Journal of Cellular Biochemistry 103:1183–1197 (2008)

S100A6 (Calcyclin) Enhances the Sensitivity to Apoptosis Via the Upregulation of Caspase-3 Activity in Hep3B Cells

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Abstract S100A6 (calcyclin) is a small calcium-binding protein which has been implicated in several cellular processes such as cell cycle progression, cytoskeleton rearrangement, and exocytosis. Also the upregulation of S100A6 has been reported in a variety of tumors and linked to metastasis. However, exact intracellular roles of S100A6 related with apoptosis have not been clarified yet. Here we demonstrated that the upregulation of S100A6 enhances the cell death rate compared to the control under the apoptotic conditions. In exogenously S100A6 induced Hep3B cells, cell viability was significantly decreased compared with mock and S100A6-knockdown cells under calcium ionophore A23187 treatment. The exogenously introduced S100A6 significantly affected the caspase-3-like activity in programmed cell death through the enhanced caspase-3 expression, which was verified by promoter assay in wild or mutant S100A6-transfected Hep3B cells. Next, the promoter activity of caspase-3 was increased by 2.5-folds in wild-type S100A6-transfected cells compared to mutant 2 (E67K, mutant of EF-hand motif) or control. Our results suggest that S100A6 might be involved in the processing of apoptosis by modulating the transcriptional regulation of caspase-3. J. Cell. Biochem. 103: 1183–1197, 2008.

Key words: S100A6; apoptosis; Hep3B; A23187; caspase-3; EF-hand

Abbreviations used: RT-PCR, reverse transcriptase-polymerase chain reaction; PARP, poly (ADP-ribose) polymerase; HCC, hepatocellular carcinoma; FBS, fetal bovine serum; PVDF, polyvinylidene difluoride; PMS, phenazine methosulphate; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; S-ODNs, phosphorothioate-oligodeoxyribonucleotides; Z-DQMD-FMK, Z-Asp(OMe)-Gln-Met-Asp(OMe)-CH_2F; Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-AMC.

Joung Hyuck Joo and Sun Young Yoon are equally contributed to this work.

Grant sponsor: 21C Frontier Functional Human Genome Project, Korea Ministry of Science and Technology; Grant numbers: FG-3-1-04, SC13040; Grant sponsor: Korea Research Foundation; Grant number: KRF-20020015-CS004.

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Received 30 May 2007; Accepted 16 June 2007
DOI 10.1002/jcb.21496

S100A6 (calcyclin) is a low molecular weight calcium-binding protein and belongs to one of the S100 family proteins that have two "EF-hand" calcium-binding pockets [Filipek and Kuznicki, 1993]. It has been reported that the proteins played an important role in cell growth, differentiation, and motility through calcium-dependent signal pathways [Schafer and Heizmann, 1996]. S100A6 had been identified and isolated as a cDNA clone regulated by serum and was later recognized as a cell growth-associated gene [Hirschhorn et al., 1984; Calabretta et al., 1985]. The expression pattern of S100A6 has been extensively investigated in diverse tissues and cells because its expression was significantly increased in some tumor tissues such as melanoma [Weterman et al., 1992], adenocarcinoma [Gong et al., 1992; Komatsu et al., 2000], neuroblastoma [Tonini et al., 1995], and leukemia [Calabretta et al., 1985]. Our previous studies also showed that S100A6 when assessed by dot hybridization and Northern blot was upregulated in intrahephatic tumors [Kim et al., 2002]. Besides malignant tissues and cells, the expression level of S100A6 was elevated in cells with high proliferating activity and cells undergoing differentiation. The patterns of differential expression of S100A6 with tissue and cell-type specificities aroused an interest in its intracellular roles. Several of the S100A6-associated proteins such as annexin II, VI, XI [Minami et al., 1992; Watanabe et al., 1993; Zeng et al., 1993], tropomyosin [Golitsina et al., 1996], caldesmon [Mani et al., 1992], and SIP [Matsuzawa and Reed, 2001] have been identified, and it has been reported that these proteins interact with S100A6 in a calcium-dependent manner. Many studies on the functional analysis of S100A6 have been focused on its roles in the development of malignancy and differentiation, such as cell proliferation and metastasis, but exact intracellular roles of S100A6 have not been clarified yet.

A novel aspect of the cellular function of S100 proteins relating to cell death other than cell proliferation, differentiation, and tumor metastasis has been suggested. For example, S100A1/B, a heterodimer of two isoforms of S100A1 and S100B, was found to exert a cytotoxic action that eventually led to cell death of rat pheochromocytoma, PC12 cells [Fano et al., 1993; Fulle et al., 1997]. In glial C6 cells, S100B synthesis was known to be associated with apoptosis in response to UV irradiation. In mouse embryo fibroblast (MEF) and rat embryo fibroblast (REF) cells, S100B expression was associated with a rescue of p53 apoptotic function [Scotto et al., 1998]. S100B expression stimulated iNOS activity and increased the expression level of iNOS mRNA in rat cortical astrocytes [Hu et al., 1996]. It also has been suggested that S100B expression was increased with age and might have pathogenic implications for Alzheimer's disease [Sheng et al., 1996]. It was also found that S100A6 was distinctively and selectively expressed in a mouse model for amyotrophic lateral sclerosis (ALS), a neurodegenerative disease [Hoyaux et al., 2000]. However, there has been no clear evidence which suggested the direct relationship between the expression of S100A6 and cell death, yet.

To study the role of S100A6 in the progression of cell death, we established stable cell lines derived from Hep3B expressing sense or antisense S100A6 gene, and investigated the correlation between the expression level of S100A6 and apoptosis after treatment with calcium ionophore A23187.

MATERIALS AND METHODS

Cells

Human liver cell lines, Hep3B, HepG2, Chang, and PLC/ARF/5 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Gaithersburg, MD) containing 10% FBS (v/v, Life Technologies), 40 U/ml penicillin, and 40 U/ml streptomycin (Life Technologies). The cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 and split regularly before attaining 70–80% confluence.

Plasmid Vector Construction and Cell Transfection

Plasmid vectors, pOPI3-EF/+S100A6 and pOPI3-EF/−S100A6, were constructed by the insertion of 273 bp long cDNA fragments coding for the region between nucleotides 103 and 375 of S100A6 mRNA (GeneBank accession # NM014624) into mammalian expression vector, pOPI3-EF plasmid in sense and antisense directions. Briefly, the coding region for S100A6 was amplified from Hep3B cDNA by

PCR method and purified. PCR primer was designed such that the S100A6-coding region and Not I restriction enzyme sites at 5′ and 3′-ends were included in PCR products (forward; 5′-ATACGCGGCCGCCAGC CCTCAGCCATG-3′, reverse; 5′-GTATGCGGCCGCTCAGCCC-TTGAGGGC-3′). The amplified S100A6-coding region was cleaved by Not I enzyme and purified by DNA-bead (ATGene, Korea). After purification, the coding region was ligated into pOPI3-EF vector through Not I site in the linker region, and orientation of the inserts was determined by DNA sequencing. The vectors containing the coding region in sense and antisense orientations were named pOPI3-EF/+S100A6 and pOPI3-EF/−S100A6, respectively. Hep3B cells were transfected with pOPI3-EF (mock control), pOPI3-EF/+S100A6 or pOPI3-EF/−S100A6. Transfections were done by liposome method (Geneshuttle40, QBIOgene, Carlsbad, CA) and cells stably transfected with corresponding vectors were selected by their ability to grow in 800 μg/ml geneticin (Life Technologies).

