in dark. After washing with PBS, the cells were mounted with mounting solution containing 0.1 μM DAPI. Fluorescence was observed under a fluorescence microscope (Carl Zeiss, Germany).

Measurement of reactive oxygen species. HiB5 cells, which were exposed to vehicle, DEX (10^{-6} M) and/or *N*-acetyl-L-cysteine (NAC, 10 mM), were treated with a reactive oxygen species (ROS)-sensitive fluorescence probe, dichloro-dihydro-fluorescein-diacetate (H_2DCFDA, Molecular Probes, Eugene, OR), at the final concentration of 5 μg/ml, for 30 min at 32 °C. Cells were washed twice with PBS and then fluorescence was monitored with a fluorescence microscope.

Plasmid construction and transfection experiments. The coding sequence for human HSP27 (hHSP27; GenBank Accession No.: X54079) was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using specific primers set as follows: hHSP27 *Bam*HI up: 5′-

ggatccCCAGCA TGACCGAGCGCCGCGT-3′; hHSP27 *Not*I dn: 5′-gc ggccgcCAGGTGGTTGCTTTGAAC-3′; hHSP27 *Not*I up: 5′-gcggccgc CCAGCATGACCGAGCGCCGCGT-3′; hHSP27 *Bam*HI dn: 5′-ggatcc CAGGTGGTTGCTTTGAAC-3′. PCR products were cloned into pGEM-T easy vector (Promega) and sequence identities were confirmed by a conventional chain-termination sequencing method. Expression vectors were constructed by subcloning into pRetro Tet-On vector (BD Bioscience Clontech, Palo Alto) using *Not*I/*Bam*HI linker sequences. For transfection experiments, HiB5 cells were plated in 12-well plates and grown to 70% confluence. After a brief washing with PBS, transfection was carried out using Lipofectamine-PLUS reagent (Invitrogen) with 500 ng of each plasmid per well according to the manufacturer's instructions. Stably transfected mutant cell lines were established under 3 μg/ml puromysin for 1 month.

Statistical analysis. Data for apoptotic cell populations were statistically evaluated using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test as a post hoc comparison. Statistical significance was set at $p < 0.05$.

Results and discussion

Previously, we demonstrated that HiB5 cells abundantly expressed both GR and MR, and treatment with DEX, a synthetic GC, strongly inhibited growth factor-induced neuronal differentiation of the cells [9]. In the present study, we show, for the first time that DEX can promote apoptotic cell death in proliferating hippocampal progenitor HiB5 cells in vitro, indicating a possible damaging effect of excess GC on the developing hippocampus. By flow cytometric analysis, the haploid or apoptotic cell population in control cells was less than 4% of analyzed cells, but in DEX-treated cells, the apoptotic population exceeded 15% (Fig. 1A). DEX-evoked apoptotic cell death of HiB5 cells was also indicated by fragmentation of chromosomal DNA. Under normal conditions, fragmented chromosomal DNA was barely detectable, while DNA fragmentation was apparent after treatment with DEX in dose- and time-dependent manners (Fig. 1B). More than 10^{-8} M of DEX was sufficient to induce fragmentation of chromosomal DNA, and it was apparent after 48 h of treatment. In keeping with the increase in the apoptotic cell population, DEX promoted the activations of apoptosis-mediating molecules such as p53 and caspase-3 [24]. Binding experiments revealed that treatment with DEX for longer than 24 h increased the binding activity of nuclear extracts (NE) to the consensus p53-responsive element (p53RE), indicating p53 activation (Fig. 1C). In addition

