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PRECLINICAL STUDY

Survivin plays as a resistant factor against tamoxifen-induced apoptosis in human breast cancer cells

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Abstract Tamoxifen has been the mainstay of endocrine therapy for estrogen receptor-positive breast cancer. However, approximately 40% of breast cancer patients do not respond to tamoxifen treatment. Further, most tumors eventually acquire tamoxifen resistance. Therefore, it is necessary to develop effective modalities to enhance the efficacy of tamoxifen in breast cancer treatment. In this study, we investigated the mechanism by which breast cancer cells develop resistance against tamoxifen from the viewpoint of tamoxifen-induced apoptosis. Overexpression of the anti-apoptotic molecule survivin rendered the human breast cancer cells MCF-7 resistant to tamoxifen-induced apoptosis. To examine whether the down-regulation of survivin can enhance tamoxifen-induced apoptosis, we introduced siRNA targeting the survivin gene (survivin-siRNA) into MCF-7 cells. Survivin-siRNA transfection not only induced apoptosis without tamoxifen treatment but also augmented the tamoxifen-induced apoptosis. We have previously demonstrated that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (HRIs), which are widely used to reduce the serum cholesterol levels in hypercholesterolemia patients, decreases survivin expression in colon cancer cells. To develop a pharmacological approach for improving the efficacy of tamoxifen treatment, we determined whether HRIs can enhance tamoxifen-induced apoptosis. Lovastatin, an HRI, down-regulated the expression of survivin protein in MCF-7 cells in a dose-dependent manner. In addition, the proportion of apoptotic cells induced by the tamoxifen and lovastatin combination was greater than the theoretical additive effect. These results suggest that survivin may function as a factor inducing resistance against tamoxifen-induced apoptosis, and the combined use of tamoxifen and HRI may be a novel approach to overcome tamoxifen resistance in breast cancer.

Keywords Apoptosis · Tamoxifen · Breast cancer cells · 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor · Survivin

Introduction

Tamoxifen is a nonsteroidal triphenylethylene derivative classified as a selective estrogen receptor modulator and has been the mainstay of endocrine therapy in the estrogen receptor (ER)-positive breast cancer in both pre- and postmenopausal woman for the last 20 years [1]. However, approximately 40% of patients with ER-positive breast cancer do not respond to tamoxifen treatment [2]. In addition, tamoxifen is usually effective only for a short duration, and most tumors eventually acquire resistance to tamoxifen in 2–5 years [3]. Therefore, it is necessary to develop effective modalities to enhance the efficacy of tamoxifen in the treatment of breast cancer. Although several different mechanisms, including loss of ER expression, mutation of ER, altered expression of coregulators, and increased growth factor signaling [4], have been postulated to be involved in the development of tamoxifen

Naoki Tsuji substantially contributed to this work and should also be considered a first author.

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resistance in breast cancer cells, this underlying mechanism remains to be completely elucidated.

It has been known that induction of apoptosis is one of the mechanisms by which tamoxifen inhibits the growth of breast cancer cells [5]. In addition, tamoxifen activates caspase-3, caspase-7, caspase-8, and caspase-9 in breast cancer cells and tumors [6, 7], and the general inhibitor of caspase abrogates tamoxifen-induced apoptosis in breast cancer cells [8]. These results indicate that caspase activation plays an important role in tamoxifen-induced apoptosis. Several proteins that inhibit caspase-dependent apoptotic signaling have been identified, including bcl-2 and the inhibitor of-apoptosis (IAP) families [9]. Survivin, a member of the latter family, has been shown to inhibit apoptosis through direct inhibition of caspase-3 and caspase-7, which act as terminal effectors in the apoptotic protease cascade [10]. Since survivin overexpression desensitizes cancer cells to several anticancer agents [11] and irradiation [12], both of which induce apoptosis, there is a possibility that survivin also attenuate tamoxifen-induced apoptosis.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (HRIs) are widely used to reduce the serum cholesterol level and are well tolerated by patients with hypercholesterolemia [13]. HRIs prevent the formation of mevalonate from HMG-CoA by inhibiting the enzyme HMG-CoA reductase, and consequently inhibit cholesterol synthesis [14]. The two downstream intermediates of the cholesterol synthetic pathway-farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP)-belong to a class of compounds named isoprenoids and are bound to several cellular proteins, including small guanosine triphosphatases (GTPases), such as Ras, Rho, and Rac, by a post-translational modification known as isoprenylation. This process involves the addition of a 15-carbon farnesyl chain in FPP (farnesylation) or a 20-carbon geranylgeranyl chain in GGPP (geranylgeranylation) to a cysteine sulfhydryl group near the carboxy terminus. Isoprenylation of these proteins is crucial for membrane attachment [15, 16] and the subsequent acquisition of biological activity [17]. In particular, Ras proteins are important regulators of cell survival and proliferation [18, 19]. We have previously demonstrated that HRIs decrease survivin expression by inhibiting phosphatidylinositol 3 (PI3)-kinase activation via the inhibition of Ras isoprenylation in colon cancer cells [20].

Here, we show that survivin overexpression desensitizes breast cancer cells to tamoxifen. Additionally, the HRI, lovastatin down-regulates survivin expression in the breast cancer cells and enhances tamoxifen-induced apoptosis. Taken together, these results suggest that treatment with the combination of tamoxifen and an HRI may be a novel approach to overcome tamoxifen resistance in breast cancer.

Materials and methods

Materials

Tamoxifen and lovastatin were obtained from Sigma-Aldrich (St Louis, MO). Anti-survivin antibody were from R&D Systems (McKinley Place, NE). Anti-ERα antibody was from Persus Proteomics (Tokyo, Japan). Anti-ERβ antibody, and anti-actin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

Human breast cancer cell line, MCF-7 and ZR-75-1 were obtained from American Type Culture Collection (Manassas, VA). These cell lines were cultured in RPMI-1640 Medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fatal bovine serum (FBS; Thermo Fisher Scientific Waltham, MA) and grown at 37°C in a humidified atmosphere of 5% CO_2.

