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Suppression of staurosporine-mediated apoptosis in Hs578T breast cells through inhibition of neutral-sphingomyelinase by caveolin-1

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Abstract

Caveolin-1, a 21–24 kDa integral membrane protein, is a principal structural component of caveolae *in vivo*. To investigate the roles of caveolin-1, we established stable transfectants in Hs578T breast adenocarcinoma cells that had up- and down-regulated caveolin-1 expression. In the paper, we demonstrated that caveolin-1 overexpression in Hs578T cells significantly reduced staurosporine-induced apoptosis and the levels of caveolin-1 expression positively correlated with the number of colonies and colony size in soft agar. Our findings indicate for the first time in Hs578T cells that caveolin-1 might play a pivotal role in regulating apoptosis as a suppressor rather than a facilitator through inhibition of neutral-sphingomyelinase, decrease of ceramide, furthermore, activation of Akt signaling pathway.

Keywords: Caveolin-1; Apoptosis; Colony formation; Ceramide; Akt

1. Introduction

Caveolae are surface membrane domains that have been shown to play an important role in many signal transduction processes by acting as a compartmentalization center for signaling molecules [1]. Caveolin-1, an important structural protein of caveolae, plays a key role in signaling transduction and lipid homeostasis [2,3]. Numerous experimental results have shown that caveolin-1 expression negatively regulates cell cycle progression and tumor cell growth [4,5]. Caveolin-1 gene in human is mapped to a common location in chromosome 7q31.1/D7S522, that is commonly deleted in a variety of human cancers [6]. These findings have led to the suggestion that caveolin-1 may act as a tumor suppressor protein. However, not all studies of caveolin-1 support this suggestion. Although there is clear evidence for negative growth regulation in some cancers, other studies reported caveolin-1 overexpression in aggressive stages of specific malignancies, which is believed to contribute to cancer promotion [7,8]. Disparate results have also been

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obtained in regulating apoptosis in cells and tissues either as a facilitator or suppressor [9–12].

Apoptosis is an active and physiological process of cell death that plays an important role in development, host defense, and immune regulation [13]. Besides Bcl/Bax [14], caspase family of proteins [15] and mitochondria pathway [16], sphingomyelinase (SMase)/ceramide pathway also plays a key role in cell apoptosis [17,18]. Sphingomyelin and ceramide, a product of SMase-catalyzed hydrolysis of sphingomyelin are structural lipid components of lipid rafts and caveolae [19]. Ceramide-induced apoptosis has been associated with inhibition of the PI(3)K/Akt activity [20]. PI(3)K/Akt is a major signaling pathway which is sufficient to promote cellular survival in a wide variety of cultured cell types ranging from fibroblasts to neurons [21,22]. Both acidic- and neutral-SMase, distinguishable by the pH optima, are believed to reside in caveolae [11,23], where structural protein, caveolin-1 is located. We therefore postulated that degradation of sphingomyelin to ceramide by SMase within caveolae may be regulated by caveolin-1.

To determine the mechanisms by which caveolin-1 suppressed or promoted cancer cell apoptosis, we established transfectants in Hs578T breast adenocarcinoma cells where caveolin-1 was both up- and down-regulated. We examined hallmarks of apoptosis including flow cytometry, as well as the activity of the SMase and Akt. We also detected growth of the transfectants expressing different caveolin levels in soft agar. These findings indicate that caveolin-1 expression negatively correlates with tumor apoptosis.

2. Materials and methods

2.1. Cell lines, plasmids, and reagents

Monoclonal antibody against caveolin-1 was purchased from Zymed Laboratories Inc. p-Akt and Akt antibodies were purchased from Cell Signaling and Santa Cruz, respectively. Cell culture supplies (RPMI 1640 medium, FBS, L-glutamine, Trypsin–EDTA, and penicillin/streptomycin) were purchased from Life Technologies Inc. Hoechst 33258, propidium iodide (PI), aprotinin, and leupeptin were purchased from Sigma. G418 was purchased from Invitrogen. Silica Gel TLC plates (LK6D) were purchased from Whatman. The pCI-neo, pCI-neo-cav-1 plasmids, and Hs578T/S cell lines were generous gifts from Dr. Eric J. Smart (Kentucky Medical School, USA) and Dr. Kjell Grankvist (Umea University, Sweden), respectively. Two siRNA plasmids, pBCMGneo-Sequence-2 and pBCMGneo-Shuffle-2, were generous gifts from Dr. K Liao (Institute of Biochemistry and Cell Biology, Shanghai, China). All other reagents were obtained from regular commercial sources with the highest degree of purity.