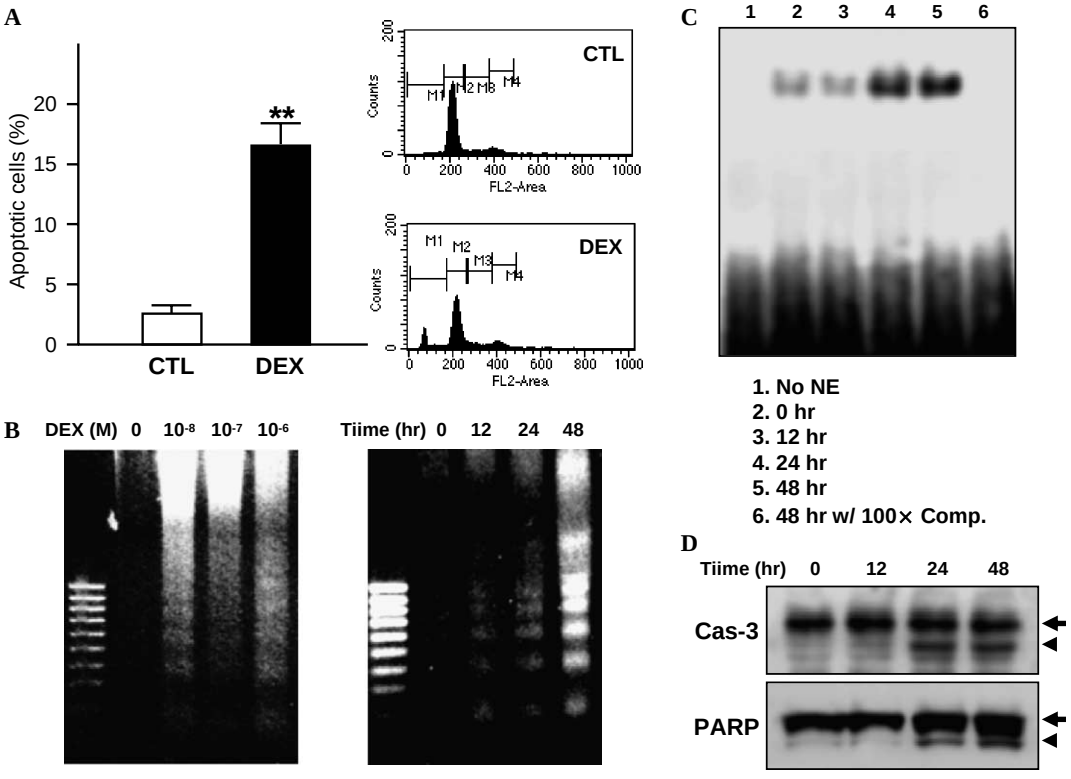

Fig. 1. DEX induces apoptotic cell death of HiB5 cells. (A) Flow cytometric analysis of apoptotic cell population. Vehicle (VEH, 0.1% ethanol)- or DEX (10^{-6} M)-treated cells were harvested 48 h after treatment, fixed with 70% ethanol, and then stained with propidium iodide. DNA content was measured and the number of apoptotic nuclei is expressed as mean % ± SE in left panel ($n = 5$; $^{**}p < 0.01$). Representative illustrations are shown in the right panel. (B) Degradation of chromosomal DNA by treatment with DEX. HiB5 cells were treated with different concentrations of DEX for 48 h (left panel), or 10^{-6} M DEX for different times (right panel), as indicated. Cells were lysed and DNA fragmentation assays were performed as described in Materials and methods. (C) HiB5 cells were treated with 10^{-6} M DEX for indicated times (0, 12, 24, and 48 h). NEs were isolated and p53RE-binding activities were examined by EMSA. Specific binding was confirmed by competition with 100-fold molar excess unlabeled p53RE oligonucleotide (100× Comp.). (D) Whole cell lysates were prepared from DEX-treated HiB5 cells and resolved on SDS–polyacrylamide gels (12% for caspase-3 and 8% for PARP). Cleavage of caspase-3 (Cas-3) and its substrate PARP was examined by immunoblot analysis with anti-caspase-3 and anti-PARP antibodies. Arrow indicates uncleaved form and arrowhead indicates cleaved form of each protein.

to p53, caspase-3 was also activated by DEX treatment. Similarly with the activation of p53, the cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP), a well-known substrate of activated caspase-3, was apparently increased in the cells that received DEX for 24 and 48 h (Fig. 1D). Considering that activations of p53 and caspase-3 pathway are regarded as indicators for apoptotic cell death, these results indicate that a long-term (more than 24 h) treatment with DEX can promote apoptotic cell death of hippocampal progenitor HiB5 cells.