Patients and frozen tissue samples

Breast cancer tissue as well as adjacent uninvolved counterparts, were obtained from 25 patients undergoing surgery. Before the acquisition of these tissues, informed consent was obtained explaining the investigational nature of the study. The samples were immediately frozen and stored in liquid nitrogen. Tissues were also stained with hematoxylin/eosin (Merck KGaA, Darmstadt, Germany), and were reviewed by well-experienced pathologists. No patient was received chemotherapy or hormone therapy.

Measurement of cell viability

Viability of MCF-7, and ZR-75-1 cells treated with tamoxifen was assessed by detection of ATP using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). 2,000 cells (MCF-7) or 3,000 cells (ZR-75-1) were cultured in 96-well culture plates (Corning, Corning, NY) in 100 µl of RPMI 1,640 containing 10% FBS and various concentration of tamoxifen (1–10 µM), and incubated until 96 h. At the end of incubation, 100 µl of assay reagent was added to be incubated for 10 min at room temparature. Luminescence was measured using GloMax™ 96 Microplate Luminometer (Promega).

DNA content analysis

Cell cycle status was assessed by DNA content analysis. Cells were resuspended in 1 ml of hypotonic fluorochrome solution (propidium iodide (PI) at 50 µg/ml in 0.1% sodium citrate and 0.1% Triton X-100) and

incubated at room temperature for 30 min in darkness. 20,000 events were measured per sample using flow cytometer (EPICS XL-MCL cytometer; Beckman Coulter, Tokyo, Japan).

Detection of apoptosis

Apoptosis was quantified by a combined staining of Annexin V and PI using MEBCYTO-Apoptosis Kit (Medical & Biological Laboratories, Nagoya, Japan). Briefly, cells were harvested and resuspended in 85 μl of 1× Binding Buffer. After adding 10 μl of Annexin V-FITC solution and 5 μl of PI solution, cells were incubated for 15 min at room temperature in the dark. At the end of incubation, 5,000 cells were analyzed by flow cytometer after adding 400 μl of 1× Binding Buffer. All experiments were performed at least three times for each experimental condition.

Quantification of survivin mRNA

The expression of survivin mRNA was determined by quantitative reverse transcription polymerase chain reaction (RT-PCR) using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) as described previously [21, 22]. The gene-specific primers and fluorescent hybridization probes for survivin used in the quantitative RT-PCR were as follows. The forward primer was 5′-AAG AAC TGG CCC TTC TTG GA-3′; reverse primer, 5′-CAA CCG GAC GAA TGC TTT T-3′; and probe, 5′-(FAM) CCA GAT GAC GAC CCC ATA GAG GAA CA (TAMRA)-3′. Survivin-2B (retaining a part of intron 2 as a cryptic exon) and survivin-ΔEx3 (lacking exon 3), which are two splice variants of survivin, were not detected by this set of primers and probe [23]. The amounts of mRNAs were normalized as ratios to the amounts of 18S ribosomal RNA (rRNA). PCR products were also confirmed by gel electrophoresis.

Immunoblot analysis

Cytoplasmic and nuclear proteins were extracted from cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL) with a protease inhibitor cocktail (Sigma-Aldrich). Equal amounts of cytoplasmic proteins (30 μg/lane) or nuclear proteins (30 μg/lane) were separated on a 4–20% gradient Tris–glycine gel (Invitrogen Carlsbad, CA) under denaturing conditions using Tris–glycine sodium dodecyl sulfate running buffer (Invitrogen). Proteins were electroblotted to a nitrocellulose membrane (Invitrogen). Proteins were detected using a WesternBreeze chemiluminescent immunodetection kit (Invitrogen) according to the manufacturer's protocol. Briefly, after incubation in blocking solution for 30 min at room temperature, the membrane was incubated with primary antibody raised against target molecule at room temperature for 1 h. The membrane was washed and incubated with secondary antibody conjugated to alkaline phosphatase at room temperature for 30 min. Alkaline phosphatase labeling was detected using chemiluminescent substrate. Chemilumonescent was detected by ChemiDoc XRS (Bio-Rad Laboratories, Hercules, CA).

Transfection of survivin gene into breast cancer cells

The plasmid encoding human survivin pcDNA3-myc-survivin and the vector control pcDNA3-myc were kindly provided by Dr. John C. Reed (The Burnham Institute, La Jolla, CA). Transfections were performed using the Lipo fectamine Plus reagent (Invitrogen) according to the manufacturer's protocol. Geneticin-resistant clones were used in this study.

Transfection of siRNA targeting survivin gene into breast cancer cells

A small inhibitory RNA (siRNA) was designed to target the coding region of the survivin gene (nucleotides 366–385, relative to the start codon). As the transfection control, we used non-silencing siRNA (QIAGEN, Valencia, CA) which has no homology to any known mammalian gene. The siRNA duplexes used in this study were 5′-GAA UUU GAG GAA ACU GCG A TT-3′ and 3′-TT CUU AAA CUC CUU UGA CGC U-5′; Transfections were performed by electroporation using Nucleofector system (Amaxa biosystems, Cologne, Germany) according to the manufacturer's protocol.

Results

Tamoxifen induced cell cycle arrest and apoptosis in breast cancer cells

We evaluated the effects of tamoxifen on the growth of human breast cancer cells MCF-7 and ZR-75-1, which express ER-α and ER-β (Fig. 1a, b). We cultured these cells with various concentrations of tamoxifen (1–10 μM) for up to 96 h and determined the number of viable cells by measuring the ATP levels. Since tamoxifen was dissolved in ethanol, cells treated with only ethanol (vehicle) at the same concentration were used as the untreated control group. The growth of both the MCF-7 and ZR-75-1 cells was inhibited by tamoxifen in a dose-dependent manner. MCF-7 cells were more sensitive to tamoxifen than ZR-75-1 cells.

