Synthetic small interfering RNA targeting heat shock protein 105 induces apoptosis of various cancer cells both *in vitro* and *in vivo*

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We previously reported that heat shock protein 105 (HSP105), identified by serological analysis of a recombinant cDNA expression library (SEREX) using serum from a pancreatic cancer patient, was overexpressed in various human tumors and in the testis of adult men by immunohistochemical analysis. In the present study, to elucidate the biological function of the HSP105 protein in cancer cells, we first established NIH3T3 cells overexpressing murine HSP105 (NIH3T3-HSP105). The NIH3T3-HSP105 cells acquired resistance to apoptosis induced by heat shock or doxorubicin. The small interfering RNA (siRNA)-mediated suppression of HSP105 protein expression induced apoptosis in human cancer cells but not in fibroblasts. By a combination of siRNA introduction and doxorubicin or heat shock treatment, apoptosis was induced synergistically in a human colon cancer cell line, HCT116. *In vivo*, siRNA inoculation into the human gastric cancer cell line KATO-3 established in the flank of an NOD SCID mouse suppressed the tumor growth. This siRNA-induced apoptosis was mediated through caspases, but not the p53 tumor suppressor protein, even though the HSP105 protein was bound to wild-type p53 protein in HCT116 cells. These findings suggest that the constitutive overexpression of HSP105 in cancer cells is involved in malignant transformation by protecting tumor cells from apoptosis. HSP105 may thus be a novel target molecule for cancer therapy and a treatment regimen using synthetic siRNA to suppress the expression of HSP105 protein may provide a new strategy for cancer therapy. (*Cancer Sci* 2006; 97: 623–632)

Heat shock protein 105 (also called HSP110)[1] is a stress protein belonging to the HSP105/110 family that is expressed constitutively in most tissues at low levels, whereas HSP105 mRNA is expressed at high levels in mouse and rat brain. At the protein level, high expression levels have been reported only in the brain of mice.[1–4] Like other heat shock proteins, HSP105 plays an important role as a chaperone under physiological conditions. HSP105 is induced by various stressors and plays an important role in protecting cells from the cytotoxic effects mediated by such stressors. HSP105 is composed of an ATP-binding domain, a β-sheet, a loop and α-helical domains similar to those observed in the HSP70 family of proteins, and it binds to non-native protein substrates, thereby preventing the aggregation of denatured protein through an interaction with the β-sheet domain of HSP105.[2,5–7]

The rat neuronal cell line PC12 transfected stably with murine *HSP105* exhibited resistance to caspase-mediated apoptosis induced by stressors.[8] In a spinal and bulbar muscular atrophy model, the transient expression of tAR containing an expanded polyglutamine tract caused aggregates of polyglutamine in COS-7 and SK-N-SH cells and then induced the cells to undergo apoptosis. In contrast, in cells cotransfected with *tAR* and *HSP105*, both the aggregation of polyglutamine and the degree of it's cell toxicity decreased.[9] In contrast, HSP105 has demonstrated apoptosis-enhancing activity during murine embryogenesis.[10–13] These observations suggest that HSP105 is involved in the regulation of apoptosis.

We previously reported that HSP105 is overexpressed in various human tumors, including colon cancer cells but not colorectal adenomas, thus suggesting that the overexpression of HSP105 is a late event in the colorectal adenoma–carcinoma sequence.[14,15] Recent studies have also demonstrated that the expression level of HSP105 is elevated in highly metastatic colon cancer cell lines, and is correlated with advanced clinical stages and positive lymph node involvement.[16]

RNA interference is used widely for manipulating biological systems, and has also been utilized successfully as a therapeutic material in experimental animals.[17–19] Synthetic siRNA strongly inhibits the expression of target proteins when they are transfected with cationic liposomes, which are thought to be safer than viral vectors for human therapy. The local injection of synthetic siRNA against VEGF [20] or sphingosine 1-phosphate receptor-1[21] into established tumors has been reported recently to suppress angiogenesis and tumor growth.

The role of HSP105 in cancer cells has yet to be elucidated. In the present study, we first transfected the *HSP105* gene

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Abbreviations: CML, chronic myelocytic leukemia; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FCS, fetal calf serum; FITC, fluorescein-isothiocyanate; GFP, green fluorescent protein; HBSS, Hanks' balanced salt solution; HSP70, heat shock protein 70; HSP105, heat shock protein 105; IRES, internal ribosomal entry site; NIH3T3-HSP105, NIH3T3 cells overexpressing murine HSP105; PARP, poly ADP-ribose polymerase; PBS, phosphate-buffered saline; PI, propidium iodide; RT-PCR, reverse transcription–polymerase chain reaction; SEREX, serological analysis of a recombinant cDNA expression library; siRNA, small interfering RNA; tAR, truncated androgen receptor; VEGF, vascular endothelial growth factor.

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into NIH3T3 cells to examine the function of those cells, while also treating those transfectants with several stressors. To investigate whether the suppression of HSP105 expression affects the growth of cancer cells, we then introduced synthetic siRNA specific to HSP105 into several human cancer cell lines both *in vitro* and *in vivo*. We herein report that HSP105 has an anti-apoptotic function and that an overexpression of HSP105 is essential for cancer cells to survive.

Materials and Methods

Cell lines and culture

The human pancreatic cancer cell line PK8 and the murine fibroblast cell line NIH3T3 were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The human hepatoma cell line SK-Hep1, human colon cancer cell line SW620, and human gastric cancer cell lines KATO-3 and MKN28 were kindly provided by Dr K. Itoh, Kurume University (Kurume, Japan). The human colon cancer cell line HCT116 was kindly provided by Dr B. Vogelstein, Johns Hopkins University (Baltimore, MD, USA). Primary normal fibroblast strains Turu and Mori were kindly provided by Dr M. Yamaizumi, Kumamoto University (Kumamoto, Japan).

NIH3T3, HCT116, SW620, Turu and Mori were all cultured *in vitro* in DMEM, and SK-Hep1, PK8, KATO-3 and MKN28 were cultured in RPMI medium supplemented with 10% FCS and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C.

Mice

C57BL/6 NOD SCID mice were kindly provided by Dr S. Okada, Kumamoto University. The mice were maintained at the Center for Animal Resources and Development of Kumamoto University and they were handled in accordance with the animal care policy of Kumamoto University.

Plasmid construction and transfection

To obtain pCAGGS-IRES-neo-R, a DNA fragment containing an IRES, the neomycin-resistance gene *neo-R* was inserted into the mammalian expression vector pCAGGS. A cDNA fragment encoding the murine HSP105 protein was inserted into pCAGGS-IRES-neo-R. Cell transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations in six-well multiplates. At 48 h after transfection, the cells were replated and were then cultured in the presence of an appropriate concentration of G418 for 1 week.

