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ASK1–JNK Signaling Cascade Mediates Ad-ST13-Induced Apoptosis in Colorectal HCT116 Cells

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ABSTRACT

ST13, a co-factor of heat shock protein, has shown potential antitumor efficacy for colorectal cancer in our previous study. However, the molecular mechanisms governing ST13-induced apoptosis are poorly understood. Here, we demonstrate that Ad-ST13 (ST13 mediated by adenovirus) activates apoptosis signal-regulated kinase (ASK1) and c-Jun N-terminal kinase (JNK) but not p38 (mitogen-activated protein kinase) in human colorectal HCT116 cells. Ad-ST13 also increases extracellular-regulated kinase (ERK) phosphorylation levels, but the change is due to adenovirus replication. Overexpression of ST13 also increases the transcription activity of AP-1. Blocking ASK1–JNK pathway affects Ad-ST13-mediated colorectal cell apoptosis, decreases the release of cytochrome *c* in cytoplasm and caspase activation. Because ASK1 is known to contain a tetratricopeptide repeat (TPR)-acceptor site and ST13 has TPR domain, we found the interaction between ST13 and ASK1. These results strongly indicate Ad-ST13 triggers colorectal cell apoptosis via ASK1–JNK signaling cascade. J. Cell. Biochem. 110: 581–588, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: ST13; HIP; ASK1; JNK; APOPTOSIS

ST13 is the gene encoding Hsp70 interacting protein (Hip), a co-chaperone of the 70-kDa heat shock proteins (Hsc/Hsp70) [Höhfeld et al., 1995; Shi et al., 2007]. Hip may facilitate the chaperone function of Hsc/Hsp70 in protein folding and repair, and in association with gesterone receptor complexes [Prapapanich et al., 1996a,b]. ST13 was cloned by subtractive hybridization analysis in normal colon tissue and colon carcinomas [Zhang et al., 1998]. Its mRNA and protein levels were down-regulated in colorectal cancer and breast cancer compared with adjacent normal tissues [Wang et al., 2005; Antiwetrueangdet et al., 2007; Williams et al., 2007]. Our previous study has proved that increased ST13 protein expression suppresses proliferation of colorectal cancer cells and induces apoptosis of colorectal cell lines SW620 and HT29 [Yang et al., 2008; Yu et al., 2009]. In addition, overexpressed ST13 also triggers apoptosis in breast cancer cells. However, the underlying molecular mechanisms behind ST13-mediated apoptosis are not completely understood.

M. Yang and M. Yu contributed equally to this work.

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Mitogen-activated protein (MAP) kinase cascades are activated in response to various extracellular stimuli, including growth factors and environmental stresses [Lee et al., 1994; Chang and Karin, 2001]. There are at least three well-defined MAP kinases in mammalian cell: extracellular signal-regulated kinase (ERK), stress-activated protein kinase (SAPK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase (p38) [Zhou et al., 1995]. Activation of MAP kinases is achieved through phosphorylation of specific threonines and tyrosines by dual specific kinases via a three-step kinase cascade. Among them, the ERK subgroup of MAP kinases is activated primarily by mitogenic stimuli such as growth factors and virus propagation [Xia et al., 1995; Schumann and Dobbelstein, 2006]. The JNK and p38 MAP kinase are activated in response to cytokines, or environmental stress resulting in cell differentiation and apoptosis [Zarubin and Han, 2005; Weston and Davis, 2007].

Apoptosis signal-regulating kinase 1 (ASK1), a MAP kinase kinase kinase (MAPKKK), is known to activate two different subgroups of MAP kinase kinases (MAPKK), SEK1 (or MKK4), and MKK3/MAPKK6 (or MKK6), which in turn activates SAPK/JNK and p38 MAP kinase, respectively [Takeda et al., 2003; Matsukawa et al., 2004; Ichijo et al., 1997]. ASK1 can be regulated by ASK1-interacting protein and tumor necrosis factor (TNF) receptor-associated factor 2 [Nishitoh et al., 1998]. Heat shock protein (Hsp70) binds ASK1 at its amino terminus and suppresses ASK1-dependent apoptosis. Overexpression of dn-ASK1 (ASK1-KM) can inhibit TNFα [Ichijo et al., 1997] and Fas ligand-induced cell death [Chang et al., 1998]. Moreover, constitutively active ASK1 (ASK1ΔN) overexpression has been demonstrated to trigger apoptosis through mitochondrial-dependent caspase activation [Hatai et al., 2000]. Activation of caspases is one of the most generally recognized features of apoptosis through the mitochondria pathway [Hengartner, 2000]. Caspases are activated following the release of cytochrome *c* from the mitochondria into the cytoplasm [Kluck et al., 1997].