Western Blotting

To prepare protein samples needed for immunoblotting, cells were lysed in modified cell lysis buffer (150 mM NaCl, 40 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 5 mM EGTA), and protein concentrations were determined by Bradford method [Bradford, 1976]. Equal amounts of cell lysates were resolved by 12% SDS–PAGE gel electrophoresis, and then electrophoretically transferred onto PVDF membrane (Millipore, Billerica, MA). The membrane was blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) and incubated overnight at 4°C with primary antibodies specific to human S100A6 (Sigma, St. Louis, MA), caspase-3 (R&D Systems, Minneapolis, MN), PARP (Pharmingen, San Diego, CA), or α-tubulin (Sigma, St. Louis, MA). Subsequently, the membrane was washed with PBST buffer and incubated with appropriate secondary antibodies. Protein bands were determined done by enhanced chemiluminescence kit (ECL kits, Amersham, Uppsala, Sweden).

MTS Assay

Cell viability was determined by MTS assay that could detect the cellular capacity to convert MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) tetrazolium salt to formazan [Goodwin et al., 1995]. MTS powder (Promega, Madison, WI) was dissolved in PBS at the concentration of 1.71 mg/ml, and the pH was adjusted to the range between 6.0 and 6.5. Phenazine methosulphate (PMS) powder (Sigma) was dissolved in PBS at the concentration of 1.53 mg/ml. Prior to MTS assay, a reaction mixture was prepared by combining 25 μl of PMS with 975 μl of MTS. Then 50 μl of the mixture was added to 200 μl of cell suspensions prepared from ionophore A23187-treated or -untreated cells in 96-well plates. After incubation for 1 h at 37°C, absorbance of each well was determined at 490 nm using an E_{max} precision microplate reader (Molecular Devices, Sunnyvale, CA). The percent relative cell viability was defined as [(A_{490} of A23187 treated)/(A_{490} of untreated)] × 100.

DNA Fragmentation Assay

Total genomic DNA was isolated from cell pellets by a method described elsewhere [Gavrieli et al., 1992]. In brief, cells were suspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA, 100 μg/ml proteinase K, 1% SDS), and incubated overnight at 55°C. DNA was extracted with phenol/chloroform (1:1, v/v) followed by precipitation with 0.3 M sodium acetate in ethanol. It was then washed with 70% aqueous ethanol. The DNA pellet was resuspended in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA), and incubated for 2 h at 37°C with 0.5 mg/ml DNase-free RNase (Roche, Mannheim, Germany). After phenol/chloroform re-extraction by the same method, DNA was analyzed on 1% (w/v) agarose gel and then visualized with ethidium bromide.

FACS Analysis

Cell cycle distribution was analyzed by flow cytometry. When necessary, each S100A6-transfected cells were pretreated with 10 μM Z-DQMD-FMK (Calbiochem, San Diego, CA), caspase-3 inhibitor, for 1 h, before treatment of 2 μM A23187 for 24 h. Cells were washed twice with cold-PBS, and fixed in cold 80% ethanol for 1 h. Subsequently, cells were rinsed with PBS, followed by the treatment of propidium iodide (PI) buffer (100 μg/ml PI, 0.1 mM EDTA, 0.1% Triton X-100 in PBS, pH 7.4) containing 1 mg/ml RNase A for 30 min, and analyzed by FACScan

flow cytometer. The distribution of the cells in different phases of cell cycle was calculated using the Cell-FIT software (Becton-Dickinson Instruments, Beverly, MA).

RT-PCR and Primers

Total RNA was extracted from *S100A6*-transfected cells as described before [Chomczynski and Sacchi, 1987]. For the first-strand cDNA synthesis, 10 μg of total RNA was reverse transcribed using a ProSTAR™ First-strand RT-PCR kit (Stratagene, La Jolla, CA). *Taq* PCR reactions were performed using the first strand cDNA as the template in the presence of specific oligonucleotide primers for caspase-3, -6, -7, -8, -9, Bcl-2, Bax, and β-actin as shown, Caspase-3, (Fwd) 5′-GAACTGGACTGTGGCATTGA-3′, (Rev) 5′-CACCAACCAACCATTTCTT-3′; Caspase-6, (Fwd) 5′-GTTTACACGCTGCCTGCT G-3′, (Rev) 5′-CTGGCTGAGAAAGCCATTTT-3′; Caspase-7, (Fwd) 5′-AGGGACCGAGCTTGATGAT-3′, (Rev) 5′-CCCTGATATGGCTATTGACTGA-3′; Caspase-8, (Fwd) 5′-GGCTTGTCAGGGGGATAACT-3′, (Rev) 5′-CACCATCAATCAGAAGGGAAG-3′; Caspase-9, (Fwd) 5′-AGCAGCAAAGTTGTCGAAGC-3′, (Rev) 5′-CCTGGGAAAGTAGAGTAGG-3; Bcl-2, (Fwd) 5′-GGAAGGATGGCGCACGCTGG-3′, (Rev) 5′-GTAGCGGCGGGAGAAGTCGT-3′; Bax, (Fwd) 5′-CAGCTCTGAGCAGATCATGAAG-3′, (Rev) 5′-CCATCTTCTTCCAGATGGTGA-3′; β-actin, (Fwd) 5′-GCCATGTACGTTGCTATCCAGGCTG-3′, (Rev) 5′-AGCCGTGGCCATCTCTTGCT CGAAG-3′.

Treatment of S100A6 Antisense Oligodeoxynucleotides

The following two different phosphorothioate-oligodeoxyribonucleotide (S-ODN) were used. Eighteen-mer antisense S-ODN, AnCACY having the complementary nucleotide sequence to the region (+642∼+659, 5′-ATGCCATGGCTGAGGGCT-3′) in S100A6 mRNA was synthesized. And, for the control experiment, scrambled 18-mer S-ODN (5′-GGT-ACGTCGAGCTATGGC-3′), ScCACY was also synthesized. Underlined sequences of each S-ODN denoted the modified sequences with phosphorothioate-oligodeoxyribonucleotide. All S-ODN were designed in this experiment and custom made by GenoTech (Korea). The cells, stably transfected with the sense S100A6 gene (pOPI3-EF/+*S100A6*), were seeded in a 96-well culture plate at 1×10^4 cells in 50 μl DMEM (10% FBS). After culture for 24 h, the mixture of 0.5 μM S-ODN (AnCACY or ScCACY) and 1.8 μl of GeneShuttle 40 was added into each well. After the 12 h culture, S-ODN-treated cells were stimulated with A23187 (0−4 μM) for 36 h and viability was measured by MTS assay.

Measurement of Caspase-3-like Activities

Activity of caspase-3 was determined with fluorescence peptide substrates AC-DEVD-AMC (Calbiochem) [Nagase et al., 2001]. In brief, cells treated with 2 μM calcium ionophore A23187 for 24 h. The cells were washed with PBS buffer and then resuspended in the lysis buffer (50 mM Tris-HCl, pH 7.5; 1 mM EDTA; 10 mM EGTA; 20 μM digitonin). After incubation at 37°C for 10 min, the supernatant was obtained. The supernatant was mixed with glycerol to 25% and then stored at −70°C for further use. An aliquot of each sample was incubated with 100 μM of each substrate at 37°C for 1 h. Fluorescence from free 7-amino-4-methyl-coumarin (AMC) was quantified at an excitation wavelength of 360 nm and emission wavelength of 450 nm.

Luciferase Assay for Promoter Analysis of Caspase-3

The 5′-flanking DNA sequence of rat caspase-3 region (GeneBank accession # AF427079) between positions −1841 and +88 were cloned into pGL3-basic vector, encoding the modified firefly luciferase. Genomic DNA PCR were performed using DNA of PC12 cells (rat adrenal pheochromocytoma) as a template in the presence of specific oligonucleotide primers for caspase-3 5′-UTR region (forward 5′-ATCCCGGGGGATCCCCTGGAGCTAGAGT-3′, reverse 5′-AGGTGCGGAGCTTGGAACGGAGATCTAT-3′). The constructed DNA was co-transfected with the each point-mutated S100A6 gene described below. Briefly, Hep3B cells (2×10^5 cells per well) were placed the day before transfection onto 6-well culture plates and grown to approximately 70% confluence. In 48 h after transfection, luciferase activities were measured, and transfection efficiency was normalized by β-galactosidase activities.