RT-PCR analysis

Total RNA was isolated using either TRIZOL reagent (Gibco BRL, Rockville, MD, USA) or RNeasy spin column kits (Qiagen, Valencia, CA, USA). Total RNA from normal human colon tissue was purchased from Clontech (Palo Alto, CA, USA). RT-PCR analysis was carried out as described previously.[22] Briefly, 1 μg of total RNA was converted into cDNA in 20 μL of reaction buffer. Each PCR regime involved an initial denaturation step at 94°C for 5 min followed by 24–33 cycles for each type of cDNA. All samples were then processed at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min

The primer sequences were as follows: *HSP105* forward 5′-ATGAAGTGATGGAATGGATG-3′ and reverse 5′-TTTGGTTTCGGTTGTGTTAC; *NOXA* forward 5′-AGATGCCTGGGAAGAAG-3′ and reverse 5′-AGTCCCCTCATGCAAGT-3′; *PUMA* forward 5′-TGTAGAGGAGACAGGAATCCACGG-3′ and reverse, 5′-AGGCACCTAATTGGGCTCCATCTC-3′; *Bax* forward 5′-AGCGGCGGTGATGGACGGGTC-3′ and reverse 5′-TCCAAGGCAGCTGGGGCCTCA-3′; and *p53* forward 5′-CCATGGCCATCTACAAGCAG-3′ and reverse 5′-AGGGTGAAATATTCTCC-3′. PCR products were visualized by ethidium bromide staining after separation on a 2% agarose gel.

Detection of cell apoptosis

Cells in the early phase of apoptosis were detected by staining with annexin V, which binds to a phosphatidyl serine marker specific for the early phase of apoptosis, using an annexin V-FITC apoptosis detection kit (BioVision, Mountain View, CA, USA). The NIH3T3-mock cells and NIH3T3-HSP105 cells were incubated at 45°C for 90 min or with 200 ng/mL doxorubicin. The cancer cell lines were treated with either 100 nM or 200 nM siRNA, with 100 nM siRNA and 200 ng/mL doxorubicin or with 100 nM siRNA and heat shock (45°C, 30 min). The cells were harvested at the times indicated and were then stained with FITC-conjugated annexin V for flow cytometric analysis according to the manufacturer's recommendations. In the caspase inhibition assay, 100 μM Z-VAD-FMK (Sigma, St Louis, MO, USA) was added 1 h before siRNA transfection.

To detect the late phase of apoptosis, DNA fragmentation in the cells was evaluated by staining with PI and flow cytometric analysis as follows: NIH3T3-mock cells and NIH3T3-HSP105 cells treated with doxorubicin were collected by tripsinization, washed with PBS, fixed in cold 70% ethanol, and stored at −20°C until staining. After fixation the cells were washed in PBS and incubated with 100 μg/mL of RNaseA for 30 min at room temperature, before staining with 25 μg/mL of PI. Flow cytometry was carried out using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) and the data were analyzed using CellQuest software (BD Biosciences).

Immunoprecipitation and western blot analysis

The cell samples were lysed in appropriate amounts of lysing buffer (200 mM NaCl, 20 mM Tris [pH 7.4], 1% Nonidet P-40, 1 mM sodium orthovanadate [WAKO, Osaka, Japan], 10% glycerol, plus one protease inhibitor tablet [Roche Applied Sciences, Penzberg, Germany]). Hsp105 and p53 were immunoprecipitated with rabbit polyclonal anti-HSP105 antibody and mouse monoclonal anti-p53 antibody (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively, together with protein A beads (Pierce, Rockford, IL, USA). The proteins were analyzed using 7% or 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis and were then transferred onto a nitrocellulose membrane. HSP105 was blotted with the above-described antibody and PARP was blotted with rabbit polyclonal anti-PARP antibody (Santa Cruz Biotechnology). p53 was blotted with DO-1 or DO-1 and labeled with biotin using the Mini-biotin-XX Protein Labeling Kit (Molecular Probes, Eugene, OR, USA). Phospho-p53 (Ser46) and phospho-p53 (Ser15) were blotted with rabbit polyclonal antibody specific to phospho-p53 (Ser46) and mouse monoclonal antibody specific to phospho-p53 (Ser15) (New England Biolabs, Beverly, MA, USA), respectively, and then with horseradish peroxidase-conjugated rabbit antimouse IgG or donkey antirabbit IgG

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(Amersham Biosciences, Piscataway, NJ, USA), respectively, as secondary antibodies. The bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

siRNA and *in vivo* siRNA treatment

The siRNA duplexes were purchased from Dharmacon Research (HSP105-siRNA and luciferase; Lafayette, CO, USA), Qiagen (GFP) and Invitrogen (HSP105-siRNA-2). The siRNA sequences used were as follows: HSP105, UUGGCUGCAACUCCG-AUUGTT; HSP105-siRNA-2, UGUACAUUACCUUUAUU-CCAACUCC; luciferase, CGUACGCGGAAUACUUCGATT; and GFP, GCAAGCUGACCCUGAAGUUCA. The transfection of siRNA oligonucleotides was carried out using oligofectamine (Invitrogen) according to the manufacturer's recommendations in a six-well plate.

KATO-3 cells (2×10^6) were suspended in 100 μL of HBSS solution (Gibco, Langley, OK, USA), injected subcutaneously into the dorsal skin of NOD SCID mice and were then allowed to grow. After 10 days, siRNA solutions were injected locally into tumors every third day. siRNA solutions were prepared by incubating 1 nmol of siRNA and 20 μL of oligofectamine in 5% glucose solution for 15 min at room temperature. The tumor volume was calculated using the equation

$$V = (L \times W^2) \times 0.5,$$

where V = volume, L = length and W = width.

Statistical analysis

The two-tailed Student's *t*-test was used to determine the statistical significance of differences in the percentage of the cell fraction evaluated by flow cytometric analysis, and in tumor size between the treatment groups. A value of $P < 0.05$ was considered to be significant. Statistical analyses were carried out using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA, USA).

Results

NIH3T3-HSP105 cells acquired resistance to apoptosis induced by stressors

To elucidate the biological function of HSP105 in cancer cells, we first transfected the murine HSP105 expression vectors or an empty vector into murine embryonal fibroblast NIH3T3 cells, which are known to express smaller amounts of HSP105 than cancer cells. Subsequently, NIH3T3-mock cell lines and NIH3T3-HSP105 cell lines overexpressing HSP105 protein were established after selecting cells with G418 (Fig. 1A). There was no difference in either the morphology or the proliferative characteristics between these two cell lines cultured in DMEM supplemented with 1, 5 or 10% FCS (data not shown).