To better understand how elevated ST13 induces apoptosis, we investigate which MAP kinase signaling pathway is involved Ad-ST13-mediated cell death and apoptosis. Our results suggest that the ASK1–JNK signaling cascade mediates Ad-ST13-induced apoptosis via mitochondria pathway in HCT116 cells.

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MATERIALS AND METHODS

CELL LINES AND REGENTS

Human colorectal cells HCT116 (Shanghai Cell Collection, Shanghai, China) were maintained in DMEM supplemented with 5% heat-inactivated fetal bovine serum (FBS). HEK293T cells (Microbix Biosystens Inc. Toronto, Ontario, Canada) were cultured in DMEM containing 10% FBS. JNK inhibitor SP600025, ERK inhibitor PD98059, and p38 MAP kinase inhibitor SB203580 were obtained from Calbiochem (San Diego, CA). Stock solutions of the inhibitors were prepared in dimethyl sulfoxide (DMSO). pcDNA3.1–ST13 was provided by Prof. Shu Zheng (Zhejiang University, Hangzhou, Zhejiang, China). pFlag-CMV-1-ST13 was constructed by inserting ST13 cDNA at the *Hin*dIII–*Eco*RV site of the pFlag-CMV-1 expression vector (Sigma, St. Louis, MO). pcDNA3.1–ASK1 was a generous gift from Dr. Gang Pei (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China). siRNA sequences are 5′-CAAAUACUGGAAGGAUUAAdTdT-3′ (sense) and 5′-UUAAUCCUUCCAGUAUUUGdTdT-3′ (antisense). JNK siRNA sequences are 5′-GACTTAAAGCCCAGTAATAdTdT-3′ (sense) and 5′-UAUUACUGGGCUUUAAGUCdTdT-3′ (antisense). Negative control siRNA sequences are 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACACGUUCGGAGAATT-3′ (anti-sense). All siRNAs sequences were synthesized in GenePharma Company (Shanghai, China).

VIRUS CONSTRUCTION AND VIRUS INFECTION

The construction and purification of Ad-ST13 and Ad-EGFP were performed as described previously [Yang et al., 2008]. Ad-EGFP expressing EGFP was used as a vector control. Ad-EGFP is an E1A and E3 areas-deleted and proliferation-limited adenovirus [Liu and Gu, 2006]. Virus titre was determined as described previously [Yang et al., 2008].

AP-1-TRANSACTIVATION ACTIVITY ASSAY AND TRANSFECTION ASSAY

Plasmid pcDNA3.1–ST13 was purified using Qiagen Plasmid Purification Maxi Kit (Qiagen, Hilden, Germany). HCT116 cells were plated at a density of 1.0×10^5 cells/ml in a 12-well plate and co-transfected with pAP-1-TA-Luc, an activator protein-1 response element (AP-1) reporter plasmid, pRL-TK (Clontech, CA), β-gal plasmid and pcDNA3.1–ST13 or pcDNA3.1 empty vector. After 48 h, cells were lysed for 15 min at room temperature (Passive Lysis Buffer; Promega, Madison, WI). The lysate was analyzed using the Dual-Luciferase Reporter Assay System (Promega) and measured in an illuminometer (Lumate LB 9507, Germany). The transfection of siRNA knockdown of ASK1 or JNK to HCT116 was performed according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). The negative siRNA acted as a control.

CELL VIABILITY ASSAY

HCT116 cells were placed in 96-well plates and treated with various inhibitors or siRNAs before adenoviruses were added. At the indicated time points, medium was removed and fresh medium containing 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml) was put in each well. After 4-h incubation at 37°C, cells were lysed with 100 μl lysis buffer (50% SDS and 50% 2, 4-dinitrofluorobenzene). Absorbance from the plates was read on a Bio-Rad (Hercules, CA) microplate reader at 595 nm.