It is well established that excess GC can contribute to neuronal atrophy or loss in the adult hippocampus. For example, it was reported that patients with Cushing's syndrome or post-traumatic stress disorder with elevated plasma GC levels exhibited reduced hippocampal volumes [25,26]. Similar hippocampal atrophy was also described in several animal models. Prolonged exposure to GC or excess GC was reported to induce massive neuronal loss in the hippocampus [27–30]. It is worthwhile to note that GC-mediated neuronal loss in the adult hippocampus has been thought to be achieved by the enhancement of a variety of neurotoxic insults, rather than a direct damage to hippocampal neurons. Disruption of Ca^{2+} homeostasis caused by disregulation of excitatory synaptic transmission and glucose utilization was proposed to be responsible for GC-mediated neuronal loss in the adult hippocampus [31]. However, proliferating HiB5 cells at 32 °C are nestin-positive, undifferentiated cells [32], which express negligible levels of receptors for excitatory amino acids (data not shown). The present study did not use any additional cytotoxic reagents in combination with DEX, indicating that it has a direct cytotoxicity on hippocampal progenitor cells.

Since it was shown that induction and nuclear translocation of HSP27 played crucially protective roles in heat shock-induced cellular damage and necrotic cell death in HiB5 cells in our previous study [15], we then focused on HSP27 during DEX-induced apoptotic cell death of these progenitor cells. At first, we examined mRNA and protein levels of HSP27 in response to DEX treatment by Northern and Western blot analyses, respectively. HSP27 mRNA and protein were expressed at a basal level under normal conditions, in accordance with our previous report [15]. They were, however, induced profoundly by DEX treatment in dose- and time-dependent manners (Figs. 2A and B). Higher