In a previous study, Hatayama *et al*. reported that rat neuronal cells overexpresing murine HSP105 were able to avoid undergoing apoptosis induced by several kinds of stressors.[8] We therefore examined the anti-apoptotic effects of HSP105 overexpression in NIH3T3-HSP105 cells. We exposed NIH3T3-mock cells and NIH3T3-HSP105 cells to heat shock at 45°C for 90 min and then detected annexin V-positive early apoptotic cells by flow cytometric analysis. The proportion of annexin V-positive NIH3T3-HSP105 cells was smaller than that of NIH3T3-mock cells ($P < 0.01$) (Fig. 1B).

We further treated these two cell lines with 200 ng/mL doxorubicin, which is known to be an inducer of apoptosis. After incubating NIH3T3-mock cells with doxorubicin for 12 h, annexin V-positive cells were detected by flow cytometric analysis. The number of annexin V-positive cells increased gradually thereafter, and almost all cells were stained with annexin V by 24 h after the treatment. However, even after 36 h incubation of cells with doxorubicin, only a small fraction (<18%) of NIH3T3-HSP105 cells were stained with annexin V. The difference in the number of annexin V-positive cells at 48 h after treatment was statistically significant ($P < 0.001$) between NIH3T3-mock and NIH3T3-HSP105 (Fig. 1C).

We next examined the DNA fragmentation of those two cell lines, which is observed during the late phase of apoptosis by staining with PI. The percentage of NIH3T3-HSP105 cells exhibiting DNA fragmentation was less than that of NIH3T3-mock cells, and the difference was statistically significant at both 36 h ($P < 0.001$) and 48 h ($P < 0.0001$) after doxorubicin treatment (Fig. 1D). These data suggest that HSP105 has an anti-apoptotic effect against apoptosis induced by heat shock and doxorubicin.

HSP105 siRNA induced various human cancer cell lines to undergo apoptosis

We previously reported that HSP105 protein is overexpressed in various human tumors and in the testis of normal adult men, but not in colon adenoma, by immunohistochemical analysis.[15] We thus examined the function of HSP105 protein in cancer cells by downregulating *HSP105* gene expression with siRNA. We used two human colon cancer cell lines, HCT116 and SW620, in which the expression of HSP105 mRNA was significantly elevated in comparison to that in normal human colon epithelium (Fig. 2A). At approximately 24 h after transfection, the adherent HCT116 cells treated with HSP105 siRNA started peeling off, and almost all of the cells had peeled off and were observed as dying cells at 48 h after transfection. In contrast, most of the HCT116 cells treated with luciferase siRNA proliferated normally (Fig. 2B). In a western blot analysis of HCT116 cells, the expression of HSP105 protein was markedly suppressed after treatment with two different HSP105-specific siRNA (HSP105-siRNA and HSP105-siRNA-2), and those cells were significantly stained with FITC–annexin V based on flow cytometric analysis (Fig. 2B,C).

Similarly, in SW620 cells after treatment with HSP105-siRNA, the expression of HSP105 protein was suppressed and a significant number of annexin V-positive cells were detected in proportion to the concentration of siRNA administered to the SW620 cells. The siRNA effect was more notable at 24 h than at 48 h after siRNA treatment in SW620 (Fig. 2C). This finding may be associated with the fact that the expression of HSP105 in SW620 was much higher than any of the other cancer cell lines tested (Fig. 3A). In addition, in other cancer cell lines, including the human hepatoma cell line SK-Hep1, human pancreatic cancer cell line PK8, and two human gastric cancer cell lines KATO-3 and MKN28, HSP105 protein expression was suppressed and all of these cell lines underwent apoptosis at 48 h after transfection of HSP105 siRNA (Fig. 2D). These data indicate that the suppression of HSP105 protein expression by siRNA can thus induce human cancer cells originating from various tissues to undergo apoptosis.