2.2. Cell culture and generation of stable cell lines

The parental human breast cancer cell line Hs578T was maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and cultured in humidified atmosphere of 5% CO_2 at 37°C. The entire cDNA sequence of caveolin-1 was cloned into the EcoRI site of pCI-neo to generate pCI-neo-cav-1. The pCI-neo-cav-1 and its empty vector were transfected into Hs578T cell lines as described [24]. After confirming caveolin-1 expression by Western blot, we counted cells, diluted them at 1 cell/200 μl and plated 100 μl per well in 96-well plates. We then picked the well which contained only monoclonal population for propagation [25]. Two siRNA expression plasmid vectors from Dr. Liao, pBCMneo-sequence-2, and pBCMneo-shuffle-2 were transfected into Hs578T using GeneJammer Transfection reagent according to the manufacturer's instruction [26]. Monoclonal cells were selected in the same way as the Hs578T/neo-cav-l. The four stable monoclonal transfectants were maintained in culture medium containing 500 μg/ml G418 for future experiments.

2.3. Analysis of cell apoptosis

Cell apoptosis was quantitatively determined by flow cytometry analysis. Hs578T transfectants washing with ice-cold PBS were fixed with ice-cold 70% ethanol and stored at −20°C overnight. Cells were pelleted and ethanol was discarded. The pellet was washed with PBS and 1×10^6 cells were resuspended in PI solution (50 μg/ml in PBS, pH 7.4) containing 50 μg/ml RNase A and incubated in the dark at room temperature for 30 min. The DNA content of the cells was then analyzed by Becton–Dickinson FACS-420.

2.4. Detection of acidic- and neutral-SMase activity

The sample was prepared as described by Zhang and coworkers [27]. Briefly, for acidic-SMase, Hs578T cells (5×10^6) were pelleted, washed and lysed in 0.6 ml of buffer (pH 5.0) containing 50 mM sodium acetate, 1% Triton X-100, 1 μg/ml aprotinin, 1 mM EDTA, and 100 μg/ml PMSF for 60 min on ice. Insoluble cell debris and nuclei were pelleted. The protein concentration in the supernatant fraction was measured with the Bio-Rad Protein Assay. Samples for neutral-SMase were prepared as above with slight modifications (0.1 M Tris–HCl, pH 7.4, was used instead of 50 mM sodium acetate). SMase

activity in whole-cell lysates was measured with the Amplex Red reaction kit (Molecular Probes Inc). One hundred and sixty micrograms of protein lysates was used to determine by two-step and continuous assay for acidic- and neutral-SMase activity in 50 mM sodium acetate (pH 5.0) and 0.1 M Tris buffer (pH 7.4), respectively.

2.5. Determination of ceramide

Ceramide was quantified by the diacylglycerol kinase assay as ^{32}p incorporated upon phosphorylation of ceramide to ceramide-1-phosphate [28]. The ceramide-1-phosphate was resolved by TLC using $CHCl_3$:CH_3OH:CH_3COOH (65:15:5, v/v) as solvent as described. Spots were visualized by autoradiography and relative content was scanned by Typhoon Trio+ instrument.

In parallel, the ceramide-1-phosphate standard was prepared enzymatically as described [28].

2.6. Soft agar assay

The *in vitro* growth characteristics were tested by colony formation assay [29]. Briefly, 2×10^4 viable Hs578T transfectant cells were suspended and plated in 0.32% agar containing RPMI 1640 supplemented with 17% fetal calf serum in the presence of staurosporine or DMSO vehicle control, and layered over a 0.5% agar base with complete medium containing 20% serum in six-well plates. The culture dishes were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Colonies were photographed at 10× magnification after 4 weeks. The number of colonies formed in soft agar was counted from one sample in three random fields. Average values presented ±SD, which was determined in each set of nine values from three-independent experiments.

2.7. Western blot

Procedures for preparation of whole cell lysates and Western blot analysis were described in our previous report [30].