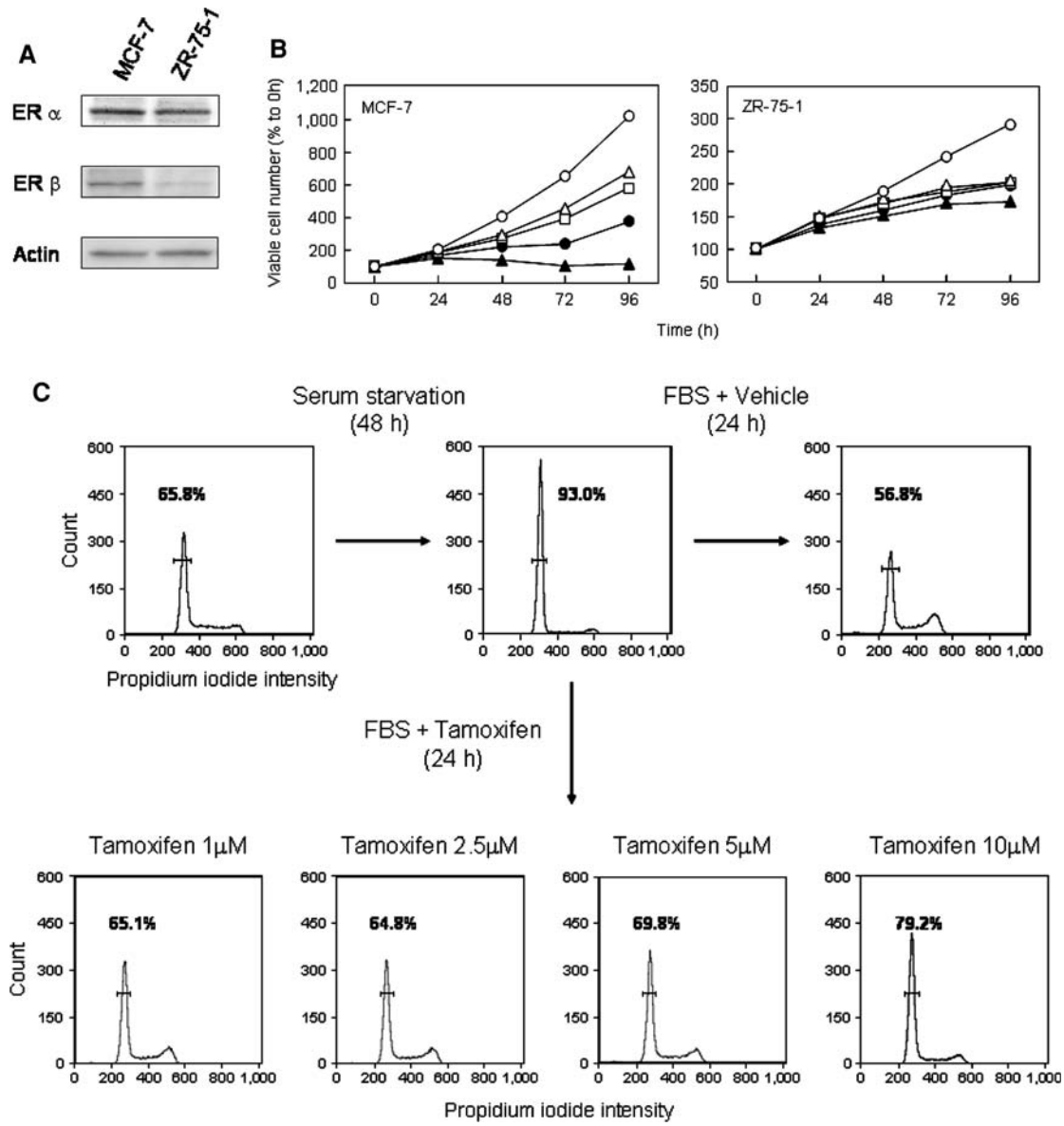

Fig. 1 Tamoxifen induced cell cycle arrest and apoptosis in breast cancer cells. (**a**) ER-α and ER-β expressions in MCF-7 and ZR-75-1 cells. The expressions of ER-α and ER-β proteins were examined by immunoblot analysis. (**b**) Number of viable cells in tamoxifen-treated MCF-7 and ZR-75-1 cells; viable cells were counted by measuring the ATP level. The number of viable cells in each experimental condition is represented as a percentage of the number of cells at time 0 h. *Open circle*, vehicle; *open triangle*, 1 μM of tamoxifen; *open square*, 2.5 μM of tamoxifen; *closed circle*, 5 μM of tamoxifen; *closed triangle*, 10 μM of tamoxifen. (**c**) Tamoxifen inhibited G0-G1→S phase progression in breast cancer cells. MCF-7 cells were serum starved for 48 h. The cells were then cultured for an additional 24 h in 10% FBS with or without the indicated concentration of tamoxifen. Cell cycle status was assessed by DNA content analysis as described in the 'Materials and methods' section. Numbers represent the proportion of cells in the indicated areas. (**d**, **e**) Proportion of apoptotic MCF-7 cells after tamoxifen treatment. MCF-7 cells were treated with the indicated concentrations of tamoxifen for 48 h (**d**) or 72 h (**e**). Apoptosis was quantified by combined staining with Annexin V and PI. The percentage of annexin V-positive cells is indicated for each condition tested. Since tamoxifen was dissolved in ethanol, cells treated with only ethanol (vehicle) at identical concentrations were used as the untreated control group

It has been reported that tamoxifen induces cell cycle arrest at the G1 phase [24, 25]. To confirm these observations, we first studied the effect of tamoxifen on G0-G1→S phase progression by using MCF-7 cells synchronized at the G0-G1 phase of the cell cycle. As shown in Fig. 1c, serum starvation for 48 h increased the estimated percentages of cells in the G0-G1 phase from 65.8% to 93.0%, indicating cell cycle arrest at the G0-G1 phase. When the cells were cultured for an additional 24 h after the addition of 10% FBS and the vehicle, the proportion of cells in the G0-G1 phase of the cell cycle decreased to 56.8%. In contrast, when the cells were cultured for an