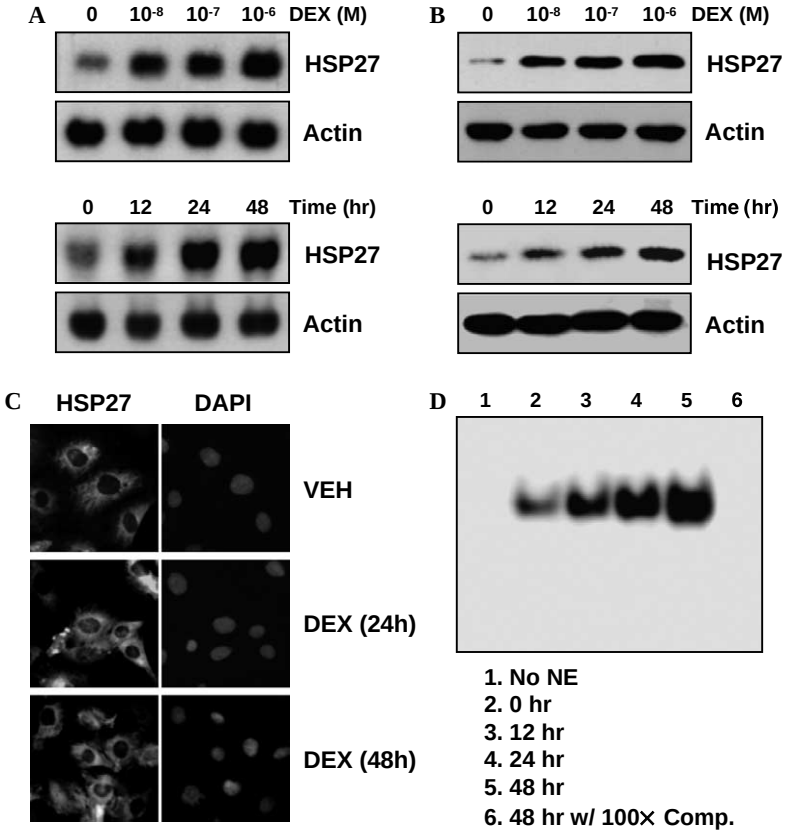

Fig. 2. Induction of HSP27 and HSE-binding activity in response to DEX. (A) HSP27 mRNA expression after DEX treatment. HiB5 cells were treated with vehicle or DEX as indicated. Northern blot hybridization was performed using ^{32}P-labeled cDNA probes, complementary to rat HSP27 mRNA (upper panel) or β-actin (lower panel). β-Actin was used as an internal control to show the equivalent loading of RNA. (B) Expression of HSP27 protein. HiB5 cells treated as indicated were harvested, and whole cell lysates were prepared. Immunoblotting was performed with anti-HSP27 or anti-actin antibody. (C) Subcellular localization of HSP27 in response to DEX treatment was examined by ICC. Cells were treated with either vehicle (VEH) or DEX (10^{-6} M, for 24 or 48 h) as indicated and fixed in 4% paraformaldehyde-containing PBS for 15 min. ICC was carried out as described in Materials and methods. DAPI was used for staining the nuclei. (D) HSE-binding activities of NE from DEX-treated HiB5 cells. EMSA was performed with ^{32}P-labeled HSE oligonucleotide. Specific binding was confirmed by competition with a 100-fold molar excess unlabeled HSE oligonucleotide (100× Comp.).

concentrations than 10^{-8} M of DEX were sufficient to increase HSP27 expression within 48 h. Expression of HSP27 constantly increased after treatment with 10^{-6} M DEX; the induction was apparent at 24 h and increased up to 48 h after treatment, suggesting that the accumulative effect of DEX is required for the expression of HSP27. However, in contrast to heat shock, DEX failed to promote apparent nuclear translocation of HSP27 in HiB5 cells (Fig. 2C), although it did strongly induce expression of the protein, similarly with heat shock. Interestingly, heat-shock element (HSE)-binding activities of HiB5 NE, which were constantly increased up to 48 h after DEX treatment, showed a similar time-dependent profile with that observed in HSP27 expression (Fig. 2D). As HSE is known as a binding element for heat-shock factors (HSFs) [23], the result indicates that HSFs in the HiB5 cells were increased or activated by DEX.

There is a growing body of evidence showing that excess GC is cytotoxic to a certain type of cultured cells including muscle cells, lymphocytes, thymocytes, neuroblastoma, and Leydig cells [33–37]. In these cells, GC-induced cell death was commonly accompanied by the mitochondrial events associated with apoptosis, which involve a reduction in mitochondrial membrane potential and the generation of ROS that precedes nuclear DNA degradation. This was also the case in the GC-evoked apoptotic cell death of HiB5 cells. As shown in Fig. 3A, the number of cells containing fluorescent signals from H_2DCFDA, which was capable of sensing ROS, was apparently increased by treatment with 10^{-6} M DEX in a time-dependent manner. Simultaneous treatment with 10 mM NAC, a potent ROS scavenger, inhibited DEX-evoked ROS production. Interestingly, inhibition of ROS production with NAC completely blocked both the induction of HSP27 and the activation of HSFs by DEX (Figs. 3B and C). In consideration of the delayed, but prolonged, increment of HSP27 level and HSE-binding activity as shown in Fig 2, these results also support the notion that accumulated cellular damage is primarily responsible for the induction of HSP27, presumably via the activation of HSFs, rather than corticosteroid receptor-mediated transcriptional induction of the protein. Actually, it is well established that oxidative damage to cells could initiate various cellular defense mechanisms including HSPs [38,39]. It was demonstrated that nuclear translocation and DNA-binding activity of HSF-1 were evidently augmented in response to treatment of neuroblastoma with H_2O_2 [23]. Furthermore, it is of interest that reduced cellular glutathione, a tripeptide with ROS detoxification and regulation of cell death, enhanced the induction of small HSPs including HSP27 with an increase of HSE-binding activity of HSFs, indicating that the expression of HSP27 acts as a buffer against oxidative stress [39,40]. In addition to HSP27 and HSFs, activation of p53 by DEX is also dependent on an increase in intracellular ROS (Fig. 3C), strongly suggesting that a direct cytotoxicity of GC on hippocampal progenitor cells is most likely mediated by the generation of ROS with a potential mitochondrial dysfunction, leading to the induction of HSP27 as a defense mechanism.