ANNEXIN V BINDING

To perform Annexin V binding, HCT116 cells were trypsinized and washed with PBS. Aliquots of cells (5×10^5) were resuspended in 500 μl of binding buffer and stained with fluorescein isothiocyanate (FITC)-labeled annexin V (KeyGEN Biotechology, Nanjing, China) according to the manufacturer's instructions. A fluorescence-activated cell-sorting (FACS) assay was performed immediately after staining.

IP AND WESTERN BLOT ANALYSIS

pFlag-CMV-1 or pFlag-CMV-1-ST13 was transfected in addition to vector pcDNA3.1–ASK1 expressing hemagglutinin (HA) tagged-ASK1. After 48 h of incubation, the transfected HCT116 cells were lysed in cold radioimmunoprecipitation assay buffer [50 mM buffer Tris (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, aprotinin (1 μg/ml), and pepstatin (1 μg/ml)] on ice and homogenized. The lysates were precleared with protein A/G-Sepharose beads (Santa Cruz Biotechnology, CA), and incubated first with antibodies against ST13 or HA (1 μg) in immunoprecipitation (IP) buffer [20 mM Tris–HCl, pH 8.0, 10% glycerol, 5 mM $MgCl_2$, 0.1% Tween 20, 0.1 M KCl, 1× protease inhibitor cocktail (Cell Signaling Technology, Boston, MA), and 0.5 mM dithiothreitol] overnight at 4°C with constant rotation and then with protein A/G (50/50)-Sepharose beads for 1 h. Beads were washed three times with IP buffer and resuspended in 40 μl of 2× SDS buffer. The sample was subjected to SDS–PAGE. Western blotting was performed as described previously [Yang et al., 2008]. The primary antibodies included ST13 (1:2,000, mouse monoclonal, provided by Prof. Zheng), ASK1, and pASK1 (1:1,000 in both cases; Signalway Antibody, Pearland, TX), p38, pp38, ERK1/2, pERK1/2, SAPK/JNK, p-SAPK/JNK, c-Jun, and c-Jun phosphorylation (pc-Jun, 1:1,000 in all assays; Cell Signaling Technology), HA, caspase 3, and cytochrome *c* (1:1,000; Santa Cruz Biotechnology), Rabbit