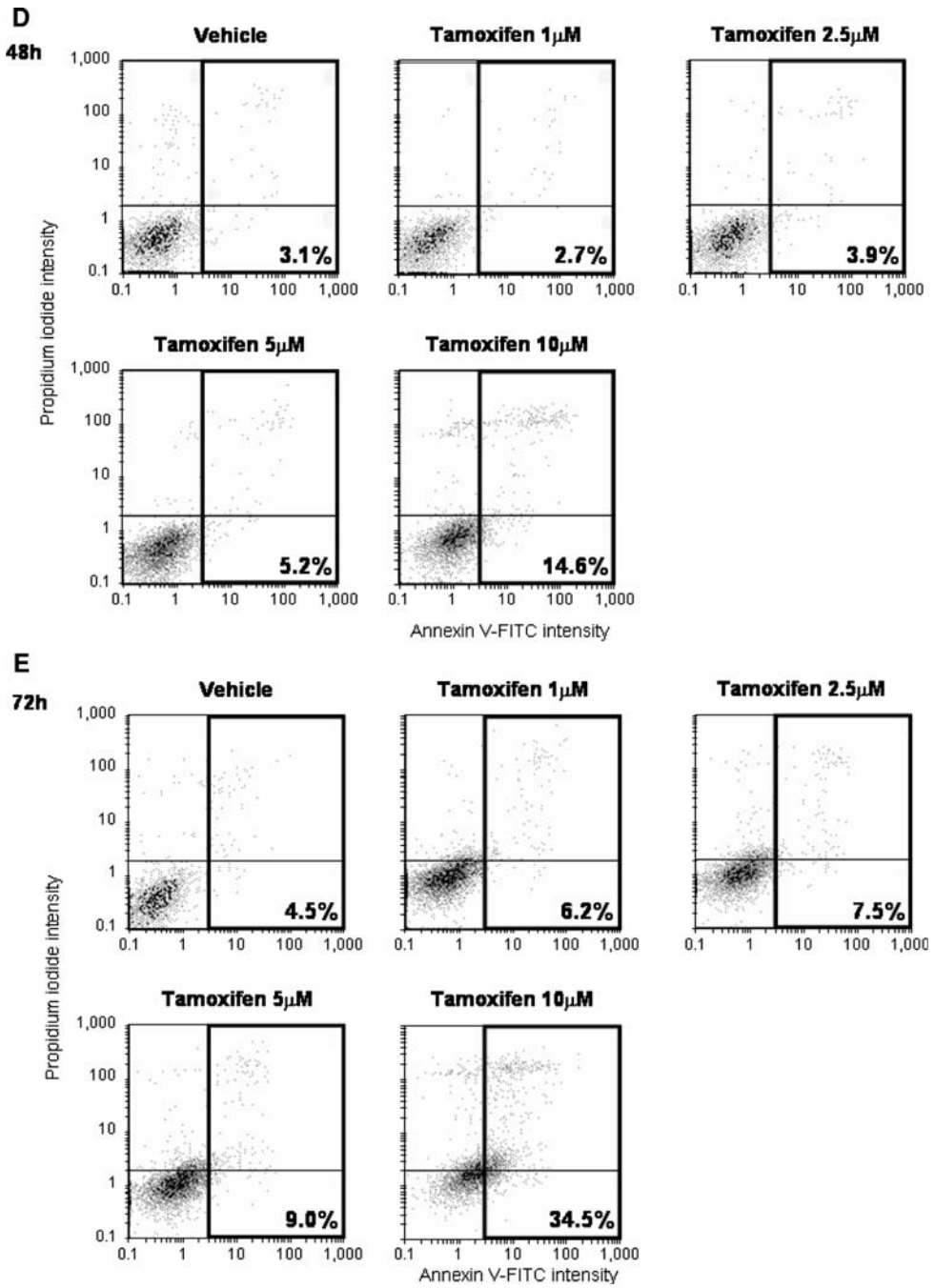

Fig. 1 continued

additional 24 h after the addition of 10% FBS and tamoxifen, the proportion of the cells in the G0-G1 phase of the cell cycle increased in a dose-dependent manner, i.e., 79.2% of the cells in the G0-G1 phase were detected in a 10 μM tamoxifen-treated group.

Induction of apoptosis is another mechanism by which tamoxifen inhibits the growth of breast cancer cells [5]. Therefore, by using annexin V-PI staining, we determined the proportion of apoptotic cells after treating MCF-7 cells with tamoxifen (1–10 μM) for 48 or 72 h. As shown in Fig. 1d, e, the proportion of annexin V-positive cells, i.e., apoptotic cells, increased in a dose-dependent manner: 34.5% of MCF-7 cells had undergone apoptosis following the 72 h treatment with 10 μM tamoxifen.

We then focused on the enhancement of tamoxifen-induced apoptosis in order to improve its efficacy.

Survivin attenuated tamoxifen-induced apoptosis

We first determined the relative levels of survivin mRNA expression in breast cancer tissues by quantitative RT-PCR. As shown in Table 1, the expression level of survivin mRNA was significantly greater in cancer tissue than in uninvolved tissue. In particular, survivin mRNA was not detected in 20 out of 23 uninvolved tissues, while only 4 out of 25 cancerous tissues showed undetectable levels of survivin mRNA (data not shown). Survivin mRNA and protein expressions were observed in MCF-7 and ZR-75-1 cells (Table 1 and Fig. 2a left).

Next, we examined whether survivin up-regulation could confer protection against tamoxifen-induced growth inhibition and apoptosis. We introduced the survivin gene expression vector, pcDNA3-myc-survivin into MCF-7 cells. The survivin gene transfectants expressed myc-tagged survivin protein in addition to the endogenous survivin protein (Fig. 2a right). Using the ATP assay, we determined the cell viability in the survivin gene transfectants treated with tamoxifen (2.5–10 μM) for 48 or 72 h (Fig. 2b). Survivin gene transfectants showed more resistance against the growth-inhibitory effect of tamoxifen than control vector transfectants. We then examined the proportion of apoptotic cells in the survivin gene transfectants by using annexin V-PI staining after treating the cells with 10 μM of tamoxifen for 48 or 72 h. As shown in Fig. 2c, d, treatment of control vector transfectants with tamoxifen for 48 and 72 h resulted in 11.1% and 35.2% more apoptotic cells than in the untreated cells, respectively. On the other hand, in the survivin gene transfectants, tamoxifen increased the number of apoptotic cells by only 5.6% and 10.0% after 48 and 72 h treatments, respectively. These results indicate that survivin is a key molecule that renders the breast cancer cells resistant to tamoxifen-induced apoptosis.