To access a functional relevance of induced HSP27 during DEX-evoked cell death, we generated mutant cell lines,

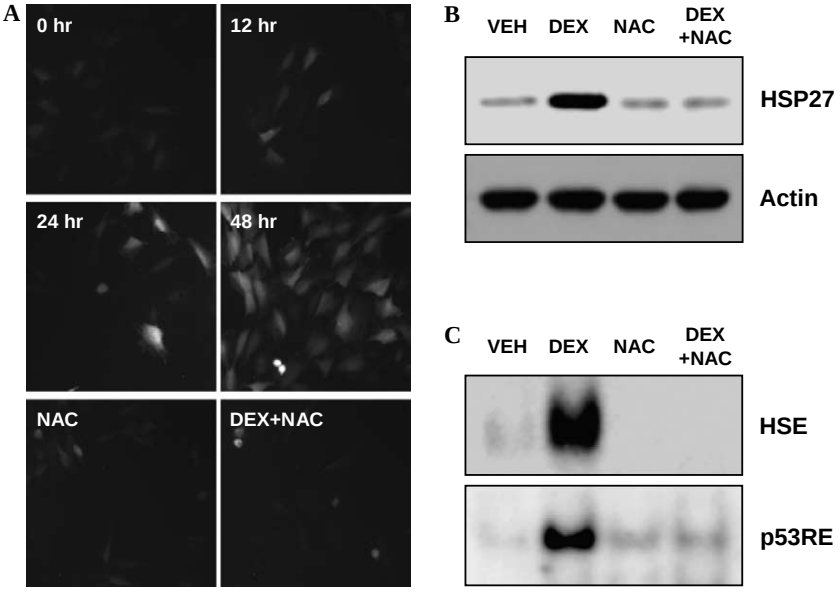

Fig. 3. Involvement of ROS in the induction of HSP27. (A) Production of ROS by treatment with DEX. HiB5 cells received appropriate treatments as indicated. H_2DCFDA was applied to each group of cells for 30 min. After washing, fluorescent signals were monitored and photographs were taken with the same exposure time (VEH: 0.1% ethanol for 48 h; 12, 24, and 48 h: 10^{-6} M DEX treatment for the indicated time; NAC: 10 mM NAC for 48 h; DEX + NAC: simultaneous treatment with DEX and NAC for 48 h). (B) Expression of HSP27 in the presence of DEX and/or NAC. HiB5 cells were treated with VEH, DEX, and/or NAC for 48 h. Whole cell lysates were prepared from these cells and resolved by SDS–PAGE. Immunoblot analysis was performed with anti-HSP27, and anti-actin antibodies. (C) Effect of NAC on DEX-evoked p53RE and HSE-binding activities. NEs from HiB5 cells receiving indicated treatments were prepared and EMSA was performed with ^{32}P-labeled p53RE or HSE oligonucleotide.