3. Results

3.1. Stable caveolin-1 expression in various cell lines

In the present study, we obtained four stable monoclonal cell lines: Hs578T/cav-1 as transfectant cells over expressing caveolin-1 and its control cells, Hs578T/vector, and Hs578T/siRNA as transfectant cells with caveolin-1 knockdown and its control cells, Hs578T/shuffle. Fig. 1 showed detection of caveolin-1 by Western blot analysis. The level of caveolin-1 expression in Hs578T/cav-1 cells was higher than that in Hs578T/vector and Hs578T/shuffle cells. The caveolin-1 protein level in siRNA-transfected

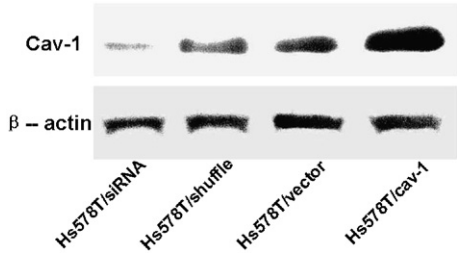

Fig. 1. Caveolin-1 expression levels were determined in Hs578T transfectants by Western blot analysis. Twenty five micrograms of whole cell lysate were separated by SDS–PAGE and proteins were transferred onto a nitrocellulose membrane. The detection of caveolin-1 was performed with ECL method. β-Actin was used as a loading control.

cells was significantly reduced. The amount of caveolin-1 in Hs578T/cav-1 was about 15-fold higher than in Hs578T/siRNA cells as detected by densitometry.

3.2. Suppression of staurosporine-mediated apoptosis by caveolin-1

The changes in the apoptosis of Hs578T cells containing various caveolin-1 levels following staurosporine treatment at 1.0 μM for 11 h by flow cytometry analysis were shown in Fig. 2A, indicating the percentage of cell apoptosis increased with the decrease of caveolin-1 levels. The amount of apoptosis in Hs578T/siRNA was about 4-fold higher than that in Hs578T/cav-1 cells (deducting control). The order of sensitivity to staurosporine was Hs578T/siRNA > Hs578T/shuffle = Hs578T/vector > Hs578T/cav-1. Induction of apoptosis by staurosporine also appeared to be time- and dose-dependent (Fig. 2B and C). In addition, we obtained nuclear morphological alteration and DNA ladder data (not showed in the paper) which are also hallmarks of cell apoptosis. These results indicated that the lower caveolin-1 expression, the higher apoptotic image in Hs578T cells, in other words, the reduction of apoptosis parallels the increase of caveolin-1.

3.3. Caveolin-1 promotion of Hs578T cell growth and clonogenic survival

To determine whether the altered levels of caveolin-1 in the tumor cells affected the cell transforming capability. Here, we observed cell growth of four types of Hs578T cells described above by colony formation in soft agar. We found that there was no obvious difference in soft agar colony formation between two control cell lines, Hs578T/vector and Hs578T/shuffle. (Fig. 3Aa,e). However, overexpressing caveolin-1 cells, Hs578T/cav-1, formed numerous large colonies after 4 weeks of growth in soft agar (Fig. 3Ab). Knockdown caveolin-1 cells, Hs578/siRNA, exhibited a reduced