Site-Directed Mutagenesis of S100A6

The S100A6 sequence in pOPI3-EF/+S100A6 was used as the template for single-tube

megaprimer PCR method in vitro mutagenesis [Ling and Robinson, 1997]. Two sets of complimentary primers were used. First set, R1 (5′-ATGAATTCCCCTCAGCCATGGCATGCCCCC-3′) and M1 (5′-CTTGCTCAGGGCGTGCTTGTC-3′), was used to alter the Thr (T) at amino acid position 28 of S100A6 to Ala (A), incorporating a 5′ *EcoR* I. Second set, R1 and M2 (5′-GAAGTTCACCTTCTGGTCCTT-3′), for Glu (E) at position 67 to Lys (K), incorporating a 5′ *EcoR* I. Underlined sequences of each primer denoted the target sequence of mutagenesis. Cycles for first amplification were, 95°C × 4 min, 50°C × 1 min, and 72°C × 1 min followed by 24 cycles of 95°C × 1 min, 50°C × 1 min, and 72°C and 1 cycle of 95°C × 1 min, 50°C × 1 min, and 72°C × 5 min. After completion of the above first PCR, primer F2 (5′-GCTCTAGATTTTCAGCCCTTGAG GGCTTC-3′) for incorporating a 3′ *Xba* I, *Taq* DNA polymerase (2.5 units), and 3 μl dNTP (2.5 mM) were added in the each resultant PCR tube. The second PCR reaction has performed for 25 cycles (95°C × 1 min, 50°C × 1 min, and 72°C × 1 min). The resultant PCR mixtures were separated and purified in 1.5% agarose gel and digested with *EcoR* I and *Xba* I restriction enzymes. Digested mutant S100A6 genes were ligated with pcDNA3.1 vector. The sequences of all mutated products were confirmed by automated DNA sequencing.

RESULTS

Modulation of S100A6 Expression in Hep3B Cells

Hep3B cells were transfected with mammalian expression vectors containing the 273 bp long *S100A6* cDNA sequences in sense (pOPI3-EF/+*S100A6*) or antisense (pOPI3-EF/−*S100A6*) orientations, respectively (Fig. 1A). Cells transfected with each plasmid vector were colonized and established as stable cell lines. The established cell lines were named Hep3B/+*S100A6* cell line for the cells containing S100A6 sequence in sense orientation and Hep3B/−*S100A6* for the cells containing the sequence in antisense orientation. As shown in Figure 1B, Hep3B/+*S100A6* cells produced higher level of S100A6 protein compared to control cells transfected with empty vector (pOPI3-EF, mock control) and Hep3B/−*S100A6* cells produced lower level of S100A6 protein compared to mock control.

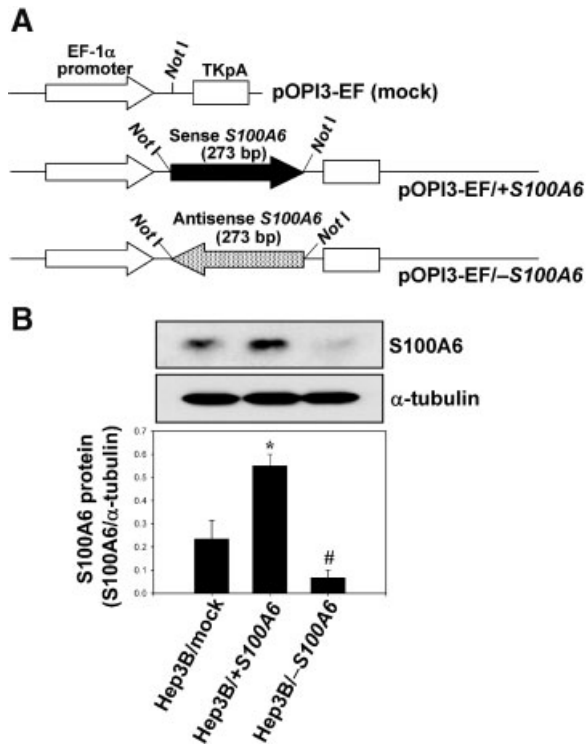


Fig. 1. Modulated expression levels of S100A6 protein by transfection of sense and antisense S100A6 gene in Hep3B cells. Hep3B cells were transfected with plasmids, pOPI3-EF for mock, pOPI3-EF/+*S100A6* for sense *S100A6* (273 bp), and pOPI3-EF/−*S100A6* for antisense *S100A6* (273 bp), respectively (**A**). Transfected cells with each plasmid were cloned and established as stable cell lines such as S100A6 over-expressing cell line (Hep3B/+*S100A6*) or cell line with reduced S100A6 level (Hep3B/−*S100A6*). The expression level of S100A6 protein was assessed by Western blot analysis of the cell lysates collected from the established clones (**B**). The relative levels of S100A6 protein were determined by densitometric analysis (**lower panel**) and presented as mean ± SD (bars) values of three independent experiments. $^{*}P < 0.004$ and $^{\#}P < 0.02$ versus Hep3B/mock control.

Increased Level of Intracellular S100A6 Sensitized Hep3B Cells to Cell Death Induced by Calcium Ionophore A23187

After stable cell lines (Hep3B/mock, Hep3B/+*S100A6*, and Hep3B/−*S100A6*) were established, the cells were exposed to different concentrations of calcium ionophore A23187. Viability of the cells was evaluated by MTS assay, and the change of cell morphology was observed by microscopic examination. As shown in Figure 2A, the treatment of A23187 resulted in a significant decrease in viability of the Hep3B/+*S100A6* compared to the mock control. By contrast, the viability of Hep3B/−*S100A6* was increased compared to the mock control.