Table 1 Survivivn mRNA expression was up-regulated in the breast cancer tissues and cell lines

	Survivin mRNA expression[a] (mean ± SD)	
Breast tissues		
Uninvoled tissues ($n = 23$)	0.000 ± 0.001	
Cancer ($n = 25$)	0.012 ± 0.168	$P < 0.001$*
Breast cancer cell lines		
MCF-7	2.611 ± 0.280[b]	
ZR-75-1	3.411 ± 0.388[b]	

[a] Survivin mRNA expression was determined by quantitive RT-PCR. The amounts of survivin mRNA were normalized as ratios to the amounts of 18S rRNA

[b] Data represents mean ± SD of three independent experiments

* Statistical analysis was performed using the Mann–Whitney *U*-test

Down-regulation of survivin enhanced tamoxifen-induced apoptosis

To examine whether survivin down-regulation enhances tamoxifen-induced apoptosis, we introduced siRNA targeting the survivin gene (survivin-siRNA) into MCF-7 cells. Figure 3a depicts the survivin protein expression in survivin-siRNA-transfected cells revealed by immunoblot analysis. The introduction of survivin-siRNA completely abrogated survivin protein expression at 48 h after transfection. No reduction in survivin protein expression was observed in the cells transfected with non-silencing siRNA, which has no homology to any known mammalian gene.

We then examined the sensitivity of survivin-siRNA-transfected cells to tamoxifen-induced apoptosis. We spread equal number of viable survivin-siRNA-transfected and non-silencing siRNA-transfected cells in a six-well culture plate at 24 h after siRNA transfection. After an additional 24 h incubation, the cells were treated with tamoxifen for 48 or 72 h, and the proportion of apoptotic cells was determined using annexin V-PI staining. In agreement with previous reports [26, 27], introduction of survivin-siRNA induced apoptosis without tamoxifen treatment (Fig. 3b, c). However, the proportion of apoptotic cells induced by the combination of tamoxifen treatment and survivin-siRNA transfection was greater than the theoretical additive effects; this indicated that down-regulation of survivin could enhance tamoxifen-induced apoptosis.

Lovastatin augmented tamoxifen-induced apoptosis

We have previously demonstrated that HRI decreases survivin expression by inhibiting PI3-kinase activation via the inhibition of Ras isoprenylation in colon cancer cells [20]. Then, by using MCF-7 cells, we determined whether the HRI lovastatin enhances the tamoxifen-induced apoptosis via survivin down-regulation.

Using immunoblot analysis, the expression levels of the survivin protein were examined for 48 h in MCF-7 cells treated with 1–20 μM of lovastatin. As shown in Fig. 4a, lovastatin down-regulated survivin expression in a dose-dependent manner.

MCF-7 cells were treated for 48 or 72 h with tamoxifen (5 μM), lovastatin (1 or 5 μM), or combinations of tamoxifen and lovastatin, and the proportion of apoptotic cells, which were detected by using annexin V-PI staining, were compared among the treatments. Figure 4b, c present representative examples of annexin V-PI staining. Treatment of MCF-7 cells with the tamoxifen and lovastatin