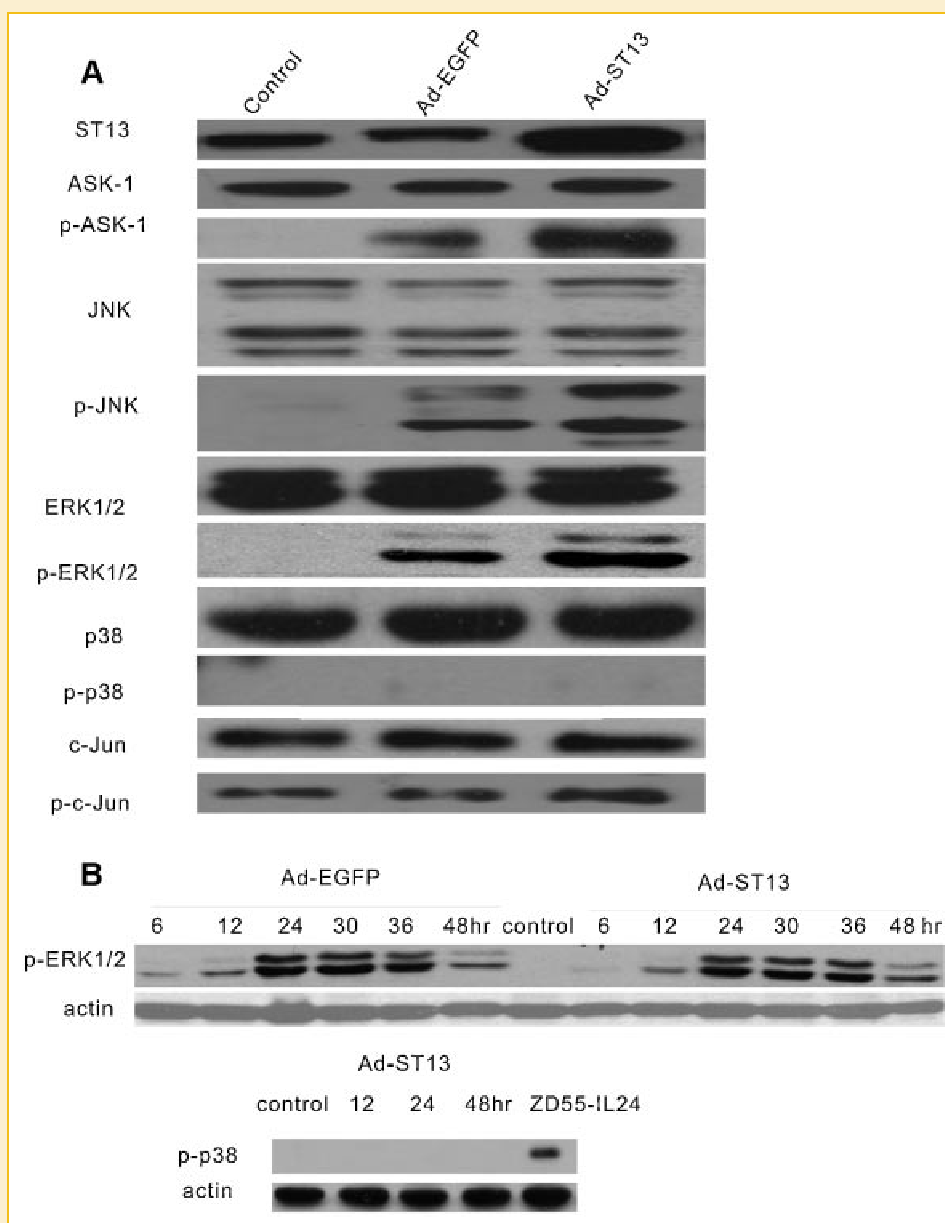

Fig. 1. Ad-ST13 activates ASK1 and JNK pathways in HCT116 cells. A: Levels of phosphorylated and nonphosphorylated ASK1, MAP kinases, and c-Jun at 48 h of 10MOI Ad-ST13-treatment. Ad-EGFP acted as vector control. B: Levels of phosphorylated MAP kinases at different time points of 10MOI Ad-ST13 induction. ZD55-IL24 acted as positive control.

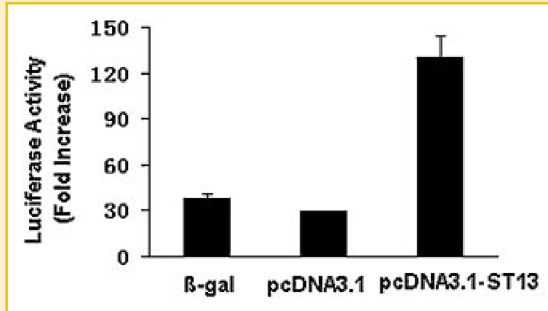

Fig. 2. Elevated ST13 activates AP-1 in HCT116 cells. AP-1 reporter construct (pAP-1-TA-Luc) was cotransfected with empty vector pcDNA3.1 or pcDNA3.1-ST13 expression vector. Cell lysates were prepared 48 h after transfection. Reporter activity was determined by luciferase assay. The data shown are mean ± SD from three independent experiments, each done in triplicate.

polyclonal anti-Flag (F7425, 1:1,000; Sigma). For determination of translocation cytochrome *c* from mitochrondria to cytosol, the method was performed as described previously [Yang et al., 2008].

RESULTS

AD-ST13 ACTIVATES ASK1 AND JNK PATHWAYS

We measured the expression levels of ASK1, JNK, and p38 phosphorylation in Ad-ST13-infected HCT116 cells by Western blotting. As shown in Figure 1A, ST13 expression levels were markedly elevated after Ad-ST13 infection. Overexpressed ST13 increased ASK1 and JNK phosphorylation levels, while p38 phosphorylation levels were not observed. ERK phosphorylation (pERK) levels also increased. However, levels of pERK in the control Ad-EGFP-infected cells had the same increase. Further, we did time course to check the change of pERK levels. We found that pERK levels increased from 6 to 36 h after Ad-ST13 infection and then decreased at 48 h. The trend was similar to the treatment of Ad-EGFP, which suggested that the activation of ERK was related to the viral propagation (Fig. 1B). To confirm whether Ad-ST13 activated p38 expression, we tested phosphorylated p38 expression levels at different time points after Ad-ST13 infection. As expected, Ad-ST13 treatment did not activate phosphorylated p38 expression (Fig. 1B).