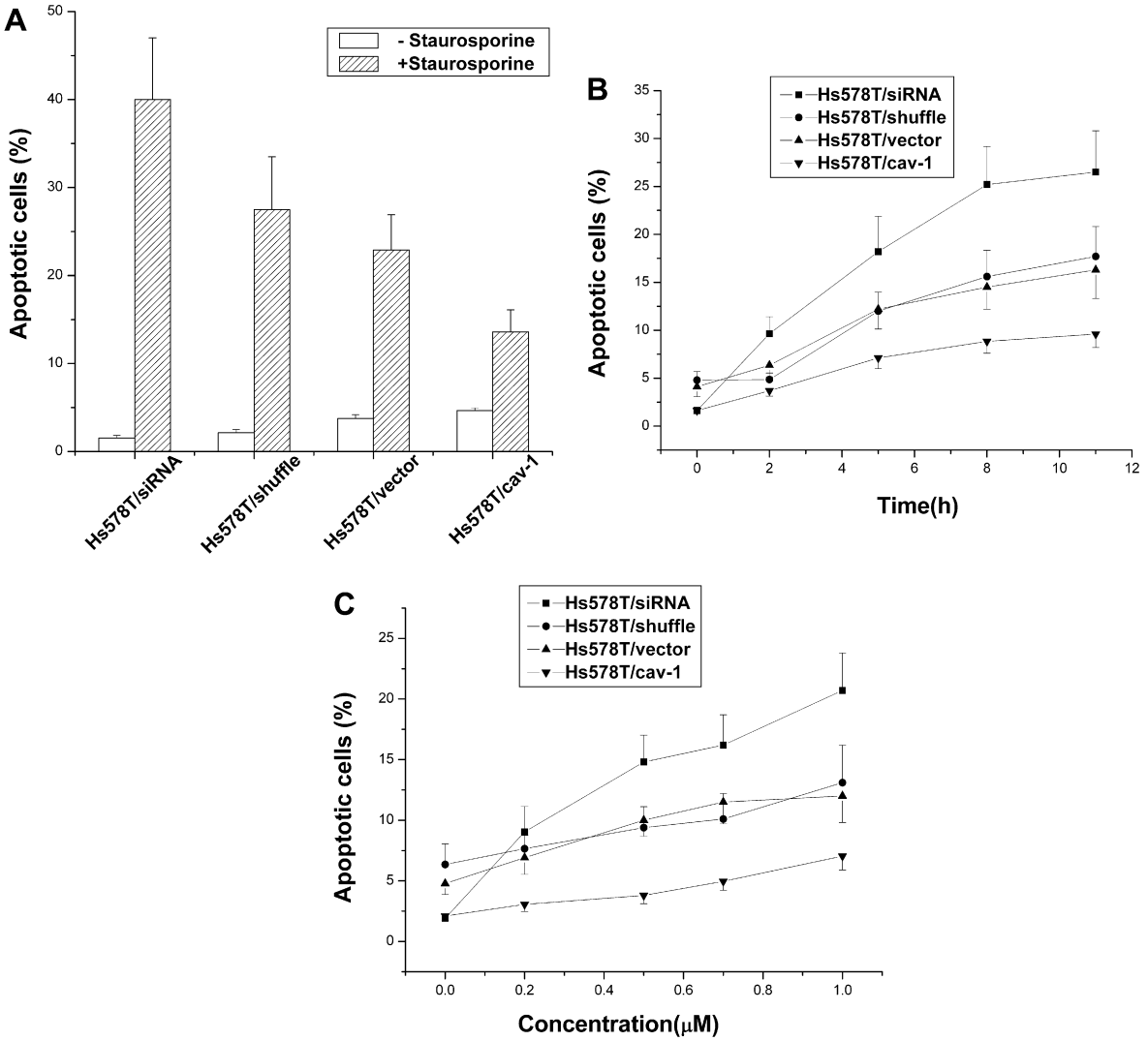

Fig. 2. Caveolin-1 suppression of Hs578T cell apoptosis was quantitatively determined by flow cytometry analysis. Hs578T cells were treated with 1.0 μM staurosporine and incubated at 37 °C for 11 h (A). The time- and dose-dependence of staurosporine apoptosis was assessed in Hs578T cell lines. The cells were pre-incubated at 37 °C at 1.0 μM staurosporine for the indicated times (B) and for 11 h at the indicated staurosporine concentrations (C). Data are expressed as the mean ± SD from three-independent experiments.

colony formation capability, with a lower number of colonies formed and reduced colony size (Fig. 3Af). Importantly, the number of colonies and colony size in soft agar dramatically decreased in two control cell lines (Fig. 3Ac,g) when the apoptosis inductor, staurosporine, was added. In particular, little colonies can be observed in soft agar for Hs578T/siRNA cells (Fig. 3Ah). Nevertheless, cell survival for Hs578T/cav-l was only moderately altered (Fig. 3Ad). The relative values of the number of colonies in soft agar were shown in Fig. 3B. These results clearly confirmed the inhibition of colony formation occurring in Hs578T cells with down-regulated caveolin-1.

3.4. Caveolin-1 reduction of ceramide levels and increase of Akt phosphorylations

As shown in Fig. 4A, an increase of ceramide was observed in knockdown caveolin-1 cells. Ceramide release in overexpressed caveolin-1 cells, Hs578T/cav-l, was decreased by 3-fold as compared with Hs578/siRNA (Fig. 4B).

Since the products of sphingomyelin hydrolysis are ceramide and phosphocholine, the enzyme involved in this reaction is SMase. To conform that ceramide content in these cells was due to alteration of SMase activity, we measured both acid- and neutral-SMase activity *in vitro*.