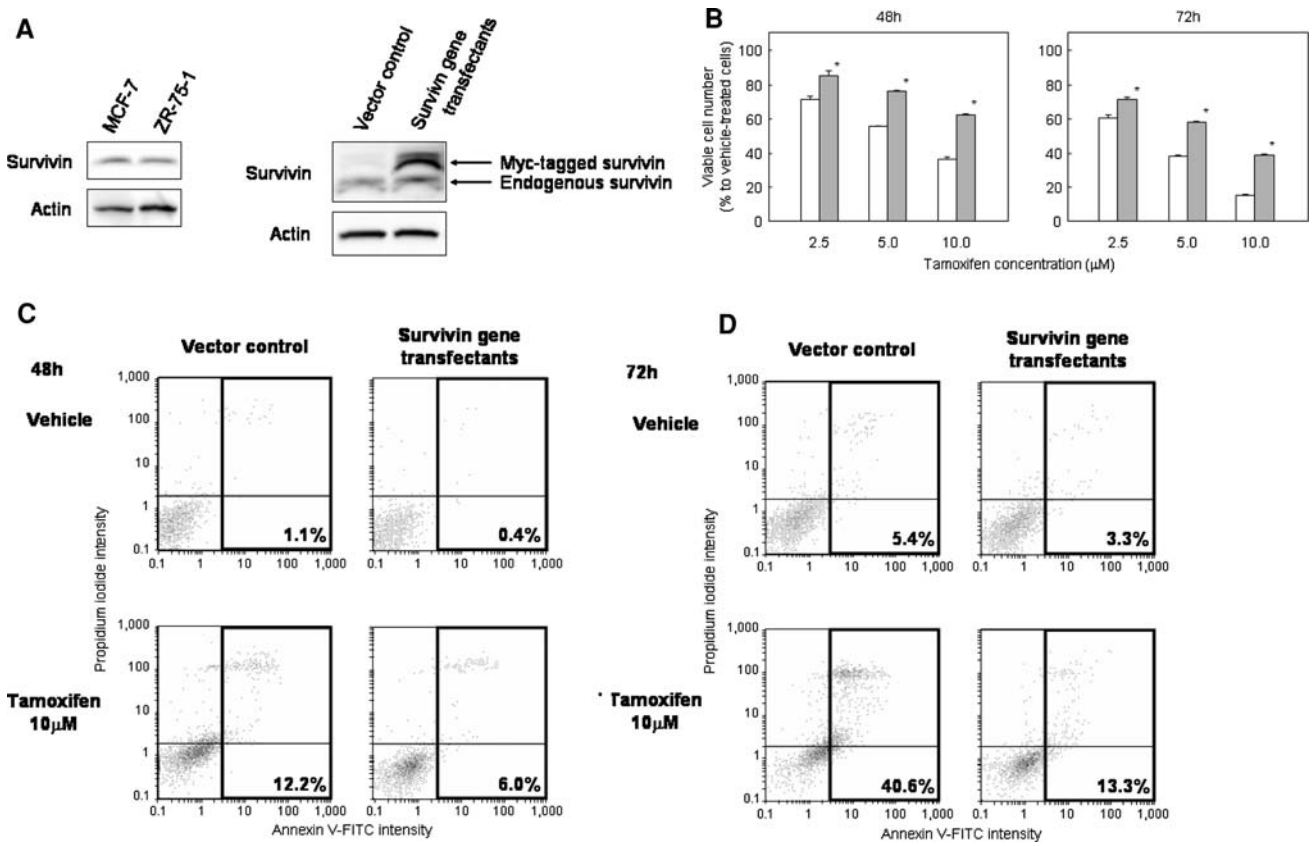

Fig. 2 Overexpression of survivin in breast cancer cells attenuated tamoxifen-induced apoptosis. (**a**) The expression of survivin proteins in MCF-7, ZR-75-1 cells, and survivin gene transfectants. Expression of survivin proteins was examined by immunoblot analysis. MCF-7 cells were transfected with the survivin gene expression vector pcDNA3-myc-survivin. (**b**) Cell viability of the survivin gene transfectants treated with tamoxifen. The control vector transfectants (white bar) and survivin gene transfectants (shaded bar) were cultured with the indicated concentrations of tamoxifen for 48 or 72 h. The number of viable cells was assessed by measuring the ATP levels. Data represent mean + SD values of three independent experiments. $*P < 0.01$ by *t*-test. (**c**, **d**) Proportion of apoptotic cells in tamoxifen-treated survivin gene transfectants. Survivin gene transfectants were treated with 10 μM of tamoxifen for 48 h (**c**) or 72 h (**d**). Apoptosis was quantified by combined staining with Annexin V and PI. The percentage of annexin V-positive cells is indicated for each condition tested. Since tamoxifen was dissolved in ethanol, cells treated with only ethanol (vehicle) at identical concentrations were used as the untreated control group

combination resulted in more apoptotic cells than tamoxifen or lovastatin treatment alone. In addition, statistical analysis using two-way analysis of variance (ANOVA) revealed that the combination of tamoxifen and lovastatin has a synergistic effect on the induction of apoptosis (Table 2).

Discussion

In this study, to discover a method for enhancing tamoxifen-induced apoptosis, we explored the mechanism by which breast cancer cells acquire resistance against tamoxifen-induced apoptosis. It has been demonstrated that activation of caspase-3, caspase-7, caspase-8, and caspase-9 was observed in tamoxifen-treated breast cancer cells and tumors [6, 7]. In addition, tamoxifen-induced apoptosis was blocked by the general inhibitor of caspase in breast cancer cells [8], indicating that the activation of caspase plays an important role in tamoxifen-induced apoptosis. Therefore, we hypothesized that the up-regulation of the anti-apoptotic protein survivin, which inhibits caspase-3 and caspase-7 may be a mechanism by which breast cancer cells acquire resistance to tamoxifen-induced apoptosis. As we expected, the number of apoptotic cells induced by tamoxifen was clearly decreased by over-expression of survivin. This observation indicated that survivin functions as a factor conferring resistance against tamoxifen-induced apoptosis.

It has been reported that mitochondria play an integral role in tamoxifen-induced caspase activation [28]. Insults by toxic molecules, such as chemotherapeutic agents induce a collapse of mitochondrial transmembrane potential (MTP) [29]. This triggers the release of cytochrome c