Next, we analyzed pc-Jun expression levels. We found that pc-Jun expression levels increased after Ad-ST13 infection (Fig. 1A). However, the pc-Jun levels in Ad-ST13-infected cells were merely 1.5 times compared with Ad-EGFP-infected cells. pc-Jun can lead to elevate transcriptional activity of AP-1 [Weston and Davis, 2007; Sabapathy et al., 2004]. Therefore, we further explored the effects of ST13 on transcriptional activity of AP-1 using luciferase assay. To

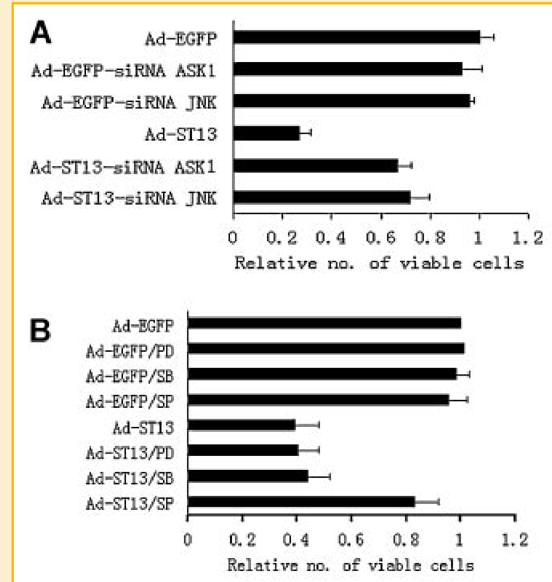

Fig. 4. The ASK1 and JNK knockdown or JNK inhibitor SP600025 protects cells against Ad-ST13-induced antiproliferative effect. Viable cells were measured by MTT assay at 48 h (A) and 36 h (B) after Ad-ST13-treatment in the present of siRNAs or various inhibitors. SB, SB20358; SP, SP600025; PD, PD98059.

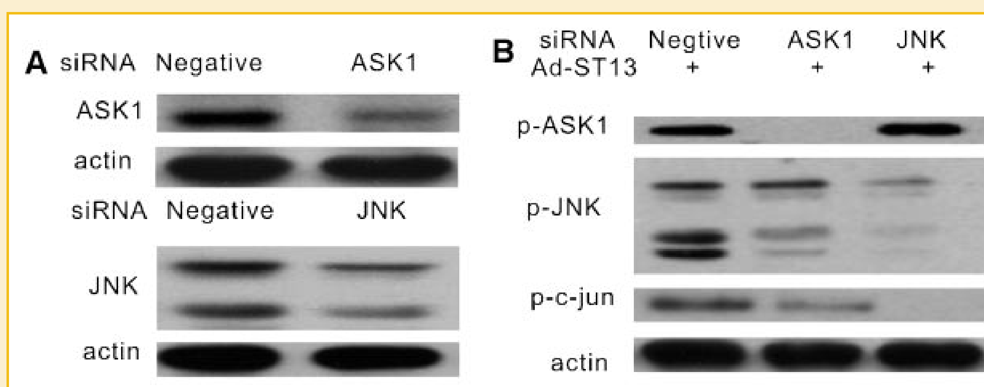

Fig. 3. Blocking ASK1 and JNK pathway protects cells against Ad-ST13-mediated antiproliferative effects. A: Detection of levels phosphorylated ASK1 and JNK after treatment of siRNA knockdown of ASK1 and JNK, respectively. B: Levels of phosphorylated JNK and c-Jun in Ad-ST13-treatment cells were measured after siRNA knockdown of ASK1. Levels of actin are shown as a loading control.

exclude the influence of virus vector, we use pcDNA3.1–ST13 to test transcriptional activity of AP-1. We observed that pcDNA3.1–ST13 caused a 4.4-fold transcriptional activation of AP-1 compared with pcDNA3.1 ((Fig. 2). Above data indicate the elevated ST13 expression activates ASK1 and JNK pathway and increases the transcriptional activity of AP-1.