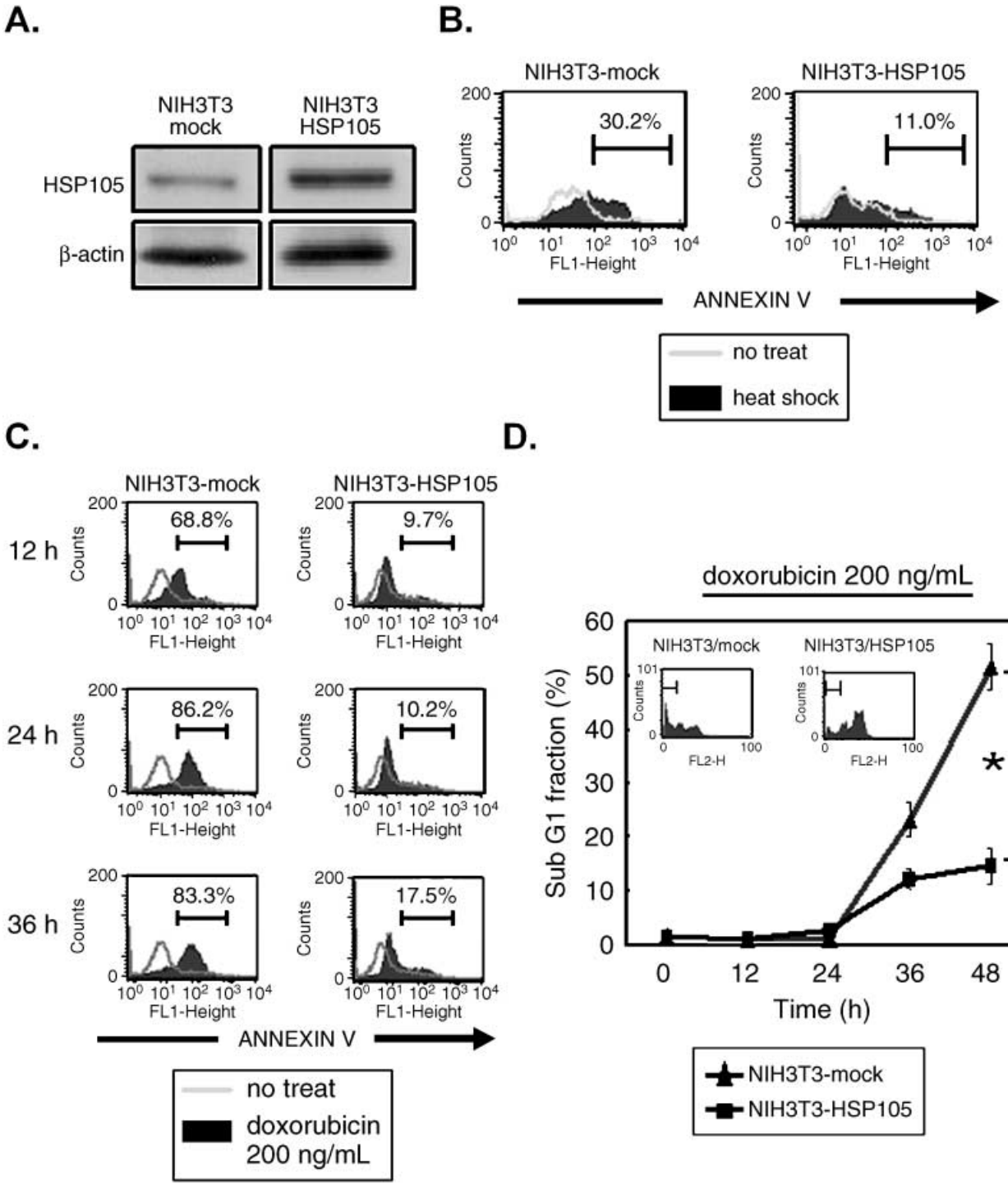

Fig. 1. Anti-apoptotic effect of HSP105 overexpression on NIH3T3 cells. (A) Western blot analysis of HSP105 expressed in NIH3T3 cells transfected with pCAGGS-IRES-neo-R or pCAGGS-IRES-neo-R-HSP105. β-Actin is shown as a control for the equal loading of protein. (B–D) Flow cytometric analyses of apoptotic cells. NIH3T3-mock cells and NIH3T3-HSP105 cells were treated at 45°C for 90 min (B) or with 200 ng/mL doxorubicin (C,D). To detect early apoptotic cells, the cells were harvested at the times indicated, stained with fluorescein-isothiocyanate–annexin V and analyzed by flow cytometry (C,D). These data are representative of at least three independent experiments. Percentages shown in the panel indicate percentage of annexin V-positive cells in heat-treated cells (B) and doxorubicin-treated cells (C). (D) Detection of DNA fragmentation by propidium iodide staining. The percentage of sub-G_1 fractions at the times indicated is shown, and the representative data of flow cytometric analysis at 48 h is shown in the panel. Data are mean ± SD ($n = 3$). The asterisk indicates that the difference in the percentages of the sub-G_1 fractions is statistically significant between the two values indicated by lines ($P < 0.001$).

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HSP105 siRNA did not induce human fibroblasts to undergo apoptosis

HSP105 is a stress-induced protein that is usually expressed ubiquitously at low levels, except in the brain and testis. To investigate the effect of HSP105 siRNA on normal cells, we applied HSP105 siRNA to human fibroblasts, Turu and Mori, generated from healthy donors. In western blot analysis, the level of HSP105 protein expressed in the Turu and Mori cells was lower than in the cancer cell lines (Fig. 3A). The expression of HSP105 protein was suppressed with HSP105 siRNA treatment, as shown in Fig. 3B,C, and the shape of fibroblast cells changed from fusiform to a round shape, but those cells were not induced to undergo apoptosis. Approximately 10 days after HSP105 siRNA transfection, both the expression of HSP105 protein and cell shape were restored (data not shown). These results indicate that the suppressive effect of HSP105 siRNA on the HSP105 expression is transient and reversible, and that the marked reduction of HSP105 protein does not have any harmful effect on normal fibroblasts under non-stressed conditions.

HSP105 siRNA treatment suppressed the growth of tumors overexpressing HSP105 *in vivo*

Small interfering RNA treatment using liposomes suppresses tumor growth *in vivo*.[21,23] To elucidate the effects of HSP105 siRNA on growing tumors *in vivo*, we injected either HSP105 siRNA or irrelevant siRNA locally into 5–7 mm KATO-3 tumors transplanted in NOD SCID mice every 3 days. As shown in Fig. 4, HSP105 siRNA suppressed tumor growth significantly in comparison to the irrelevant siRNA ($P < 0.01$). On day 15 after the first injection of HSP105 siRNA into tumors, the volumes of the tumors remained almost the same as those on day 0. During this observation period, neither abnormal behaviors nor neurological abnormalities were observed in these HSP105 siRNA-treated mice, and the expression of HSP105 protein in the brain was not suppressed by immunohistochemical analysis (data not shown).

Synergistic effects of siRNA and doxorubicin or heat shock on the *in vitro* induction of apoptosis in tumor cells

For the treatment of cancer patients with advanced, unresectable or recurrent focus, chemotherapy, radiation and other therapies are applied singly or in combination. To investigate the feasibility of combined treatment of tumor cells with HSP105 siRNA and other treatments, we treated HCT116 cells with HSP105 siRNA and either heat stress or doxorubicin. At 12 h after siRNA transfection into HCT116 cells, we added 200 ng/mL doxorubicin or treated the cells with heat shock at 45°C for 30 min, and detected the presence of apoptotic cells by staining with annexin V after 36 or 24 h incubation, respectively. As shown in Fig. 5, both combined treatments synergistically induced HCT116 cells to undergo apoptosis in comparison with the single treatment ($P < 0.001$).

Apoptosis induced by HSP105 siRNA was dependent on the caspase cascade but not on the p53 pathway

To investigate whether caspases are involved in apoptosis induced with siRNA, we examined PARP cleavage by western blot analysis using HCT116 cells with wild-type p53. PARP, a nuclear enzyme involved in DNA repair, is a well-known substrate for caspase-3, and is cleaved from a 112-kDa protein to an 85-kDa protein by the activation of caspase-3. As shown in Fig. 6A, cleavage of PARP was observed in those cells transfected with HSP105 siRNA. Moreover, this cleavage was completely inhibited by adding a pan-caspase inhibitor, Z-VAD-FMK. As a result, the apoptosis induced by HSP105 siRNA was also inhibited by Z-VAD-FMK.

We next examined whether the p53 tumor suppressor protein is involved in HSP105 siRNA-induced apoptosis. The p53 protein is located upstream of the caspase cascade and is associated with heat shock proteins such as HSP70 and HSP90.[24] As shown in Fig. 6C, the HSP105 and p53 proteins were coimmunoprecipitated with anti-HSP105 antibodies and the DO-1 in non-treated HCT116 cells. These results indicate that a proportion of HSP105 protein is bound to p53 protein in non-treated HCT116 cells. Furthermore, the expression of p53 protein decreased with HSP105 siRNA treatment at the post-transcriptional level (the mRNA expression of p53 was not suppressed), and p53 protein was not phosphorylated at serine 15 or serine 46 by this treatment (Fig. 6D,E). Furthermore, to confirm the suppression of p53 transcriptional activity, the mRNA expression of Bax, NOXA and PUMA, which are the transcriptional targets of p53-mediated apoptosis, were assessed by RT-PCR and found to be suppressed (Fig. 6E). These data suggest that HSP105 protein is thus associated with wild-type p53 protein in HCT116 cells under non-stress conditions, and HSP105 siRNA-induced apoptosis is dependent on caspases but independent of the p53-mediated apoptosis pathway.