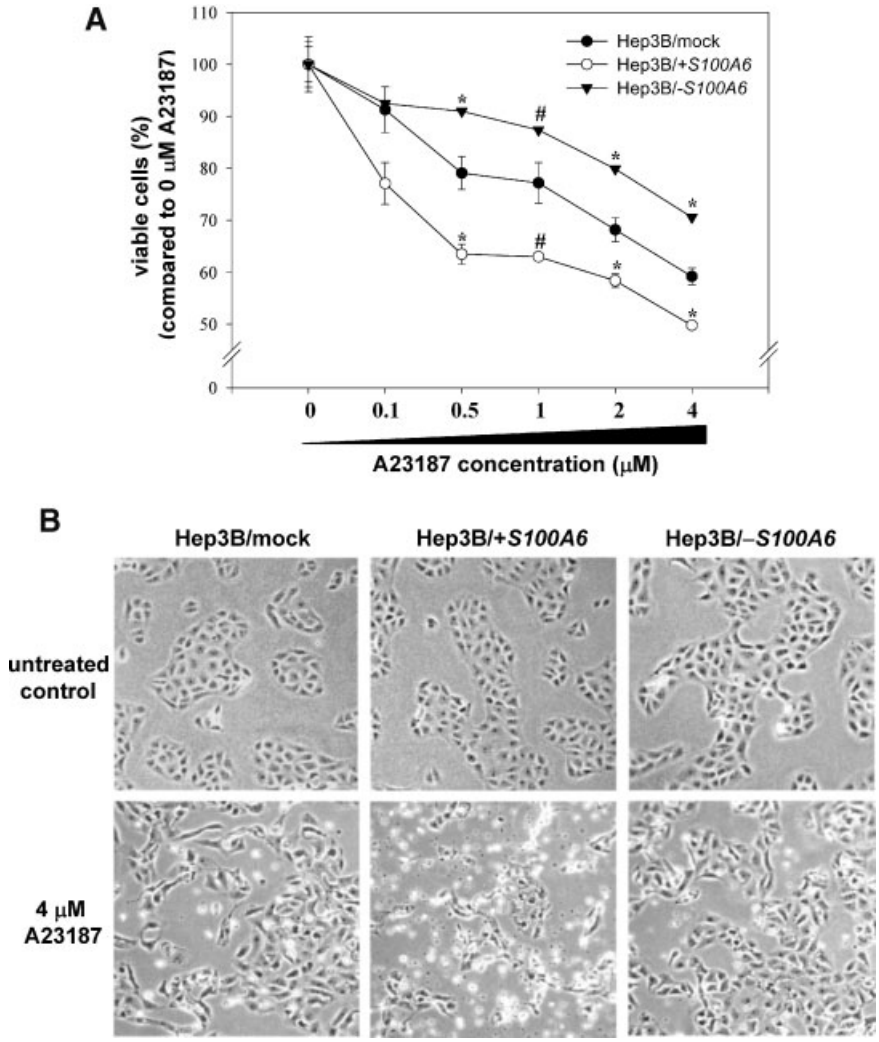


Fig. 2. S100A6 upregulated cells were susceptible to apoptosis induced by calcium ionophore A23187 treatment. S100A6-modulated cells with antisense and sense *S100A6* and mock control plasmid vectors were independently seeded in 96-well culture plates with 1×10^4 cells/well. Apoptosis was induced by adding gradually from 0 to 4 μM concentrations of calcium ionophore A23187, independently, and cell viabilities were measured by an MTS assay after 24 h. Each figure represented the mean ± SD of three independent experiments. $^*P < 0.001$ and $^\#P < 0.01$ versus mock control (**A**). The morphological changes of S100A6-modulated cells were examined after 36 h treatment with 4 μM calcium ionophore A23187 by 100-fold magnified microscopic observation (**B**).

When 0.5 μM of A23187 was added simultaneously to the Hep3B/+*S100A6* and Hep3B/−*S100A6* cells, the viability of the cells was 63.5% and 91.0%, respectively. This result suggests that an increased level of intracellular S100A6 enhances cell death when cells were stimulated with A23187.

The morphological change of the cells was evaluated by microscopic inspection as shown in Figure 2B. Most of the cells over-expressing S100A6 (Hep3B/+*S100A6*) died and the cells were eventually detached from the culture plate after 36 h in culture media containing 4 μM A23187. However, cells transfected with antisense *S100A6* (Hep3B/−*S100A6*) or mock control maintained relatively intact cell morphology under the same condition.

Cell Death in S100A6 Over-Expressing Cells Was Increased by Enhanced Sensitivity to Apoptosis

A significant decrease of cell viability in S100A6 over-expressing cells was caused by the increased sensitivity to apoptosis, which was verified by the studies on DNA content profiles and DNA fragmentation assay. DNA fragmentation assay has been known as a tool for evaluation of apoptotic progression. According to gel electrophoresis data, it

was revealed that internucleosomal DNA fragmentation with the smallest fragment of 180–200 bp in the ladder was distinctively detected in Hep3B/+*S100A6* cells when exposed to 2 μM of A23187 for 36 h (Fig. 3A). On the contrary, the intensity of the DNA fragment of Hep3B/−*S100A6* was lesser than other experimental group. Measurement of DNA content was also performed to determine whether S100A6 might have effects on DNA content which was associated with apoptosis. Each group of stable cell lines were exposed to 0–4 μM of A23187 for 24 h, and the change of DNA content during cell cycle progression was calculated by flow cytometric assay. According to the histogram of DNA content being presented in Figure 3B, Figure 3C shows that the presence of cells with fragmented DNA was indicated by a sub-G_1/G_0 peak of DNA content. Sub-G_1/G_0 population increased proportionally to the concentration of A23187 added. When treated with 4 μM of A23187, sub-G_1/G_0 population from Hep3B/+*S100A6* cells was increased up to 25.9%, but those from mock and Hep3B/−*S100A6* cells were increased by 15.3% and 8.9%, respectively. These results suggest that the upregulated expression of S100A6 makes cells more sensitive to apoptosis induced by A23187.

Sensitivity to Calcium Ionophore A23187-Induced Apoptosis of S100A6 Over-expressing Cells Was Reduced by Treatment With Antisense Oligodeoxynucleotides of S100A6

Cells transfected with sense DNA to *S100A6* (Hep3B/+*S100A6* cells) were more sensitive to calcium ionophore-induced apoptosis than antisense-transfected cells (Hep3B/−*S100A6*). The finding that enhanced sensitivity to cell death resulted from an increased level of intracellular S100A6 was further verified by the treatment of antisense phosphorothioate-oligodeoxyribonucleotide (S-ODN, 18-mer) of S100A6 into the Hep3B/+*S100A6* cells. Treatment with antisense S-ODN on Hep3B/+*S100A6* cells reduced the expression level of S100A6 (Fig. 4, inner box) as well as the sensitivity of the cells to apoptosis induced by A23187 (Fig. 4). The cell viability of the antisense S-ODN (AnCACY)-treated Hep3B/+*S100A6* group was increased compared to that of the scrambled S-ODN (ScCACY, control)-treated Hep3B/+*S100A6* group as calculated by 7–12%. This result again showed that the A23187-induced apoptosis could be influenced by expression level of S100A6.

Activity of Caspase-3 Was Increased in Proportion to the Expression Level of S100A6

The activities of capase-3 determined by their own cleavage, PARP, and Bid cleavage were generally enhanced by the treatment of A23187 as previously reported [Lotem and Sachs, 1998; Petersen et al., 2000; Popescu et al., 2001; Sennvik et al., 2001]. In Western blot analysis of caspase-3, the levels and cleaved form was distinctively decreased in cells with reduced S100A6 level (Hep3B/−*S100A6*) after treatment with 2 μM A23187 for 36 h (Fig. 5A). When the caspase-3 activity evaluated by detection of cleaved PARP of caspase-3, substrate protein also decreased in Hep3B/−*S100A6*. On the other hand, cleavage of caspase-3 and PARP were the most processed in Hep3B/+*S100A6*, the S100A6 upregulated cell lines. To further substantiate these data, caspase-3-like activity were measured by cleavage of AC-DEVD-AMC. As shown in Fig. 5B, a significant increase in caspase-3-like activity was measured in Hep3B/+*S100A6* cells after treatment with 2 μM A23187 for 24 h. Hep3B/−*S100A6* was a half measured of Hep3B/mock, control. The processing of caspase-3 and the appearance of the cleaved small fragments (20, 18, and 16 kDa) of caspase-3 coincided with an elevation and/or reduction of caspase-3-like enzyme activity in A23187-treated cells. From the results described above, we believe that the S100A6 is involved in cellular apoptosis by modulating activation of caspase-3. To further verify this hypothesis, we used Z-DQMD-FMK, a cell-permeable peptide inhibitor that specifically inhibits caspase-3-like enzyme activities [Talanian et al., 1997]. To verify that the S100A6-stable cell lines (Hep3B/mock, Hep3B/+*S100A6*, and Hep3B/−S100A6) underwent apoptotic cell death, cell cycle distribution was analyzed by flow cytometry. The cells were pretreated with 10 μM Z-DQMD-FMK, for 1 h, before treatment with 2 μM A23187 for 24 h. According to the histogram of DNA content being presented in Figure 5C, the presence of cells with fragmented DNA (apoptotic cells) was indicated by a sub-G_1/G_0 population (percentage). Apoptotic cell death induced by A23187 was largely prevented by Z-DQMD-FMK in the Hep3B/+*S100A6* cells compared with other