BLOCKING ASK1 AND JNK PATHWAY PROTECTS CELLS AGAINST Ad-ST13-MEDIATED ANTIPROLIFERATIVE EFFECTS

To detect ASK1 and JNK pathway relating to Ad-ST13-mediated cytopathic effects, ASK1- or JNK-specific siRNA was used in the following experiments. We observed the designed siRNA ASK1 and siRNA JNK decreased ASK1 and JNK expression, respectively

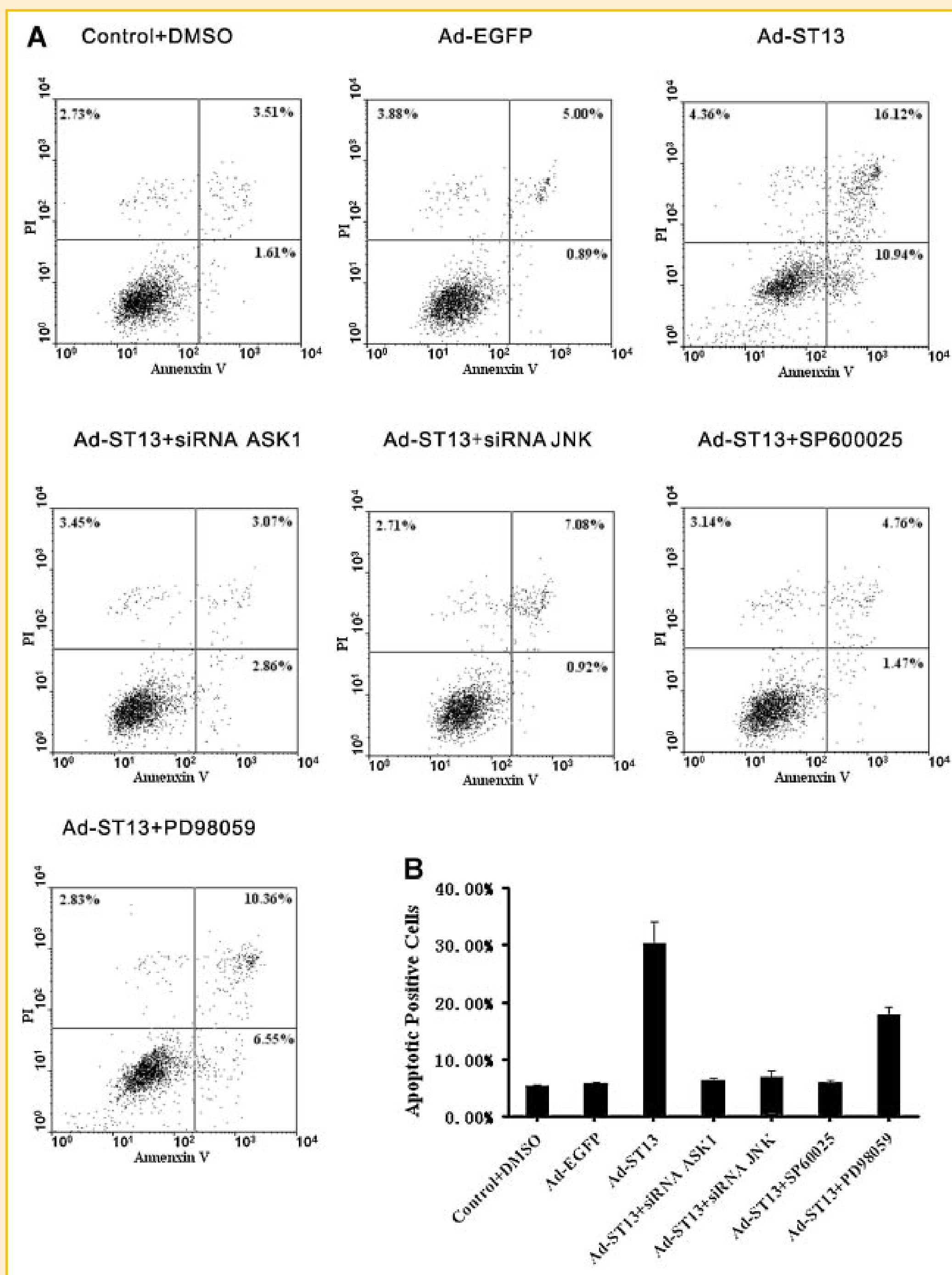

Fig. 5. Ad-ST13-induced apoptosis in HCT116 cells was mediated by ASK1 and JNK pathway. A: Blockage of Ad-ST13-induced apoptosis at 36 h by the siRNA knockdown of ASK1 and JNK or JNK inhibitor SP600025 was observed, but not by those of ERK. B: Percentage of apoptotic cells after various treatments.