Discussion

In the present study, we obtained the following results: (1) HSP105 protein protects tumor cells from apoptosis; (2) constitutive overexpression of HSP105 protein is essential for the survival of various kinds of cancer cells; and (3) apoptosis induced with HSP105 siRNA treatment is dependent on caspases but not p53.

Recent studies, including ours, have shown that HSP105 is overexpressed in various human tumors and that HSP105 is thus speculated to be involved in both tumorigenesis and protection of cells from apoptosis.[8,14,15] Our data obtained using NIH3T3-HSP105 cells are consistent with the findings of a recent study on neuronal PC12 cells in which the overexpression of HSP105 did not affect the growth rate of PC12 cells, but the apoptosis induced by stressors was inhibited in those cells.[8] These data indicate that HSP105 is involved in tumorigenesis through protection of cells against apoptosis.

Among the heat shock proteins, HSP70 is well characterized and HSP105 shares functional properties with HSP70. HSP70 also inhibits apoptosis induced by various stimuli.[25–27] Furthermore, HSP70 is also overexpressed in human breast cancer.[28–31] One difference in function between HSP70 and HSP105 is that HSP105 has an increased capacity to bind to denatured polypeptides in comparison to HSP70.[32] In addition, HSP105 suppresses the aggregation of denatured proteins under stress conditions in the presence of ADP, whereas HSP70 suppresses it in the presence of ATP.[7] In breast cancer cell lines, inhibition of HSP70 expression by antisense cDNA causes those cells to undergo apoptosis, and this action is

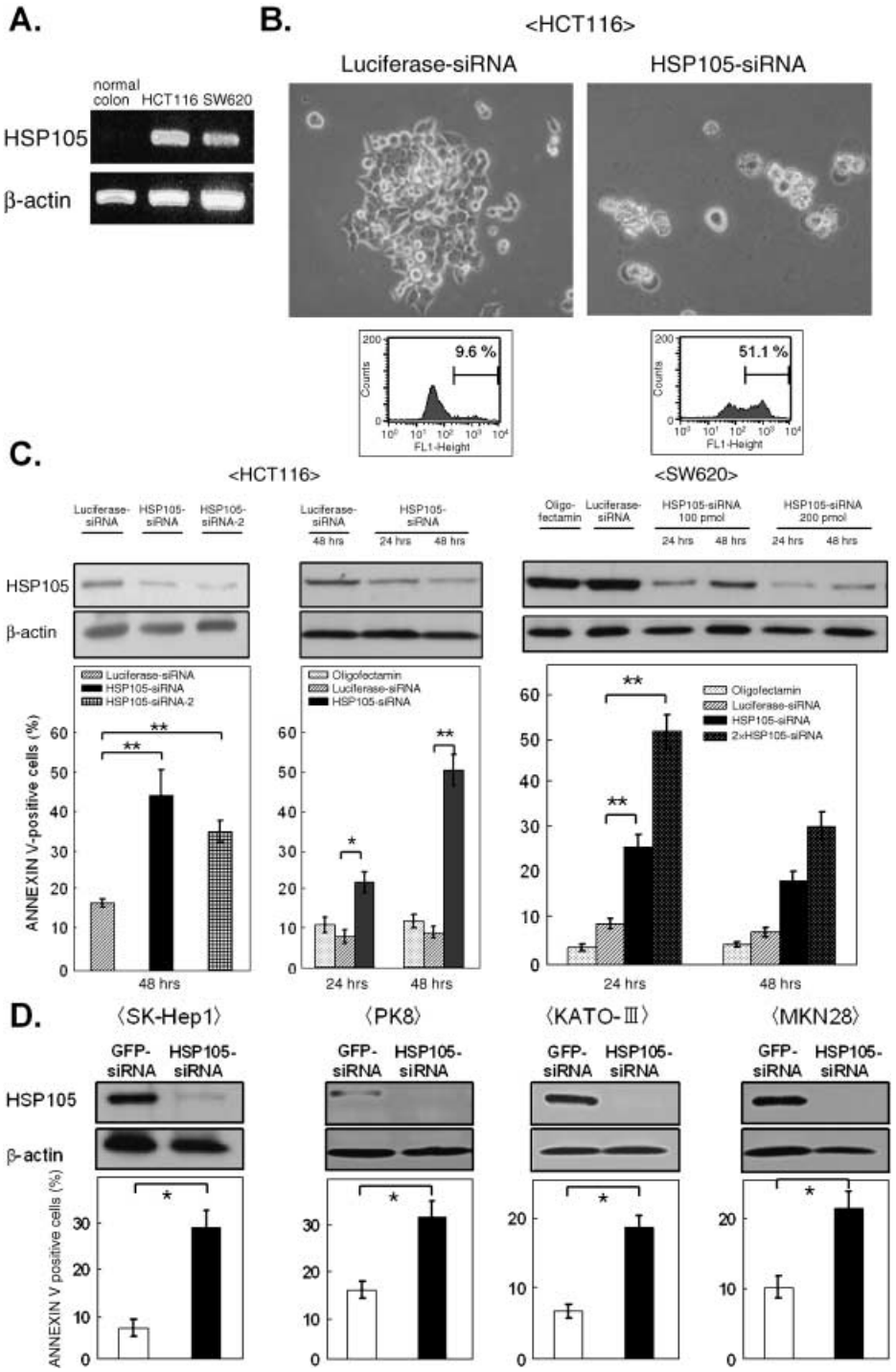

Fig. 2. Small interfering RNA (siRNA)-mediated inhibition of HSP105 expression enhanced the apoptotic cell death of human cancer cell lines. (A) Reverse transcription–polymerase chain reaction analysis of HSP105 mRNA expression in normal human colon epithelium, and in human cancer cell lines HCT116 and SW620. (B) Light microscopic pictures of HCT116 cells introduced with or without siRNA and representative flow cytometric analysis data of apoptotic cells stained with annexin V at 48 h after transfection. (C,D) Western blot analysis of HSP105 protein expression and flow cytometric analysis of apoptotic cells. HCT116 cells and SW620 cells were treated with oligofectamine, control siRNA, HSP105-siRNA (100 nM or 200 nM) or HSP105-siRNA-2 (B,C). (C) For western blot analysis, the cells were lysed at 24 or 48 h after transfection and analyzed. β-Actin is shown as a quantitative control. For flow cytometric analysis, cells were harvested at 24 or 48 h after transfection and then stained with fluorescein-isothiocyanate–annexin V and analyzed by flow cytometry. (D) Western blot analysis of HSP105 protein expression in cancer cell lines including SK-Hep1, PK8, KATO-3 and MKN28, and flow cytometric analysis of apoptotic cells at 48 h after transfection with 100 nM green fluorescent protein siRNA (□) or HSP105 siRNA (■). Data are the mean of three independent experiments ± SD. The asterisks indicate that the differences in the percentages of annexin V-positive cells are statistically significant between the two values indicated by lines (*$P < 0.01$; **$P < 0.001$).