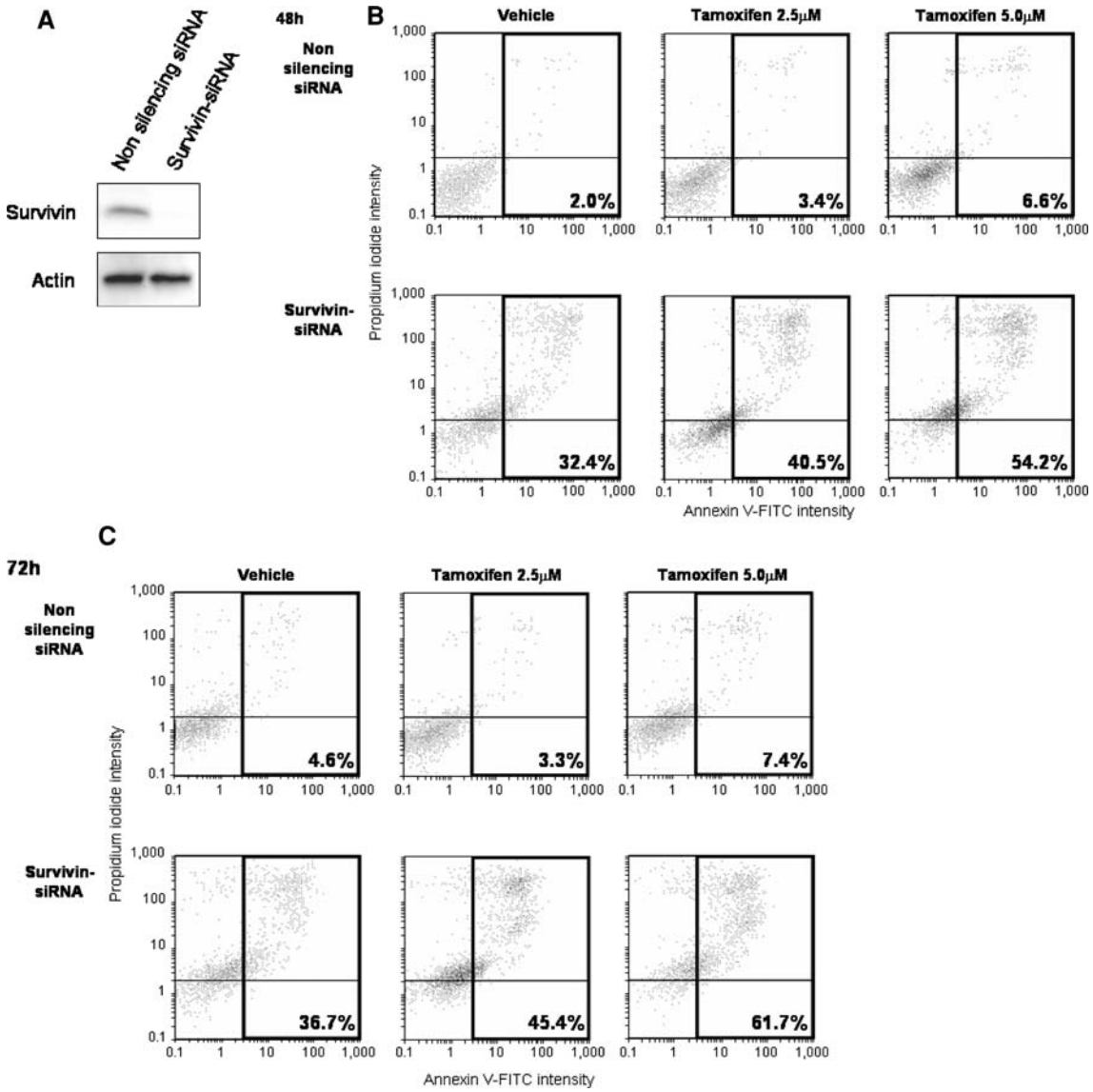

Fig. 3 Survivin down-regulation by survivin-specific siRNA enhanced tamoxifen-induced apoptosis. (**a**) Survivin protein expression in breast cancer cells-transfected with survivin-specific siRNA. MCF-7 cells were transfected with siRNA that targeted the survivin gene (survivin-siRNA), and survivin protein expression was assessed by immunoblot analysis at 48 h after transfection. Non-silencing siRNA, which bears no homology with any known mammalian gene, was used as the transfection control. (**b**, **c**) Proportion of apoptotic cells in survivin-specific siRNA-transfected cells treated with tamoxifen. Equal number of viable survivin-specific siRNA-transfected cells and non-silencing siRNA-transfected cells were plated in six-well culture dishes at 24 h after siRNA transfection. After additional incubation for 24 h, the cells were treated with tamoxifen for 48 h (**b**) or 72 h (**c**). Apoptosis was quantified by combined staining with Annexin V and PI. The percentage of annexin V-positive cells is indicated for each condition tested. Since tamoxifen was dissolved in ethanol, cells treated with only ethanol (vehicle) at identical concentrations were used as the untreated control group

from inner membrane of mitochondria into the cytoplasm [30]. Cytochrome c, together with the apoptosis-inducing factor (AIF), activates procaspase-9 to its active form, caspase-9 [31]. Caspase-9 activates the downstream or effector caspases such as caspase-3 and caspase-7 and eventually leads to the proteolytic degradation of substrates, resulting in the apoptotic morphology. Dietze et al. [28] reported that tamoxifen-induced a rapid MTP decrease, mitochondrial condensation, and caspase activation prior to the morphological appearance of apoptosis. The mechanisms by which tamoxifen induces MTP collapse and caspase-9 activation remain to be studied. However, since tamoxifen-induced caspase-9 activation in ER-negative breast cancer cells [32], it may also affect the mitochondria via the ER-independent pathway. Inhibition of apoptotic signaling by survivin involves the inhibition of caspase-3 and caspase-7 activation [10]. Janicke et al. [33] reported that procaspase-3 was absent in MCF-7 because of