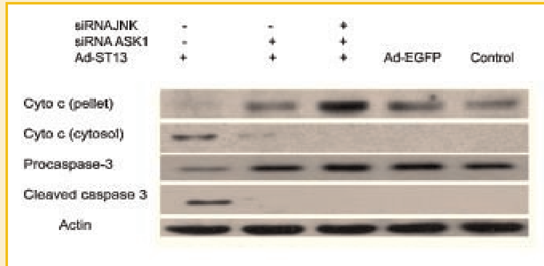

Fig. 6. Blocking of ASK1 and JNK pathway prevents Ad-ST13-mediated apoptosis. The release of cytochrome *c* and caspase-3 expression level by the ASK1 and JNK knockdown were measured.

(Fig. 3A). ASK1 knockdown decreased JNK phosphorylation, and ASK1 or JNK knockdown blocked pc-Jun (Fig. 3B). These results indicate that ASK1 is associated to JNK, and c-Jun is the target of ASK1 and JNK cascade. Next, we used the MTT assay to detect the effects of ASK1 and JNK pathway on cell viability after Ad-ST13 infection. HCT116 cells were transfected with ASK1 or JNK siRNA or added with different inhibitors. We found Ad-ST13 caused 75% cell death at 72 h (Fig. 4A). However, this inhibitory effect was reversed by ASK1 or JNK knockdown. The same inhibition result was also obtained after treatment with 1 μM JNK inhibitor SP600025. However, treatment of p38 MAP kinase inhibitor SB203580 or ERK1/2 inhibitor PD98059 had no such effect (Fig. 4B).

BLOCKING OF ASK1 AND JNK PATHWAY PREVENTS Ad-ST13-MEDIATED APOPTOSIS

To investigate the effects of ASK1 and JNK pathways on Ad-ST13-mediated apoptosis, the inhibitors were added into Ad-ST13-infected cells. As monitored by Annexin V binding assay in HCT116 cells, ASK1 or JNK knockdown effectively blocked the Ad-ST13-induced apoptosis (Fig. 5A,B).

Cytochrome *c* and caspase-3 proteins are apoptosis-related proteins [Hsu et al., 1997]. Thus, the release of cytochrome *c* in cytoplasm and the caspase-3 expression levels were detected in HCT116 cells after siRNA transfection. As expected, the release of cytochrome *c* and the cleavage of caspase-3 mediated by Ad-ST13 were blocked (Fig. 6).

ST13 INTERACTS WITH ASK1 THROUGH THE TPR DOMAIN

To assess whether ST13 and ASK1 directly interact, we performed coimmunoprecipitation assays. Because C-terminus of Hsp70-interacting protein (CHIP) is a co-chaperone that interacts with ASK1 and Hsp70 through an amino-terminal tetratricopeptide repeat (TPR) domain (Hwang et al., 2005), we were particularly interested in whether ST13 can bind ASK1. HA–ASK1 was coexpressed with Flag-tagged ST13 or vector Flag-CMV-1 in HCT116 cells. Whole-cell extracts of the transfected cells were precipitated with anti-HA antibody, and the precipitates were analyzed by Western blotting with anti-ST13 and anti-HA antibodies. As shown in Figure 7A, ST13 efficiently coimmunoprecipated ASK1 indicating an interaction between these proteins. Importantly, ST13 was also associated with ASK1 when the reverse IP with anti-HA antibody and subsequent Western blotting with anti-Flag antibody were performed (Fig. 7B).

DISCUSSION

Our previous studies have shown that the overexpression of ST13 triggers apoptosis in several cell types including colorectal cells SW620 and HCT116 [Yang et al., 2008; Yu et al., 2009]. However, the mechanisms of ST13-induced anti-proliferation and apoptosis are not uncertain.

ASK1, a mammalian MAPKKK, may be a key element in the mechanism of stress and cytokine-induced apoptosis [Takeda et al., 2003; Matsukawa et al., 2004]. Overexpression of ASK1 activates SAPK/JNK and p38 signaling pathways, and ASK1 is activated in cells treated with TNF-α, Fas, and oxidative stress [Ichijo et al., 1997; Chang et al., 1998]. Here, we provide evidence that Ad-ST13 induces phosphorylation of ASK1 and JNK linked to activation of the cell death machinery in HCT116 cells. Knockdown of ASK1 or JNK by siRNA prevents Ad-ST13-induced apoptosis and promotes cell survival. However, p38 MAP kinase is not activated and p38 MAP