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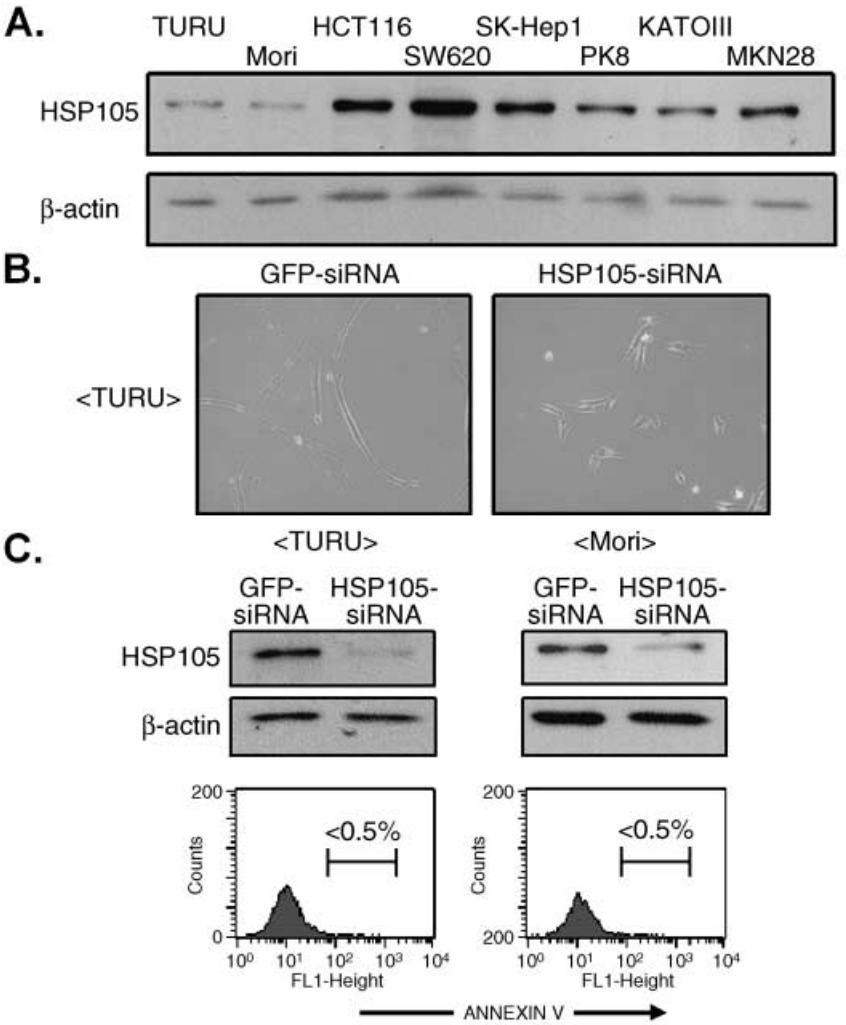

Fig. 3. No apoptosis-inducing effects of HSP105 small interfering RNA (siRNA) on human fibroblasts. (A) Western blot analysis of HSP105. The lysates of human fibroblasts Turu and Mori, and human cancer cell lines HCT116, SW620, SK-Hep1, PK8, KATO-3 and MKN28 were used and blotted with an anti-HSP105 antibody. β-Actin is shown as a quantitative control. (B) Light microscopic pictures of Turu at 72 h after transfection with 100 nM green fluorescent protein (GFP) siRNA or HSP105 siRNA. (C) Effects of siRNA on Turu and Mori. Western blot analysis of HSP105 and flow cytometric analysis of apoptotic cells detected by annexin V staining at 72 h after transfection of 100 nM GFP siRNA or HSP105 siRNA. These data are representative of at least three independent experiments. The percentages shown in the panel indicate percentage of annexin V-positive cells in HSP105 siRNA-treated cells.

independent of caspases.[33] In the present study, transfection of HSP105 siRNA caused HCT116 cells to undergo apoptosis in a caspase-dependent manner without suppressing the expression of HSP70 protein (data not shown). Our data suggest that HSP105 has a different character regarding caspase dependency in comparison to HSP70.

In the present study, HSP105 siRNA transfection induced various cancer cell lines to undergo apoptosis. These observations raise the question of how such apoptosis is induced. One possible explanation is that suppression of HSP105 activates the apoptotic pathway mediated by the p53 tumor suppressor protein. Molecular chaperones such as HSP70 and HSP90 are overexpressed in various tumor cells,[34] associating with wild-type or mutated p53 tumor suppressor proteins. Such heat shock proteins mediate stabilization, cytoplasmic sequestration and localization of p53 proteins.[35,36] In our study, HSP105 protein was bound to wild-type p53 in HCT116 cells under non-stress conditions, as shown in Fig. 6B. However, when the expression of HSP105 protein was suppressed with HSP105 siRNA, the expression of p53 protein also decreased and the p53-mediated apoptotic pathway was not activated (Fig. 6C,D). These results suggest that HSP105 stabilizes the p53 protein and protects it from degradation, but the apoptosis induced by HSP105 siRNA treatment is not mediated by the p53-dependent apoptotic pathway. We herein observed that every cancer cell line with wild-type p53 (HCT116), mutated p53 (SW620) or without p53 (KATO-3) was induced to undergo apoptosis. Further studies are needed to elucidate the biological significance of the interaction between HSP105 and p53 protein in cancer cells.