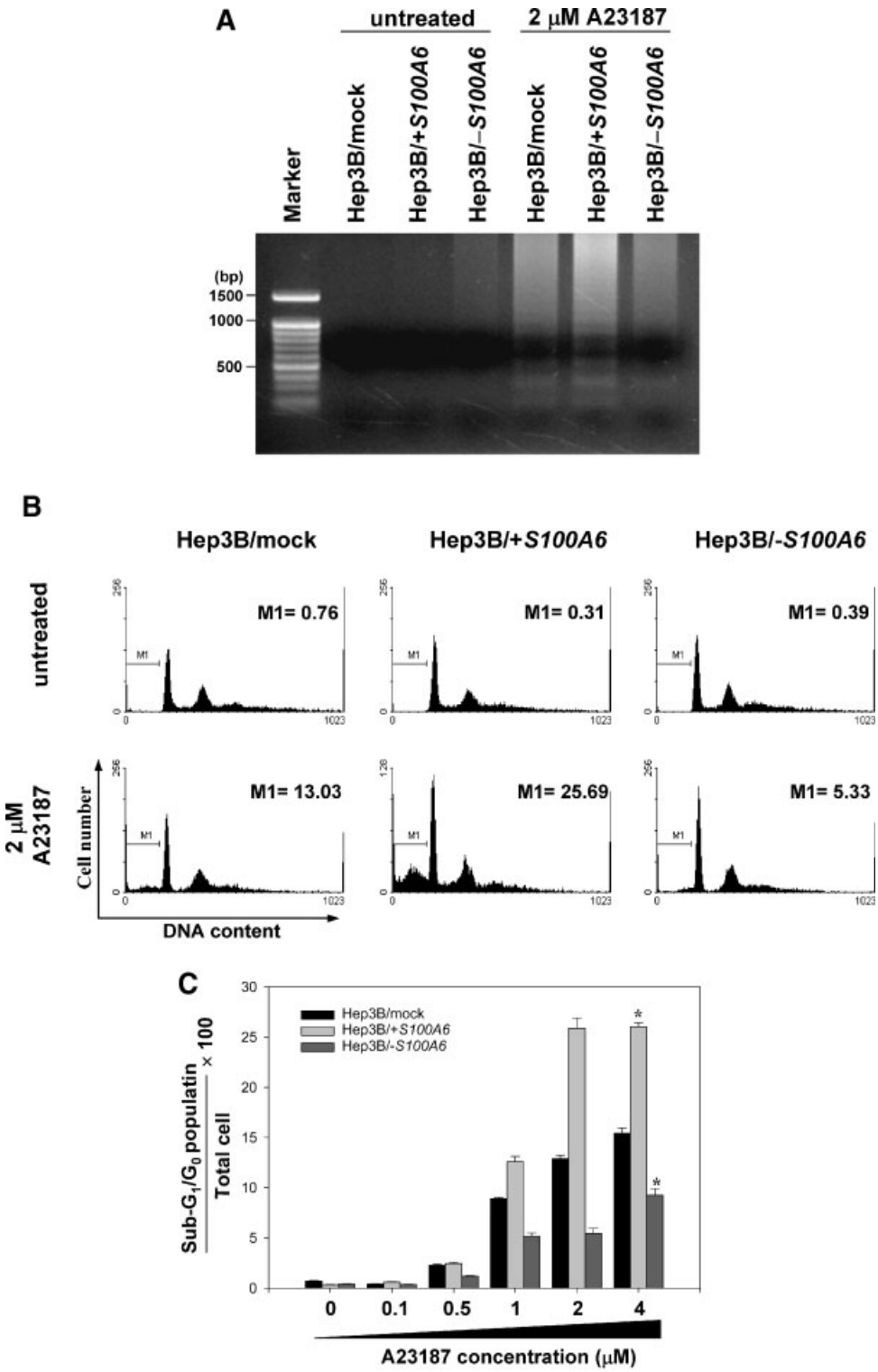


Fig. 3. DNA fragmentation assay and profiling of DNA content in S100A6-modulated cells. The cells stably transfected with plasmid vectors containing sense and antisense *S100A6* and control plasmid were treated with 2 μM calcium ionophore A23187 for 36 h, and genomic DNA was isolated from the cells (Hep3B/+*S100A6*, Hep3B/−*S100A6*, and mock). DNA fragmentation was analyzed by gel electrophoresis on 1% (w/v) agarose gel (**A**). Profiling of genomic DNA content in the established cell lines was assessed by a flow cytometer. The cells were harvested after treatment with calcium ionophore A23187 (0–4 μM) for 24 h and stained with propidium iodide. The distribution of genomic DNA was analyzed as described in Experimental Procedures. Populations of sub-G_1/G_0 phase indicating progressed apoptosis were analyzed, and the result was shown in histogram (**B**,**C**). Each value represented the mean ± SD of three independent experiments. *$P < 0.001$ versus mock control.

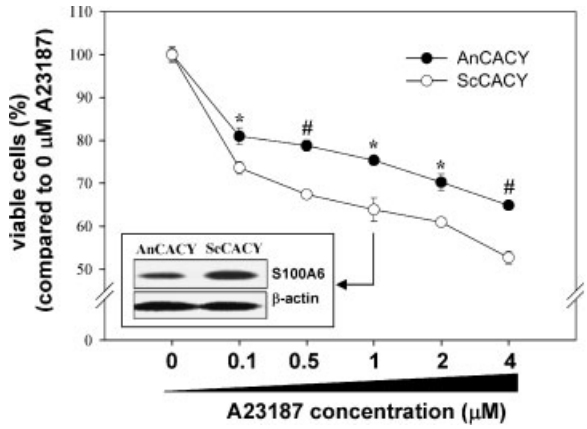


Fig. 4. The increased susceptibility of cells to apoptosis induced by over-expression of S100A6 was reduced by blocking the expression of S100A6. S100A6 over-expressed Hep3B/+*S100A6* cells were seeded in 96-well culture plates at 1×10^4 cells in 50 μl DMEM (10% FBS). After culturing for 24 h, the mixture of 0.5 μM antisense S100A6 S-ODN (AnCACY) and 1.8 μl of liposome or mixture of control scrambled S-ODN (ScCACY) and liposome was added into each well. After 12 h of culture, each S-ODN-treated cell was stimulated with calcium ionophore A23187 (0–4 μM) for 36 h, and the viability of S-ODN-treated cells was measured by MTS assay. Inset shows the levels of S100A6 mRNA of 1 μM A23187-treated group. It was measured by RT-PCR technique. The cell viability was presented as mean ± SD values of three independent experiments. $^*P < 0.001$ and $^\#P < 0.0001$ versus ScCACY-treated group.

experimental groups. These results confirm that the enhanced cell death of the cells over-expressing S100A6 results mainly from the caspase-3 activity.

Alteration of Apoptosis-Associated Molecules in S100A6-Modulated Cells: Expression Level of Caspase-3 Was Increased in Proportion to S100A6 Level

It has been found that an elevated level of intracellular S100A6 rendered higher apoptotic sensitivity to calcium stress via activation of caspase-3. However, in the cleavage pattern of caspase-3 (Fig. 5A), there are differences in the expression pattern of caspase-3 protein. To examine whether the modulation of S100A6 level affected the transcriptional activities of apoptosis-associated molecules or not, the expression level of apoptosis-associated molecules including caspase-3, -6, -7, -8, -9, Bcl-2, and Bax were studied in the cells with modified levels of S100A6 using the RT-PCR analysis. Figure 6A and B showed that the mRNA and protein levels of caspase-3 were modulated in proportion to the expression level of S100A6, of which the cellular expression level was controlled by the transfected sense or antisense S100A6 gene. Only caspase-3 exhibited meaningful differences in expression level among the molecules tested. Expression of caspase-3 was downregulated in cells transfected with antisense *S100A6* (Hep3B/−*S100A6*) and/or upregulated in sense *S100A6* (Hep3B/+*S100A6*)-transfected cells. These data suggest that the expression level of caspase-3 may be controlled by the intracellular level of S100A6. Here, we also examined whether expression of S100A6 and caspase-3 shows correlation in different liver cell lines. As shown in Figure 6C, S100A6 mRNA was highly expressed in Chang and Hep3B cells but lower in PLC/ARF/5 and HepG2 cells. The expression level of caspase-3 mRNA was in accordance with that of S100A6 mRNA.