which over-express the human HSP27 gene in either antisense or sense direction (denoted as AS-HSP27 or S-HSP27, respectively). A coding sequence for human HSP27 was inserted into a tetracyclin-inducible expression vector and transfected to HiB5 cells for the establishment of stable cell lines in puromysin-containing media (Fig. 4A). The AS-HSP27 or S-HSP27 HiB5 clone was selected by immunoblot analysis on HSP27 expression after treatment with 10^{-6} M DEX for 48 h. The expression of HSP27 was decreased by one-fourth in a selected AS-HSP27 HiB5 cell line (clone #1), and induced by 5-fold in S-HSP27 HiB5 cells (clone #5), compared with that of wild-type HiB5 cells (Fig. 4B). Using these mutant cells, we examined apoptotic cell populations and DNA fragmentation in the mutant HiB5 cells compared to wild-type cells after treatment with 10^{-6} M DEX for 48 h. The apoptotic cell population after DEX treatment of AS-HSP27 HiB5 cells was more than doubled as compared with that in wild-type cells ($13.5 \pm 3.4\%$ in normal DEX-treated HiB5 cells and $29.4 \pm 5.5\%$ in AS-HSP27 HiB5 cells). In S-HSP27 cells, it significantly decreased to $5.9 \pm 2.2\%$ (Fig. 4C). There were no significant differences in apoptotic cell populations in the absence of DEX. In accordance with the result, DNA fragmentation was greatly increased in AS-HSP27 cells and decreased in S-HSP27 cells by DEX treatment (Fig. 4D). These results clearly indicate that induced HSP27 obviously plays a protective role against DEX-induced apoptotic cell death of hippocampal progenitor HiB5 cells.

Our previous report indicated that nuclear translocation and the stabilization of cytoskeletal structures following multiple phosphorylation on serine residues were crucial for the protective functions of HSP27 against heat shock in HiB5 cells [15]. Regarding a protective function in the nucleus, it was also reported that nuclear HSP27 conferred thermo-protection by prohibiting nuclear protein aggregation as a molecular chaperone [41]. However, a dramatic nuclear translocation could not be observed during