The second possible mechanism of cancer cell apoptosis induced with HSP105 siRNA treatment is the involvement of ER stress. Heat shock proteins have housekeeping functions, such as folding and degradation of various proteins. ER stress, induced by the accumulation of unfolded or malfolded proteins, induces the unfolded protein response, characterised by the induction of chaperones, the translation block and ER-associated degradation. However, if such degradation is not sufficient, then prolonged ER stress activates various apoptotic pathways, including caspase activation.[37–39]

Abnormal protein aggregation has been suspected to cause many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and trinucleotide repeat disease. In the brain in Alzheimer's disease, HSP90 facilitates the clearance

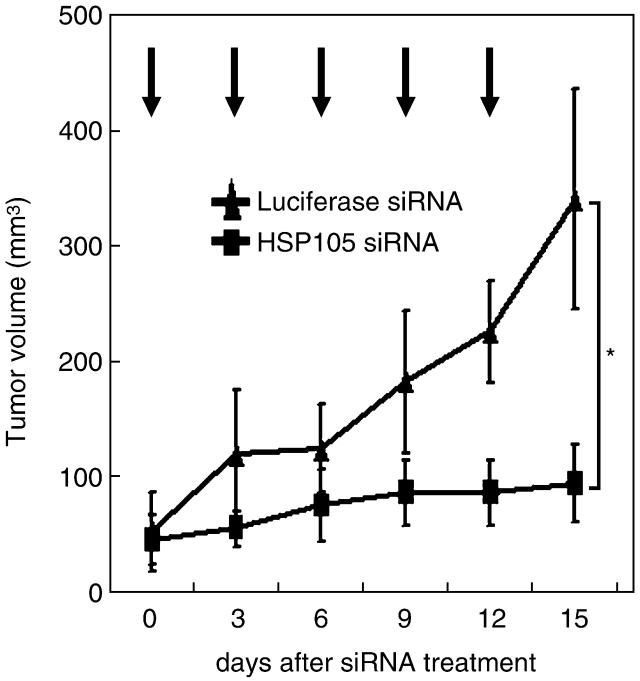

Fig. 4. The inhibitory effect of HSP105 small interfering RNA (siRNA) on the growth of established tumors in mice. (A) KATO-3 cells (2×10^6) were implanted subcutaneously into the dorsal skin of NOD SCID mice to establish growing tumors, and siRNA was injected into the tumors every 3 days (indicated by arrows). The tumor volume was measured and plotted (luciferase siRNA, ▲ HSP105-siRNA, ■). Data are mean ± SD ($n = 4$). The asterisk indicates that the difference in the tumor volume on day 15 is statistically significant between the two values as indicated by lines ($P < 0.01$).

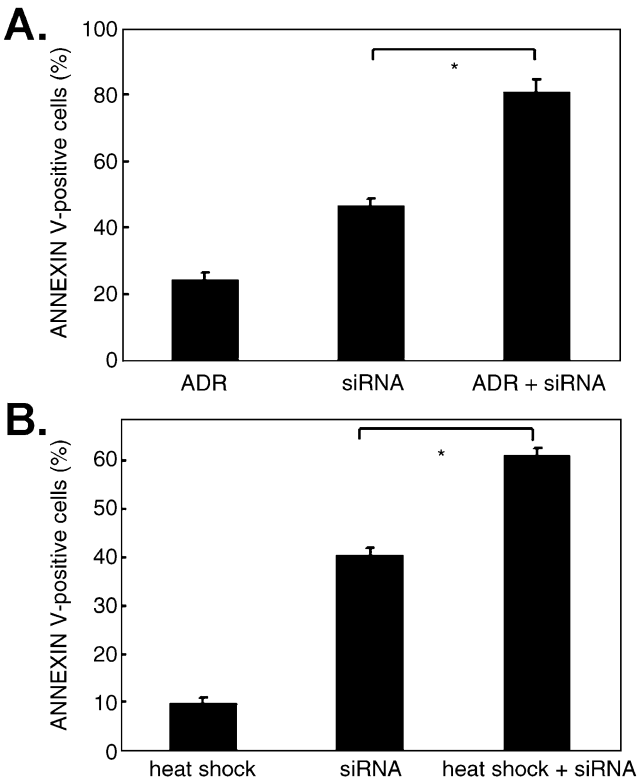

Fig. 5. The synergistic effect of HSP105 small interfering RNA (siRNA) with doxorubicin or heat shock regarding the induction of apoptotic cell death in HCT116 cells. At 12 h after transfection with 100 nM siRNA, the cells were incubated with 200 ng/mL doxorubicin (A) or treated with heat shock at 45°C for 30 min (B). Subsequently, the cells were stained with fluorescein-isothiocyanate–annexin V and analyzed by flow cytometry. Data are the mean of three independent experiments ± SD ($n = 3$). The asterisks indicate that the differences in the percentages of annexin V-positive cells are statistically significant between the two values as indicated by lines ($P < 0.001$).

of amyloid-beta.[40] In our study, HSP105 siRNA treatment induced HCT116 cells to undergo apoptosis through caspase activation. Considering these findings, we speculate that HSP105 siRNA treatment may induce aggregation of unfolded protein while also causing insufficient protein degradation, consequently leading to ER stress-mediated apoptosis, especially in cancer cells carrying mutations and aberrant expression of oncoproteins. Recent reports demonstrating that HSP105 prevents the aggregation of thermal-denatured protein *in vitro*[6] and that overexpression of HSP105 suppresses aggregation and cell toxicity in a spinal and bulbar muscular atrophy model[9] support our speculation. Regarding caspase dependency, the cleavage of PARP has been reported to be suppressed in PC12 cells overexpressing HSP105 protein and those cells were also protected from apoptosis caused by several stressors.[8] These observations are consistent with our results.

Cancer cells often have aberrantly expressed or mutated genes that lead to uncontrolled cell growth and the prevention of apoptosis, and the usage of siRNA against such targets is thus considered to be promising for cancer therapy. Several recent studies have demonstrated the effective silencing by siRNA of targets, such as β-catenin for colon cancer,[41] mutated K-ras for pancreatic carcinoma[42] and BCR/ABL fusion protein for CML.[43] In those reports, the injection of siRNA either induced target cells to undergo apoptosis or caused inhibition of their proliferation. In the present study, HSP105 siRNA induced various human cancer cell lines to undergo apoptosis both *in vitro* and *in vivo* without side effects. It is notable that the effect of HSP105 siRNA treatment was transient and not lethal in normal fibroblasts, whereas the effects of known chemical agents tend to be cytotoxic for normal cells. Indeed, human fibroblast cells treated with doxorubicin were induced to undergo apoptosis (data not shown). These data suggest that HSP105 siRNA treatment is useful for cancer therapy and it may thus be applied to various kinds of cancer patients with minimal side effects.