Activation of Caspase-3 Promoter Was Confirmed by Introduced Wild-Type and Mutant S100A6

To verify modulation of caspase-3 transcription by the exogenous S100A6, we prepared two kinds of S100A6 mutant designed as a single amino acid-mutated protein with deficiency of calcium-binding capacity. Then we introduced the mutant into Hep3B cells. S100A6 is a Ca^{2+}-binding molecule with two EF-hands. While the C-terminal EF-hand displays a canonical 12 amino acid, N-terminal EF-hand usually referred to as the S100-hand, encompass 14 amino acids, and has a lower affinity for Ca^{2+} [Schafer and Heizmann, 1996; Donato, 2001]. The mutants used in this experiment were prepared by exchange of a nucleotide sequence in calcium-binding motif as the same method previously reported (Fig. 7A) [Kawasaki et al., 1998; Otterbein et al., 2002]. Thr (T) was replaced with an Ala (A) at amino acid position 28 of calcium-binding motif for mutant 1; Glu (E) was replaced with a Lys (K) at amino acid position 67 of calcium-binding motif for mutant 2. Each point-mutated gene named as M1 and M2, respectively, was subcloned into the mammalian expression vector pcDNA3.1 (Fig. 7B). Transcriptional activity of caspase-3 was examined through promoter analysis of rat caspase-3. Because it was well known to have a cross activity in a human cell line [Liu et al., 2002]. The 5′-flanking DNA sequence of the rat caspase-3 gene, between positions −1841 and +88, was cloned into pGL3-basic vector encoding luciferase reporter gene

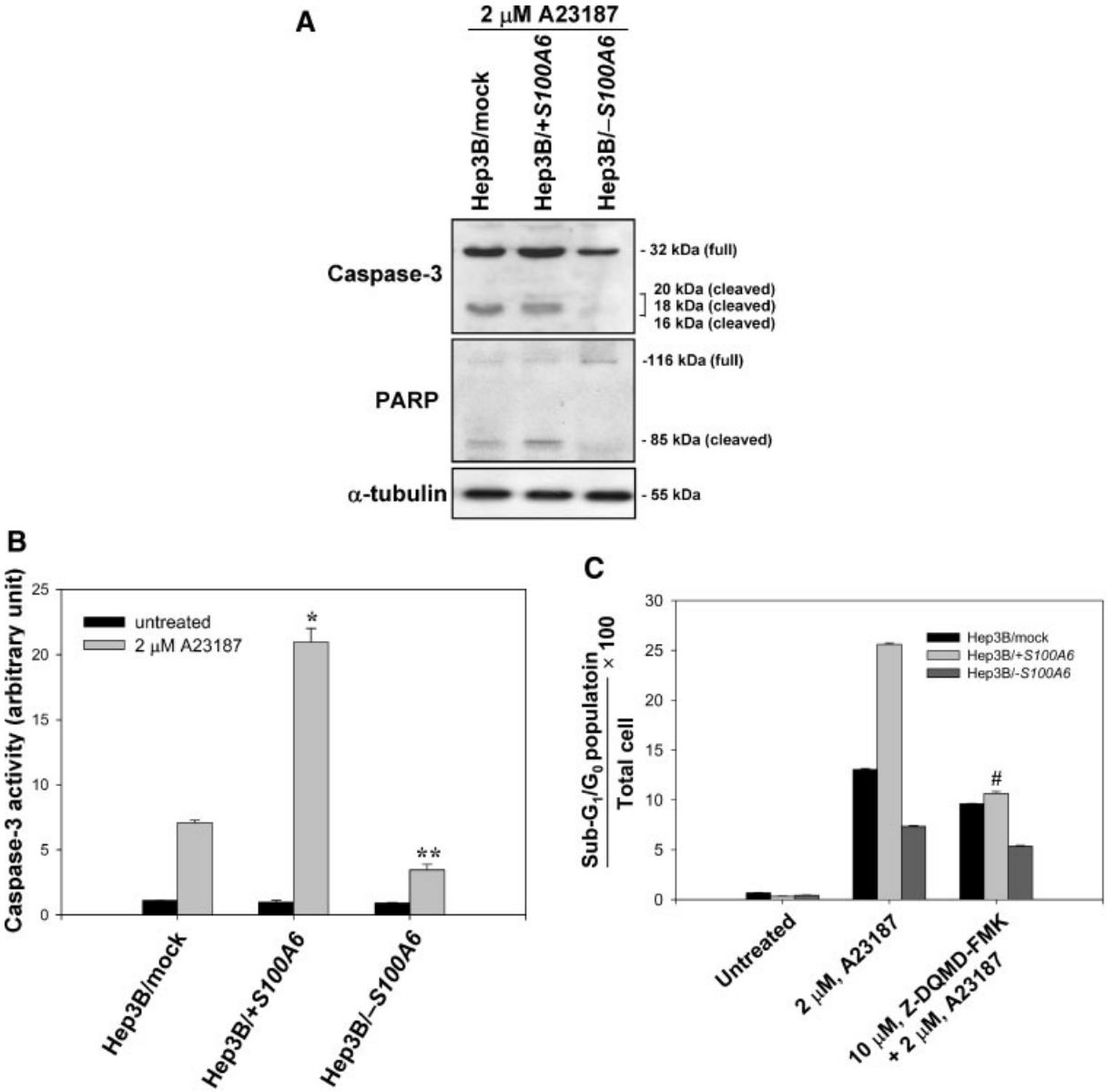


Fig. 5. Analysis of caspase-3 processing and activity induced by calcium ionophore A23187 in S100A6-modulated cells. The processing of caspase-3 determined by its own cleavage and PARP were generally enhanced by the treatment of A23187 as previously reported. Hep3B/+*S100A6*, Hep3B/−*S100A6*, and mock cell lines were treated with 2 μM calcium ionophore A23187 for 36 h and cell lysates were prepared. The cell lysates were subjected to 12% SDS–PAGE and electrophoretically transferred onto PVDF membrane. They were then incubated with specific antibodies to caspase-3, PARP, and α-tubulin (**A**). Caspase-3-like activity was determined as described in ''Materials and Methods'' using AC-DEVD-AMC (**B**). Each S100A6-modulated cells were pretreated with 10 μM Z-DQMD-FMK, caspase-3 inhibitor, for 1 h, before treatment of 2 μM A23187 for 24 h. To verify the apoptotic cell death, cell cycle distribution was analyzed by flow cytometry. The presence of cells with fragmented DNA (apoptotic cells) was indicated by a sub-G_1/G_0 population (percentage) (**C**). Data (mean ± SD) are from three independent experiments. $^{*}P < 0.0001$ and $^{**}P < 0.001$ versus Hep3B/mock (B). $^{\#}P < 0.0001$ versus 2 μM A23187-treated group (C).

(pGL3-caspase-3). The reporter gene and each mutated S100A6 gene were transiently co-transfected into Hep3B cells. As shown in Figure 7C, promoter activity of caspase-3 was elevated by 2.5-folds in wild-type S100A6 (pcDNA-S100A6/wt)-transfected cells than in the control vector (pcDNA-empty). But, single point-mutated S100A6 in calcium-binding motif could not activate efficiently the caspase-3 promoter as much as wild-type.