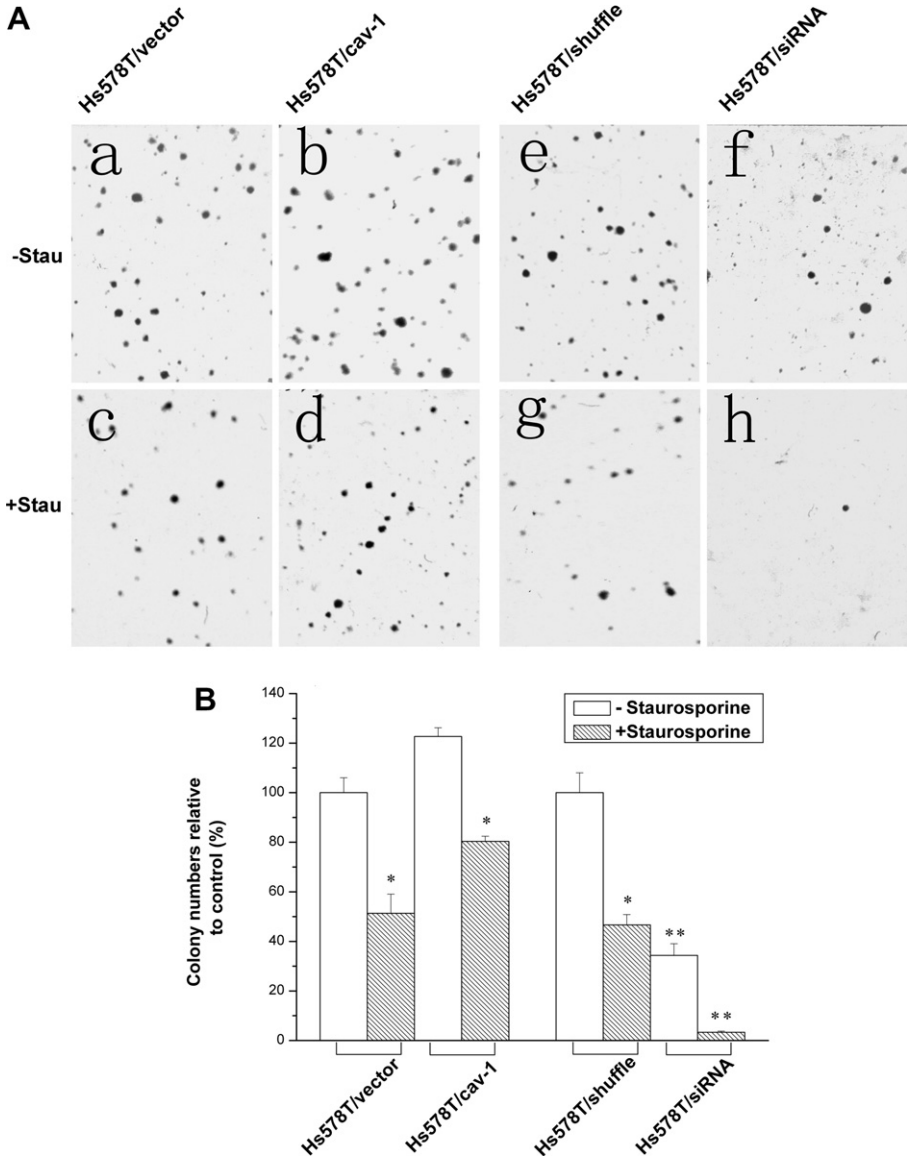

Fig. 3. Caveolin-1 promotion of growth and survival of Hs578T cells in soft agar. (A) All of the transfectants were seeded at 2×10^4 cells per well (in six-well plates) in growth medium containing RPM 1640 supplemented with 17% fetal calf serum and 0.32% soft agar in the presence or absence of staurosporine. After 4 weeks, the surviving colonies were photographed. The result was representative of three-independent experiments. (B) The number of colonies formed in soft agar (from A) was counted from one sample in three random fields. Average values presented ±SD, which were calculated in each set of nine values from three-independent experiments. The colony numbers of Hs578T/cav-l and Hs578T/siRNA cells in soft agar were expressed as percentage of levels of Hs578T/vector and Hs578T/shuffle cells in absence of staurosporine, respectively. $^{*}P < 0.05$. $^{**}P < 0.001$.