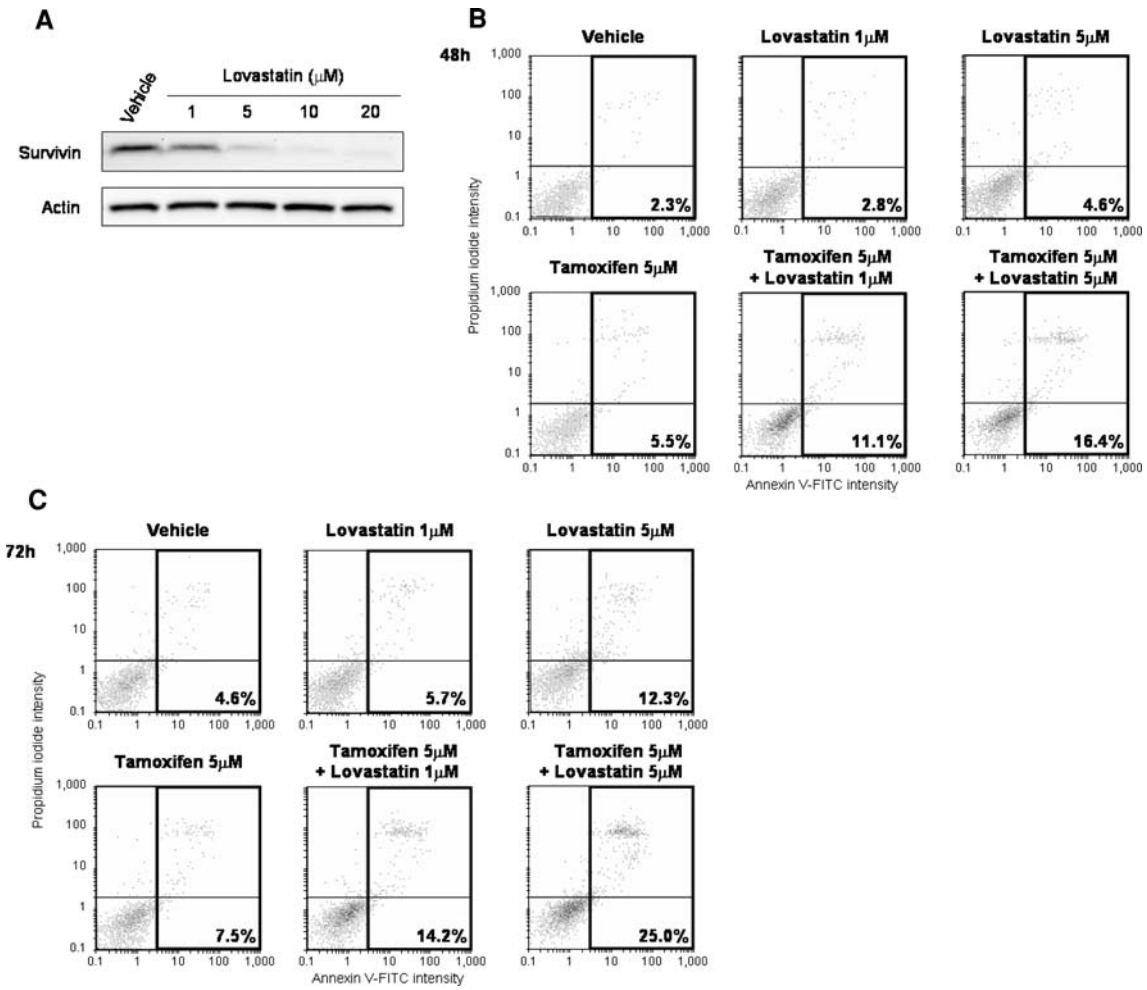

Fig. 4 Lovastatin augmented tamoxifen-induced apoptosis. (**a**) Lovastatin down-regulated survivin protein expression. MCF-7 cells were treated with the indicated concentrations of lovastatin for 48 h. Survivin protein expression was assessed by immunoblot analysis. (**b**, **c**) Proportion of apoptotic cells in tamoxifen- and lovastatin-treated breast cancer cells. MCF-7 cells were treated with tamoxifen (5 μM), lovastatin (1 or 5 μM), or both tamoxifen and lovastatin in various combinations for 48 h (**b**) or 72 h (**c**). Apoptosis was quantified by combined staining with Annexin V and PI. The percentage of annexin V-positive cells is indicated for each condition tested. Since tamoxifen and lovastatin were dissolved in ethanol, cells treated with only ethanol (vehicle) at identical concentr ations were used as the untreated control group

the partial deletion of the *CASP-3* gene. Therefore, inhibition of caspase-7 by survivin may be important for rendering MCF-7 cells resistance to tamoxifen-induced apoptosis.

Next, we showed that down-regulation of survivin by introduction of survivin-siRNA into breast cancer cells enhanced tamoxifen-induced apoptosis. However, this approach may not be feasible in routine clinical therapy for cancer unless a technique for efficiently introducing survivin-siRNA into cancer cells is established. Therefore, the development of a pharmacological approach that targets survivin is warranted for improving the efficacy of tamoxifen treatment.

Our study demonstrates that lovastatin effectively reduces survivin expression and enhances tamoxifen-induced apoptosis in breast cancer cells, indicating that lovastatin may be a pharmacological agent that targets survivin, and it could be used for the clinical treatment of breast cancer. We used 5.0 μM of tamoxifen combination with lovastatin in this study. Kisanga et al. [34] examined the tamoxifen concentration in the cancerous tissues derived from the 35 patients who had received 20 mg/day of tamoxifen for 28 days; they reported that tamoxifen concentration in the cancerous tissues ranged between 0.54 μM and 6.60 μM. Therefore, 5.0 μM of tamoxifen could be achieved in vivo.

Phase I and II clinical trials of lovastatin have been conducted in patients with cancers of various origins, including that of the lung, breast, stomach, colon, prostate, and brain [35–37]. In these clinical studies, the patients were administered oral lovastatin at doses of 2–45 mg/(kg·day) (for cholesterol control, 20–80 mg/day lovastatin

Table 2 Effect of lovastatin on the tamoxiten-induced apoptosis

Treatment	Annexin V-positive cells (%)[a] (mean ± SD)	
48th treatment		
Vehicle	2.34 ± 0.35	
Tamoxifen 5 μM	5.79 ± 0.30	
Lovastatin 1 μM	2.36 ± 0.40	
Lovastatin 5 μM	4.60 ± 0.30	
Tamoxifen 5 μM + Lovastatin 1 μM	11.21 ± 0.29	$P < 0.01$*
Tamoxifen 5 μM + Lovastatin 5 μM	16.29 ± 0.47	$P < 0.01$*
72th treatment		
Vehicle	4.75 ± 0.15	
Tamoxifen5 μM	7.77 ± 0.15	
Lovaststin 1 μM	5.54 ± 0.30	
Lovaststin 5 μM	12.13 ± 0.56	
Tamoxifen 5 μM + Lovastatin 1 μM	14.32 ± 0.19	$P < 0.01$*
Tamoxifen 5 μM + Lovastatin 5 μM	24.44 ± 0.55	$P < 0.01$*

Data represents mean ± SD of three independent experiments

[a] Annexin V positive cells were determined by flow cytometer

* Statistical analysis was performed using two-way ANOVA

is normally prescribed). When the patients were treated with lovastatin at doses 25 mg/(kg·day) and above, the average serum lovastatin concentration was 0.28 μM, indicating that the lovastatin concentration used in our study is not achievable in vivo when the drug is administered orally. However, if intra-venous or intra-arterial (tumor-feeding arteries) administration of lovastatin were possible, high lovastatin concentrations could be achieved in vivo. In addition, a new HRI, rosuvastatin exhibits greater efficacy for lowering low density lipoprotein (LDL) cholesterol than lovastatin [38]. Therefore, rosuvastatin may have the potential to enhance tamoxifen-induced apoptosis in breast cancer cells even when administered orally. Currently, we are conducting a further study to determine the effect of rosuvastatin on tamoxifen-induced apoptosis.

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