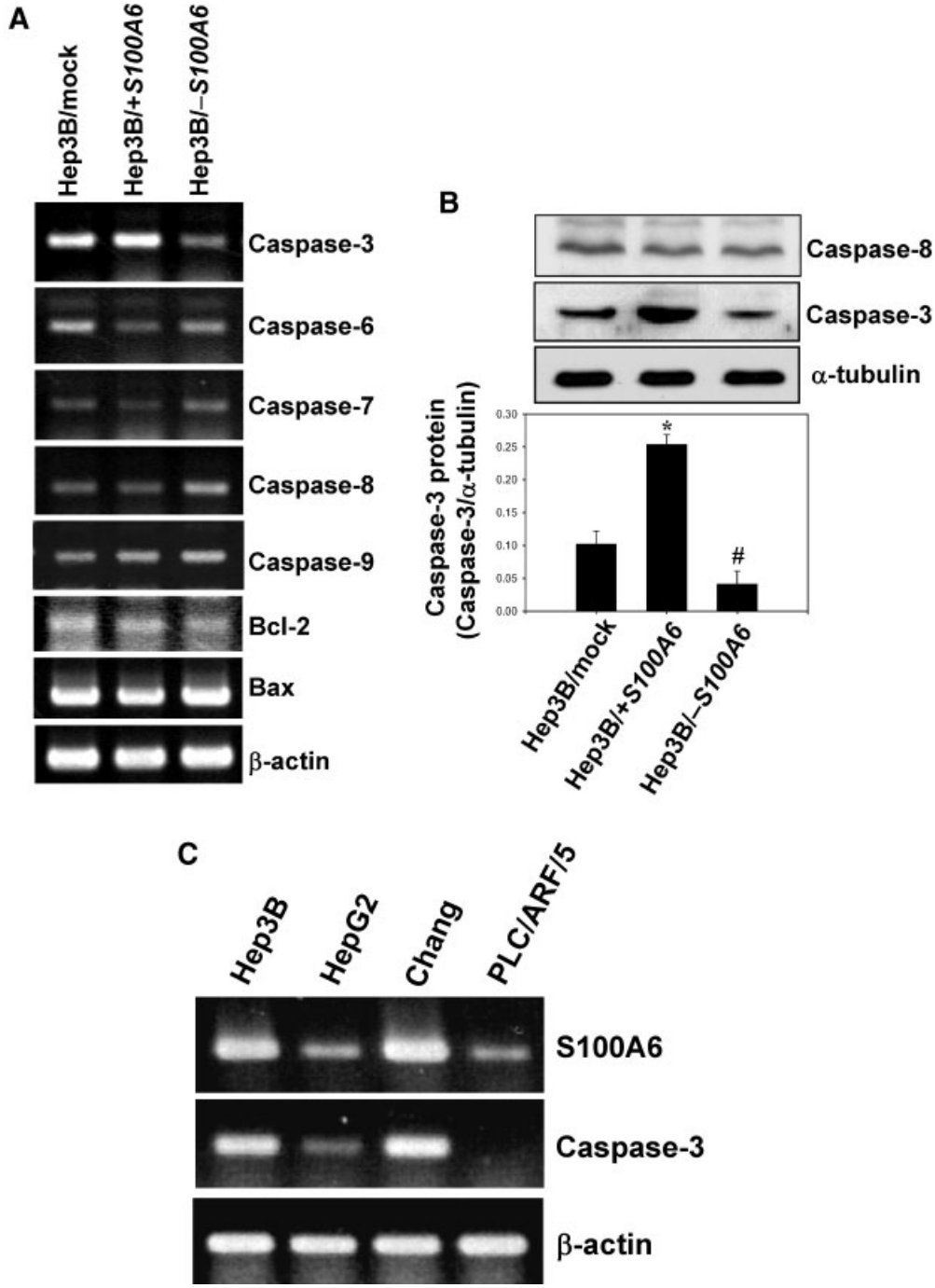
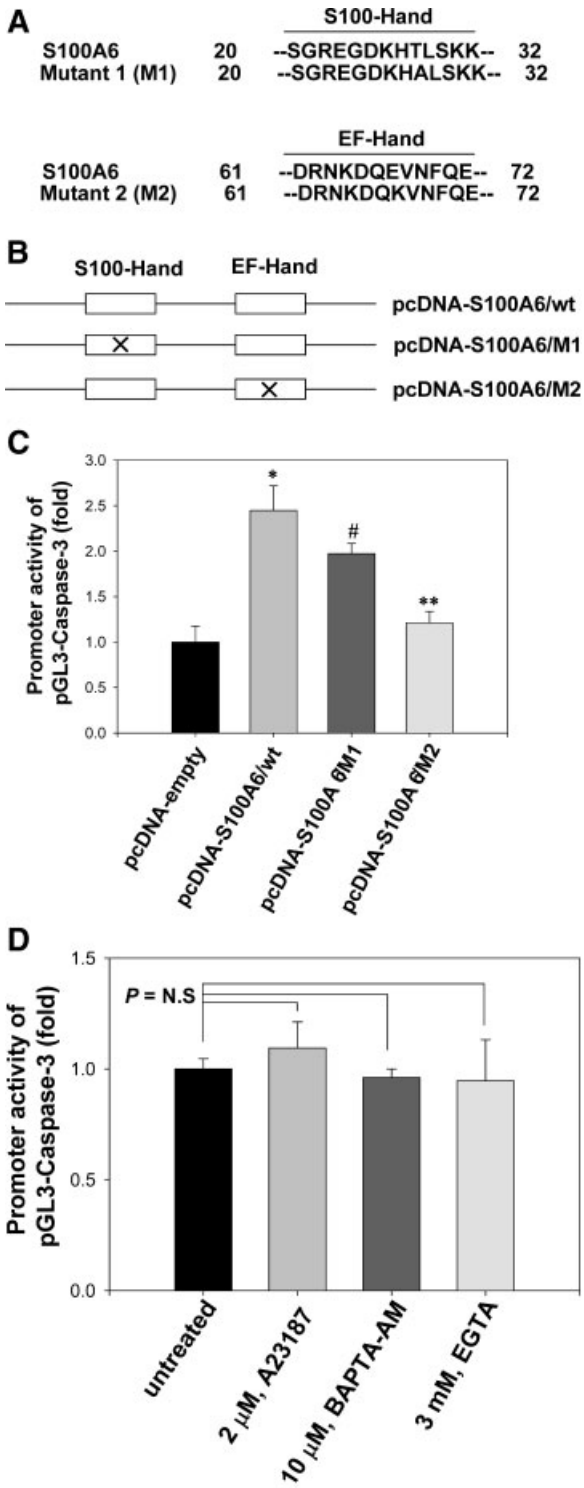


Fig. 6. Altered expression of ''executioners'' molecules of apoptosis in the S100A6-modulated cell lines. Total RNAs were extracted from the established cell lines (Hep3B/+*S100A6*, Hep3B/−*S100A6*, and mock), and reverse-transcribed to cDNA. *Taq* polymerase PCR was performed using specific PCR primer sets for the amplification of caspase-3, -6, -7, -8, -9, Bcl-2, and Bax as described in Experimental Procedures (**A**). The relative levels of caspase-3 proteins were determined by densitometric analysis of Western blot products as shown in panel (**B**). Expression level of S100A6 and caspase-3 mRNA in four human liver cell lines (Hep3B, hepatocellular carcinoma; HepG2, hepatocellular carcinoma; Chang, liver; PLC/ARF/5, hepatoma) accessed via RT-PCR. Each cell line cultured in DMEM in 10% FBS. β-Actin was employed as reference to normalize the quantity of reverse-transcribed mRNA in each cell line (**C**). The relative levels of caspase-3 protein were presented as mean ± SD (bars) values of three independent experiments. $^{*}P < 0.0001$ and $^{\#}P < 0.02$ versus Hep3B/mock control.

M2 (pcDNA-S100A6/M2) lost its ability of transcriptional activity much more than M1 (pcDNA-S100A6/M1).