Fig. 4C illustrated that down-regulation of caveolin-1 expression resulted in the activation of a neutral-SMase, whereas its activity in cells overexpressing caveolin-1 decreased. Neutral-SMase activity was about 2.6-fold higher in Hs578T/siRNA cells than in Hs578T/cav-l cells, which roughly correlates with a 3-fold increase of ceramide release. In contrast to the neutral-SMase, acidic-SMase activity remained almost unchanged.

To look into whether caveolin-1 affected Hs578T cell apoptosis through the ceramide/p-Akt signaling pathway, we also determined Akt phosphorylation in the four transfectant cells by Western blot analysis. As shown in Fig. 5A that increase of caveolin-1 expression resulted in elevated basal Akt phosphorylation. Hs578T/cav-l cells exhibited both the highest caveolin-1 levels and highest phosphorylated Akt levels, while Hs578T/siRNA cells

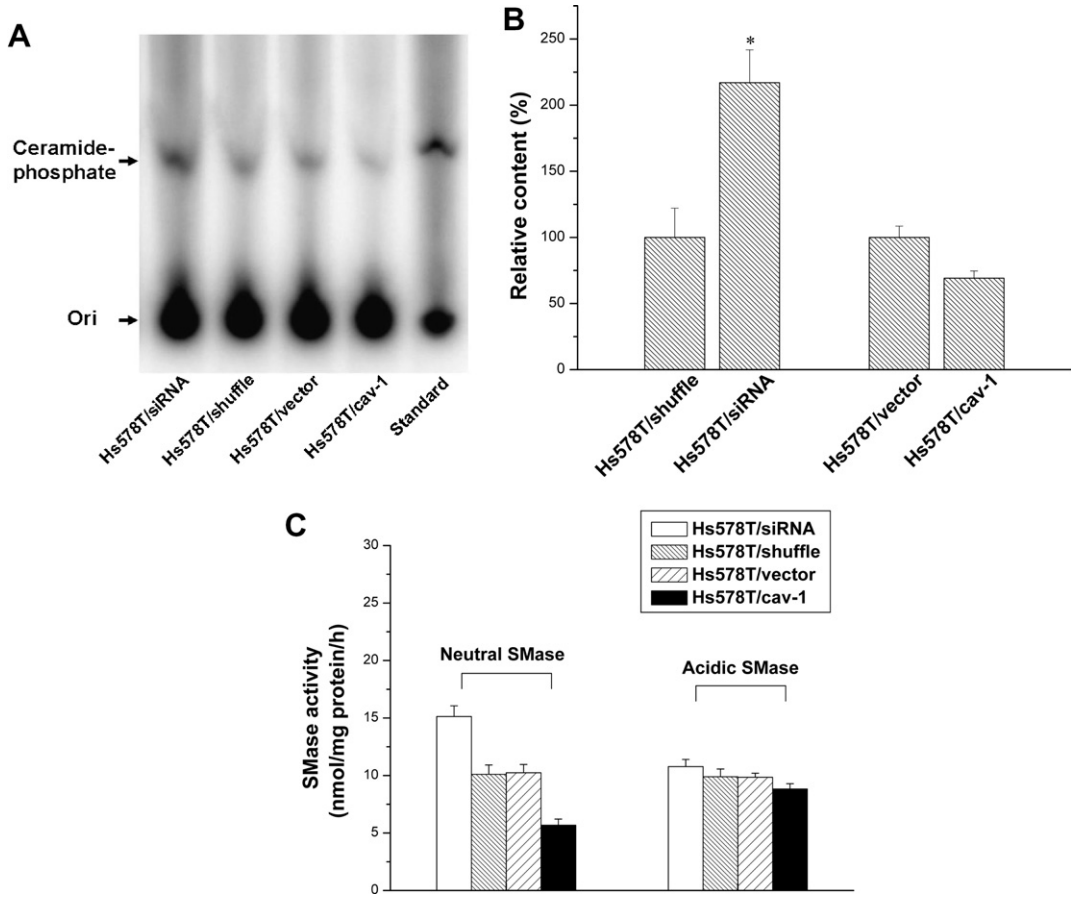

Fig. 4. Caveolin-1 reduction of ceramide release and SMase activity. (A) Transfectant cells were washed and lysed, and then the lipids were extracted with organic solution. Ceramide release was determined by incubation of the lipids with *Escherichia coli* DAG kinase in the presence of [γ-^{32}P]ATP. Lipids were resolved by TLC using chloroform:methanol:acetic acid (65:15:5, v/v) as solvent and autoradiography. Authentic ceramide 1-phosphate standard prepared enzymatically. It was representative of three-independent experiments. (B) The relative contents of ceramide 1-phosphate (densitometrical analysis) in Hs578T/siRNA and Hs578/cav-l cells were expressed as percentage of levels of Hs578t/shuffle and Hs578T/vector cells (both control cells), respectively, $n = 3$, mean $\pm$ SD. $^{*}P < 0.05$. (C) Hs578T transfectants were washed and lysed, SMase activity was determined in whole cell lysates according to manufacturer's instruction. One hundred and sixty micrograms protein cell lysates were used as sample to determine both SMase. The mean $\pm$ SD of three-independent experiments was shown.