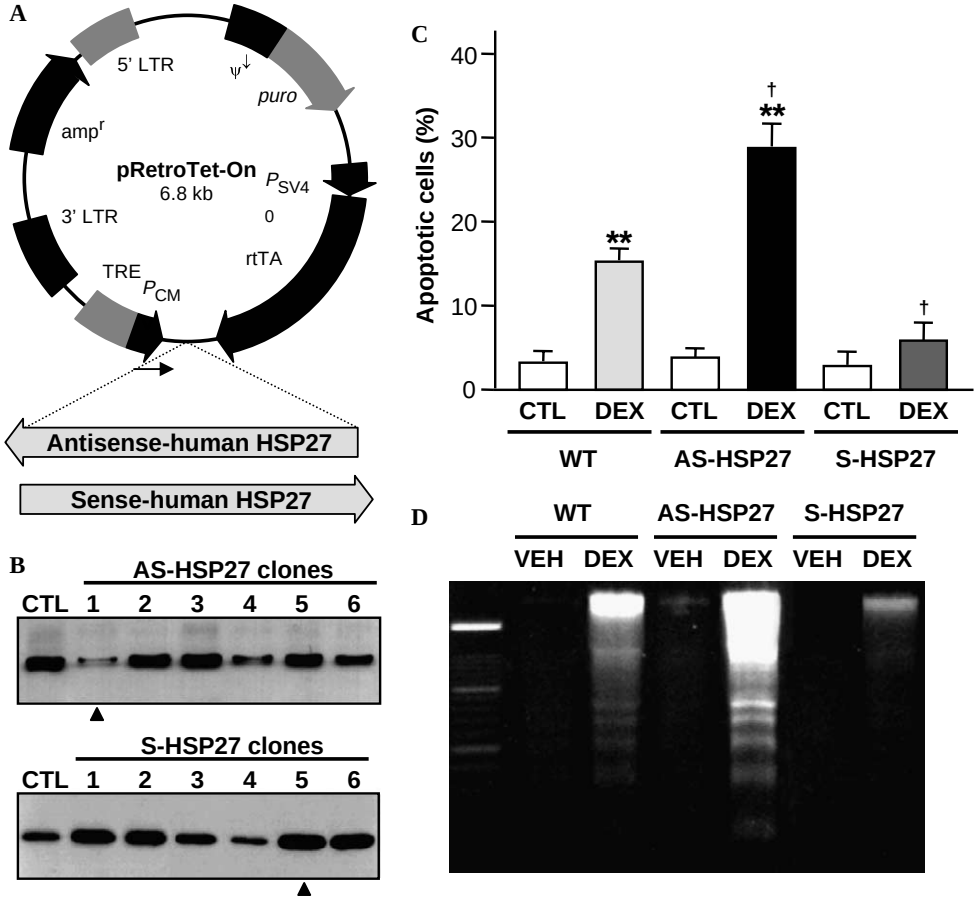

Fig. 4. The protective role of HSP27 against DEX-induced apoptotic cell death. (A) Human HSP27 cDNA was subcloned with the antisense or sense orientation into the pRetro Tet-On expression vector (named as AS-HSP27 and S-HSP27, respectively). Either AS- or S-HSP27 was transfected into HiB5 cells, and stable cell lines (denoted as AS-HSP27 and S-HSP27 HiB5, respectively) were established by a selection in the presence of 2 μg/ml puromycin for one month. (B) Wild-type HiB5 cells and several stable cell lines were treated with 10^{-6} M DEX for 48 h. Total proteins were isolated and immunoblot analysis was performed with anti-HSP27 antibody. Clone #1 among AS-HSP27 cells and Clone #5 among S-HSP27 cells were chosen for further experiments (arrowhead). (C) Wild-type, AS-HSP27, and S-HSP27 HiB5 cells were treated with vehicle (VEH) or 10^{-6} M of DEX for 48 h. Apoptotic cell death was examined with FACS analysis. The relative number of apoptotic nuclei is expressed as mean % ± SE ($n = 4$; $^{**}p < 0.01$ vs. VEH in each cell line; $^{\dagger}P < 0.05$ vs. DEX-treated normal HiB5 cells). (D) DNA fragmentation was examined in wild-type, AS-HSP27, and S-HSP27 cells as mentioned above.

DEX-evoked apoptotic cell death as shown in Fig. 2C. From this fact, it can be postulated that there can also be differential defense mechanisms in the cytoplasm, which involve the anti-apoptotic functions of HSP27 against DEX and are distinguished from those in thermo-protective role of the protein. In this context, it should be noted that HSP27 was reported to be more effective than other inducible HSPs against apoptosis-inducing stimuli on neuronal cells. Over-expression of HSP27 evidently conferred protection against apoptotic cell death in cultured primary and immortalized neuronal cells. In contrast, other HSPs such as HSP56, 70, and 90 were not as effective as HSP27, although they still play effective protective roles during thermal or ischemic stress-evoked necrotic cell death [20,42–44] This evidence strongly suggests that HSP27-specific protective mechanisms may be more critical for its anti-apoptotic functions in the nervous system, rather than being an aid in the refolding of non-native proteins, a common function among HSPs. Indeed, a variety of different roles of HSP27 during cellular stress have been proposed to account for the anti-apoptotic activity seen with increased expression of this protein. For instance, it was well established that HSP27 could associate with and activate Akt, a pivotal protein kinase as a survival promoting factor against diverse cellular stress [45]. In addition, HSP27 was reported to prevent cytochrome *c* release from mitochondria and the activation of subsequent caspase cascade [46]. Considering the importance of ROS production in the present study, it is noteworthy that HSP27 can increase intracellular levels of glutathione, although HSP27 is devoid of any endogenous ROS-detoxifying activity [47]. Collectively, it can be speculated that a diverse anti-apoptotic function of HSP27 in the cytoplasm might be involved in its protective role against DEX-evoked apoptotic cell death in HiB5 cells.

In conclusion, we clearly demonstrate that excess GC can directly damage proliferating hippocampal progenitor cells in vitro to cause apoptotic cell death. Regarding the present results and our previous study showing the differentiation-inhibiting effect of GC [9], it is of importance to consider developmental significances since excess GC due to maternal stress or pathogenic occasions may cause malfunctions in the developing fetal brain. More importantly, as DEX is often used to treat pregnant women to enhance fetal lung development [48], the clinical consequences of exposure of the developing brain to synthetic GCs should also be considered. In addition to the harmful influence of GC, we also provide significant evidence that HSP27 can act as an effective cytoprotective molecule in hippocampal progenitor cells against both necrotic [15] and apoptotic cell death, suggesting the possible therapeutic importance of HSP27.

Acknowledgments

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