In such experimental conditions, there is a possibility that modulation of calcium concentration by the transfected S100A6 could activate the caspase-3 promoter because S100 proteins have a function of intracellular calcium buffering [Donato, 2001]. To test that possibility, Hep3B cells were treated with several calcium level modifiers, A23187, BAPTA-AM, and EGTA. As shown in Fig. 7D, there are no significant differences in caspase-3 promoter activity. This result suggests that the activation of caspase-3 promoter might be resulted from S100A6 intrinsic function not from modification of intracellular calcium concentration.



DISCUSSION

In this study, we tried to elucidate the role of S100A6 in apoptotic process, apoptotic condition endowed by calcium ionophore A23187 was introduced into in vitro culture system of Hep3B cells after their modulation of cellular S100A6 level by transfection of the plasmids containing sense or antisense S100A6 genes. It was shown that the over-expression of S100A6 made Hep3B cells (Hep3B/+*S100A6*) more sensitive to apoptosis induced by calcium ionophore A23187. In contrast, the cells transfected with antisense *S100A6* (Hep3B/−*S100A6* cells) showed higher cell viability and lower degree of DNA fragmentation and sub-G_1/G_0 population than the cells transfected with mock control or Hep3B/+S100A6 cells (Fig. 3). In Figure 5b, it was shown that caspase-3-like activity was higher in Hep3B/+*S100A6* cells and lower in Hep3B/−*S100A6* cells when compared to mock control cells. The cytotoxic effect

Fig. 7. S100A6 activated caspase-3 promoter. S100A6 protein was mutated in the requisite sequence of calcium-binding motif. Thr (T) was replaced with an Ala (A) at amino acid position 28 for Mutant 1, or Glu (E) with a Lys (K) at amino acid position 67 for Mutant 2 (**A**). Each point-mutated gene (Mutant 1 and Mutant 2) was then subcloned into the mammalian expression vector pcDNA3.1, respectively (**B**). The 5′-flanking DNA sequence of rat caspase-3 region between positions −1841 and +88 were cloned into pGL3-basic vector, encoding the modified firefly luciferase as reporter gene. The reporter gene and each mutant-S100A6 (pcDNA-S100A6/M1 or pcDNA-S100A6/M2) were co-transfected into Hep3B cells, respectively. After 48 h, each cell was harvested and measured activity of caspase-3 promoter (**C**). Hep3B cells containing luciferase reporter were stimulated with several Ca^{2+}-level modifying chemicals such as 2 μM of A23187, 10 μM of BAPTA-AM, and 3 mM of EGTA for 24 h. Each cell was harvested and measured promoter activity (**D**). Data (mean ± SD) come from three independent experiments. $^{*}P < 0.001$ versus pcDNA-empty; $^{\#}P < 0.04$ versus pcDNA-S100A6/wt, $^{**}P < 0.001$ versus pcDNA-S100A6/wt. NS denotes not significant.

of calcium ionophore A23187 treatment on Hep3B/+*S100A6* cells was diminished by Z-DQMD-FMK. The results revealed that over-expressed S100A6 protein could influence the cellular apoptosis of Hep3B cells treated with calcium ionophore A23187 via regulation of caspase-3 activity. Another interesting fact observed was that the expression level of caspase-3 was increased proportionally to the increase of S100A6 level when assessed by RT-PCR, Western blot analyses (Fig. 6), and promoter analysis of caspase-3 (Fig. 7C). In the expression levels of other transcripts such as caspase-6, -7, -8, -9, Bcl-2, and Bax, there were virtually no differences found. Above results suggested a possibility that S100A6 was engaged in cellular apoptosis as a transcriptional activator of caspase-3. It has been suggested here, based on above findings that S100A6 protein had involved in the transcriptional control of caspase-3, an apoptosis-related gene. Again, these findings gave us a clue to directly relate the expression of S100A6 to cellular apoptosis of Hep3B.

The above results suggest a possibility that S100A6 is engaged in cellular apoptosis as a transcriptional activator of caspase-3. Of the 14 caspases identified in mammals, caspase-3 appears to be the major effector in cellular apoptosis triggered by various stimuli and many researchers concentrate the cleavage and activation of caspases. However, recent findings suggest that differential expression of the caspase-3 gene may underlie the regulation of apoptotic susceptibility during brain development as well as after acute injury to mature brain [Bittigau et al., 1999; Pohl et al., 1999; Hu et al., 2000; Yakovlev et al., 2001]. Furthermore, previous studies demonstrated significant increases in the transcriptional activity of the caspase-3 gene during neuronal cell apoptosis after various stimuli [Eldadah et al., 1997; Yakovlev et al., 1997]. In the breast cancer or cell lines, it was reported that over-expression of caspase-3 restores sensitivity for drug-induced apoptosis in the cell lines with acquired drug resistance [Friedrich et al., 2001]. Our results also showed that transcriptional level of caspase-3 could regulate the sensitivity for A23187-induced apoptosis in the liver cell line, and the expression level of caspase-3 could be modulated by the exogenous S100A6 introduction. Caspase-3 promoter analysis in Mutant S100A6-introduced cells also confirmed that expression of S100A6 modulate caspase-3 transcription with aids of calcium-binding moiety. As previously reported, C-terminal EF-hand has higher Ca^{2+}-affinity than that of N-terminal and it is assumed that Ca^{2+} binds first to the C-terminal EF-hand, this will probably result in a favoring Ca^{2+} binding to N-terminal EF-hand [Otterbein et al., 2002]. From our result also showed that C-terminal EF-hand of S100A6 might be more important than that of N-terminal for performing cellular functions in apoptotic process.

This study is the first report that intracellular function of S100A6 directly is involved in apoptotic process. Other S100 protein, such as S100B, induces apoptosis in PC12 cells, neuroblastoma cells, and primary embryonic rat neurons [Hu et al., 1997]. Also S100A6 was also found to show elevated expression in the brain of Alzheimer's disease (AD) patients [Hoyaux et al., 2000]. But additional studies to clarify the functional mechanism of this S100A6 over-expression were not known yet. So we thought that this report might be a clue to evaluate the concrete role of intracellular S100A6 protein in apoptotic process.

In our experiments, we induced the cell death using calcium ionophore A23187 which can activate caspase-12 or -3 after the treatment. Also calpain has a similar function on the signaling pathway [Wang, 2000]. Here, we have focused and showed the results that the increase of S100A6 protein is closely associated with the expression of caspase-3 confirmed by caspase-3 promoter analysis. Although it is still unknown whether S100A6 can directly bind to caspase-3 promoter region or not, we thought that this protein may be a co-factor or co-regulator of the key transcriptional factor regulating the caspase-3 expression.

Interestingly, we have seen that S100A6 protein was differentially expressed with tissue specificity, and its intracellular localization was apparently observed as different pattern like as expression in nucleus of HepG2 and cytoplasm in Hep3B (data not shown). Moreover, it was localized differently according to region of tumor tissue such as cells in outskirt region to be expected proliferating expressed S100A6 protein in the cytoplasm, meanwhile cells in inner region to be found apoptotic processing expressed it in the nucleus. Consistently, caspase-3 is highly expressed in the nucleus in that S100A6 localized. On the

contrary, caspase-3 expression is low when S100A6 is diffusely expressed in the cytoplasm (data not shown). From these results, we can imagine that S100A6 might be correlated to apoptotic function by caspase-3.

Next, we had a question that whether S100A6 can only regulate the expression of caspase-3 or other S100 proteins are not. When S100A1, S100A2, or S100A4 are transiently expressed in HepG2 cells, we had checked that these proteins didn't affect the expression of caspase-3 (data not shown).

The main aim of this study was to provide evidence that S100A6 is related with cell apoptosis in liver cell line. It is the first report that S100A6 has a possible role as an enhancer of apoptosis in some conditions via upregulation of caspase-3 activity and its expression. The molecular mechanism S100A6 in process of transcription activity should be elucidated.

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