which expressed low levels of caveolin-1, displayed minimal Akt phosphorylation. In order to correct loading difference for each sample, the results were also expressed as a ratio (p-Akt/β-actin) (Fig. 5B) which showed clearly caveolin-1 expression positively correlated with p-Akt.

4. Discussion

In the present study, we used both approaches of knockdown and overexpression of caveolin-1 in Hs578T breast adenocarcinoma cells, demonstrating down-regulation of caveolin-1 increases the susceptibility of the cells to staurosporine-induced apoptosis. Importantly, evidence demonstrating the anti-apoptosis action of caveolin-1 was observed in caveolin-1-overexpressing cells. Our findings demonstrated that caveolin-1 plays a key role in the negative regulation of cell apoptosis in Hs578T breast cells.

To further assess whether caveolin-1 expression is necessary for cell survival, we employed anchorage-independent cell growth (i.e. growth in soft agar). Our experiments demonstrated that up-regulation of caveolin-1 promoted cell proliferation and survival. Similarly, it has been indicated that loss or down-regulation of caveolin-1 expression in prostate cancer results in significantly reduced cancer tumorigenic and metastatic potential with increased

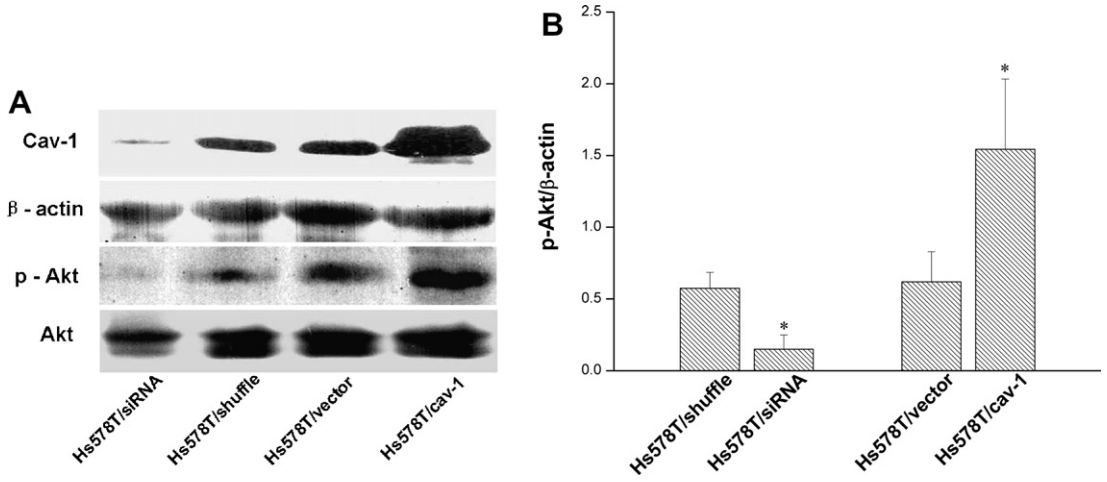

Fig. 5. Caveolin-1 expression levels and Akt-phosphate and Akt were determined in Hs578T transfectant cells by Western blot analysis. Forty micrograms of whole cell lysate was loaded in each lane. Caveolin-1, β-actin, Akt, and p-Akt were detected by ECL method. (A) It was representative of three-independent experiments. β-Actin served as a loading control. (B) Graphics represented Western blot densitometrical analysis and the results were expressed as a ratio (p-Akt/β-actin). $n = 3$, mean ± SD. $^{*}P < 0.05$ vs level of control cells, respectively.

apoptosis [31]. In contrast with our results, Engelman and co-workers showed that expression of caveolin-1 in oncogenically transformed cells abrogated their growth in soft agar and initiated apoptosis in rapidly dividing cells because its expression dramatically inhibited both Ras/MAPK and basal transcriptional activation of a mitogen-sensitive promoter [5]. A number of studies also indicated that exogenous expression of caveolin-1 in oncogenically transformed cells and cancer cell lines resulted in inhibition of cells growth in soft agar, invasion, and tumorigenesis [4,32]. Intriguingly, it was found that caveolin-1 expression in small cell lung cancers significantly inhibited soft agar colony formation, whereas caveolin-1 appears to be required for the cell survival and growth in non-small cell lung cancers [29].