For patients with advanced or metastatic cancer, combination therapies using some cytotoxic agents and radiation are now often performed clinically. We expected synergistic effects of combination therapy using HSP105 siRNA and doxorubicin, which have different mechanisms of action. siRNA suppresses the expression of targeted proteins by RNA cleavage, whereas doxorubicin a DNA intercalating agent that induces apoptosis by damaging DNA. In the present study, treatment combining HSP105 siRNA with doxorubicin synergistically induced cancer cells to undergo apoptosis. We also suspected that heat shock is effective when it is combined with HSP105 siRNA because HSP105 is essential in order to protect cells from heat stress.[8]

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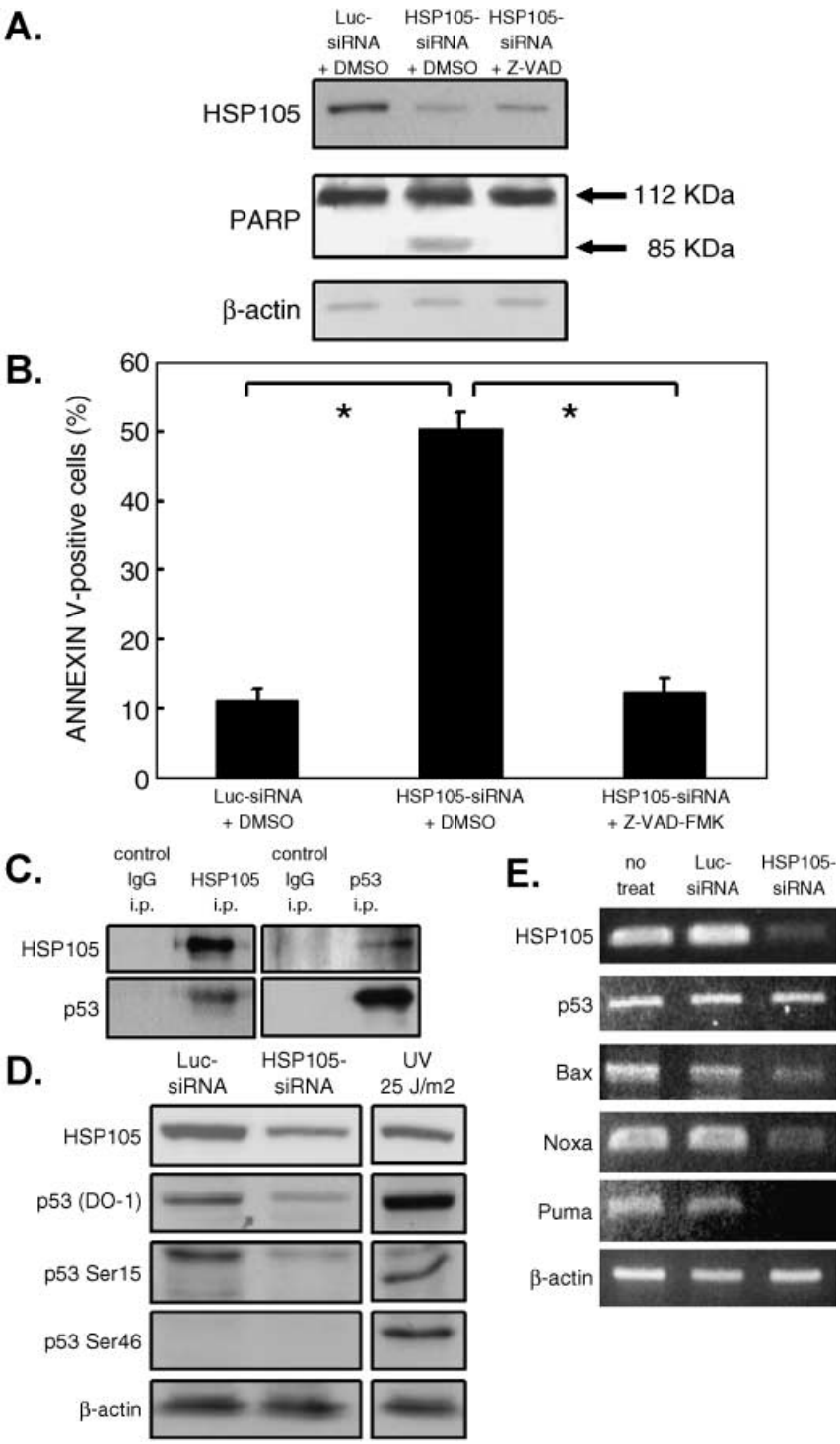

Fig. 6. Caspase-dependent and p53-independent induction of apoptosis in HCT116 cells administered HSP105 small interfering RNA (siRNA). (A) Western blot analysis of poly ADP-ribose polymerase (PARP) expression and (B) flow cytometric analysis of apoptosis induced in HCT116 cells transfected with siRNA in the presence of dimethylsulfoxide (DMSO) or Z-VAD-FMK. HCT116 cells treated with luciferase siRNA + DMSO, HSP105 siRNA + DMSO or HSP105 siRNA + 100 μM Z-VAD-FMK were cultured for 48 h and apoptotic cells were stained with annexin V. Cells were lysed and blotted with either anti-HSP105 or anti-PARP antibody. Data are the mean values of three independent experiments ± SD. The asterisks indicate that the differences in the percentages of annexin V-positive cells were statistically significant between the two values as indicated by lines ($P < 0.001$). (C,D) Western blot analysis of HSP105 and p53. HCT116 cells were lysed and immunoprecipitated with an anti-HSP105 antibody or an anti-p53 antibody (DO-1), and the proteins were blotted with either anti-HSP105 antibody or a biotin-labeled DO-1. The immunoprecipitates with rabbit polyclonal IgG and mouse monoclonal IgG2a were used as negative controls for anti-HSP105 antibody and DO-1, respectively (C). HCT116 cells transfected with luciferase siRNA or HSP105 siRNA were lysed at 48 h after transfection and blotted with the anti-HSP105 antibody DO-1, anti-phospho-p53 (Ser46) and antiphospho-p53 (Ser15). Ultraviolet light-irradiated HCT116 cell lysates were used as a positive control for p53 phosphorylation. (D) β-Actin is shown as a quantitative control. (E) Reverse transcription–polymerase chain reaction (RT-PCR) analysis of HSP105, p53, Bax, NOXA and PUMA expression in HCT116 cells transfected with siRNA. HCT116 cells transfected with luciferase siRNA or HSP105 siRNA were harvested at 24 h after transfection and the cDNAs were used for PCR analysis. cDNA extracted from the untreated HCT116 cells was used as a negative control. β-Actin is shown as a quantitative control.

As shown in Fig. 5B, the combination of HSP105 siRNA with heat shock, which is clinically applied to cancer patients as hyperthermia, exhibited a synergistic apoptotic effect in cancer cells.

In conclusion, our findings suggest that HSP105 is involved in tumorigenesis by protecting cancer cells from apoptosis, and the constitutive overexpression of HSP105 protein was found to be essential for various cancer cells to survive. We also suggest that the apoptosis-inducing effect of HSP105 siRNA is specific for cancer, therefore HSP105 siRNA may be useful as a novel therapeutic tool for patients with cancers originating from various tissues. By using effective drug delivery systems and combining this treatment with existing cytotoxic agents, an enhanced effect is thus expected.

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