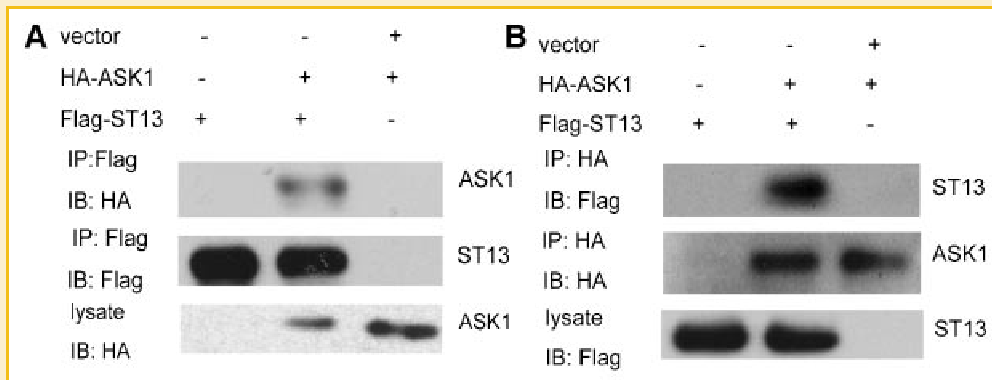

Fig. 7. Interaction of ST13 with ASK1. HCT116 cells were transiently transfected with HA–ASK1 and either vector or Flag-ST13. After 48 h, cell lysates were immunoprecipitated with anti-Flag or anti-HA. Whole cell lysates and the immunoprecipitated proteins were immunoblotted with anti-HA and anti-Flag (A,B).

kinase inhibitor SB203580 has no effect on blocking Ad-ST13-induced cell death. These data suggest that the ASK1 activates JNK but not p38 MAP kinase in the Ad-ST13-induced cell apoptotic pathway. Ad-ST13 also causes ERK1/2 activation, but the activation is mostly due to adenoviruses propagation. Adenovirus-induced pERK enhances viral progeny and induces expression of inflammatory factors [Bruder and Kovesdi, 1997; Schumann and Dobbelstein, 2006].

Mitogenic stimuli produce transient JNK induction, whereas environmental stresses such as UV and heat shock response produce sustained JNK activation and induce apoptosis [Chen et al., 1996; Bennett et al., 2001; Salh, 2007]. In this study, we observed that Ad-ST13 causes sustained JNK activation (data not shown). We also found an increase in pc-Jun levels, a JNK substrate, and involvement of AP-1 in response to overexpression of ST13. Moreover, blocking ASK1–JNK pathway affects cytochrome *c* release and caspase activation. However, how the JNK/AP1 pathway induces caspase-dependent apoptosis needs further exploration. It is possible that ST13/Hip possesses a sequence similar to Bid [Caruso and Reiners, 2006]. Bid is an essential component of many apoptotic pathways. Cytosolic proteases cleave Bid within an extended loop region, generating an activated truncated fragment which synergizes with Bax and Bak to induce release of apoptotic factors, including cytochrome *c* [Tournier et al., 2000; Van Loo et al., 2002].

The TPR domain (residues 114–215) of the ST13 protein is responsible for these protein–protein interaction [Höhfeld et al., 1995; Prapapanich et al., 1996a,b; Shi et al., 2007]. ASK1 is known to contain a TPR-acceptor site. A previous data has shown that CHIP inhibits ASK1-dependent apoptosis by interaction with the TPR repeats [Hwang et al., 2005]. This leads us to propose that ST13 may interact with ASK1 via its TPR domain. ASK1 is well known as a proapoptotic, stress-activated signaling protein under tight regulation at multiple levels. We propose a model that ST13 interacts with ASK1 and induces ASK1-dependent JNK activation and apoptosis. ST13 is a Hsp70-interacting protein. Others have demonstrated that Hsp70 represses the apoptotic pathway by direct interactions with JNK peptide-binding domain and ASK1 peptide-binding domain [Park et al., 2001, 2002]. Thus, further studies should explore whether ST13 is involved in apoptotic pathway by interaction with JNK.

In summary, we reveal a novel mechanism that Ad-ST13 mediates apoptosis by interacting with ASK1 and induces JNK activation. Activation of JNK may cause the release of cytochrome *c* from mitochondria to cytoplasm and activate the caspase cascade. The findings described here would increase our understanding of Ad-ST13 as a potential targeted antitumor.

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