The sphingomyelin pathway is a ubiquitous, evolutionarily conserved signaling system. SMase activation and the function of the released ceramide have been associated with signaling pathways in cell growth, differentiation, and apoptosis [33]. Previous reports have indicated that SMase and caveolin-1 are predominantly found in the caveolae membrane fraction [23]. In the present paper, however, we demonstrated only neutral-SMase activity was inhibited by caveolin-1. Similarly, Czarny and co-workers have also found that the mechanoactivation occurs directly at the luminal endothelial cell surface primarily in caveolae enriched with sphingomyelin and neutral-SMase, but not acidic-SMase [34]. Although Zundel and co-workers found acidic-SMase activity in caveolin-rich membrane sub fractions [11], in general, it was affirmed that some cell plasma membranes and their caveolae are enriched with functional neutral-SMase but not acidic-SMase, which is an intracellular enzyme primarily localized in the cytoplasm, lysosomes, and endosomes [34,35]. Specially, Veldman and co-workers have reported that the CSD of caveolin-1 specifically inhibits neutral-SMase, while acidic-SMase is completely insensitive to the CSD [23]. In our study, the functional inhibitory effect of caveolin-1 on neutral-SMase (but not acidic) is also observed in over-expression of caveolin-1 cells and the reduction of neutral-SMase activity parallels the increase of caveolin-1 expression, implying a direct molecular interaction between caveolin-1 and neutral-SMase may occur in caveolae.

The Akt has been shown to negatively regulate pro-apoptotic effectors and deregulation of signaling Akt pathway is dependent on the generation of lipid secondary messenger ceramide [11]. Our observations in the paper showed that reduction of ceramide levels due to inhibition of neutral-SMase in Hs578T cells overexpressing caveolin-1 result in a significant enhancement in Akt phosphorylation. Similarly, Shack and colleagues showed that the caveolin-overexpressing cells exhibited a significant activation of the Akt pathway due

to decreased neutral SMase activity and ceramide synthesis [20]. But surprisingly, this up-regulation of Akt signaling in caveolin-overexpressing 293 and HeLa cells is found to promote cell death rather than to favor survival, offering the existence of a novel Akt-mediated death pathway that is dependent on high caveolin expression [20]. However, our findings clearly demonstrate that an activated Akt pathway is mainly responsible for cell survival in Hs578T cell lines. It could be due to the use of normal cells and arsenite, as apoptotic stimuli in Shack's study, while, breast cancer cell and staurosporine were used in our experiment. Interestingly, Zundel et al. reported that overexpression of caveolin-1 can inhibit the activity of PI(3)K/Akt in fibroblasts and result in the cells more sensitive to apoptosis stimuli [11]. PI(3)K has been shown to localize in caveolae in fibroblasts [36]. So the possibility could not be excluded that the direct interaction of PI(3)K and caveolin-1 in caveolae under certain condition may result in decrease of PI(3)K activity. Currently, several controversial pieces of evidence relating to caveolin-1 as a promoter or suppressor in normal and tumor cell apoptosis and survival have been documented. Until now, there is very little explanation for the disparate results can be convinced because the mechanism of caveolin-1-mediated cellular apoptosis *in vivo* remains unknown.

It should also be noted that although we have provided evidence for the coordinated regulation of apoptosis and survival via caveolin-1 inhibition of neutral-SMase and decrease of ceramide release, furthermore, activation of the Akt pathway, this does not mean that this is the only system for suppressing apoptosis. Other mechanisms have also been found to be in existence. For example, caveolin-1 expression significantly reduces thapsigargin-stimulated apoptosis in prostate cancer cells through inhibiting serine/threonine protein phosphatases PP1 and PP2A [9], and expression of caveolin-1 enhances matrix-independent cell survival in MCF-7 breast cancer cells through stimulation of pathway of survival signaling including the Akt by up-regulation of insulin-like growth factor-1 receptor expression [37].

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