Activation of c-Jun N-terminal kinase is required for mevastatin-induced apoptosis of salivary adenoid cystic carcinoma cells

Shan Zhang[a], Xiao-Ling Wang[b], Ye-Hua Gan[a] and Sheng-Lin Li[a]

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, originally developed for lowering cholesterol. Statins also have pleiotropic effects, independent of cholesterol-lowering effects, including induction of apoptosis in various cell lines. However, the mechanism underlying statin-induced apoptosis is still not fully understood. This study aims to explore the proapoptotic effects and underlying mechanisms of statins on human salivary adenoid cystic carcinoma (SACC). Exposure of SACC cells to mevastatin resulted in cell growth inhibition and apoptosis in a dose-dependent manner, accompanied by the release of cytochrome *c* and cleavage of caspase-3. A remarkable decrease in phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and increase in phosphorylation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated kinase were observed. Furthermore, the JNK-specific inhibitor SP600125, but not the p38-specific inhibitor SB203580, abolished mevastatin-induced cell growth inhibition and apoptosis in SACC cells. This was supported by results in which the JNK inhibitor efficiently blocked mevastatin-induced JNK phosphorylation, but not p38 phosphorylation, and further decreased mevastatin-induced phosphorylation of ERK1/2. Taken together, the results suggest that the JNK pathway was required for mevastatin-induced cell growth inhibition and apoptosis in SACC cells. Statins could be potential anticancer agents for SACC chemotherapy.
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Introduction

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key rate-limiting enzyme in the biosynthesis of cholesterol [1]. In the last 20 years, statins have been used as first-line pharmacologic therapy for hypercholesterolemia. Interestingly, studies also show that statins have pleiotropic effects independent of cholesterol-lowering effects, presenting a potential benefit in different diseases such as atherosclerosis, osteoporosis, and even cancer [2,3].

Statins have anticancer activity in several types of cancer cells including malignant mesothelioma, thyroid cancer, breast cancer, C6 glioma, prostate cancer, pancreatic cancer, and HL60 cells [4–11]. Blockage of protein geranylgeranylation is believed to be the major cause of statin-induced cell apoptosis [8,11,12]. However, the molecular mechanisms of proapoptotic effects of statins are still not fully understood. Several different signal pathways are reported to be involved in the proapoptotic effects of statins. The decrease in Bcl-2 expression, dephosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), and increase of p21, p27, and proapoptotic Bax are involved in statin-induced apoptosis [13,14]. Data show that signaling pathways of mitogen-activated protein kinases (MAPKs) play important roles in the response of tumor cells to statins [7,15]. The involved signaling pathways in statin-induced apoptosis seem to be cell type-specific or related to different biological features of cell types.

Salivary adenoid cystic carcinoma (SACC) is a malignancy that arises in the major and minor salivary glands, and has a tendency toward prolonged clinical course with local recurrence and distant metastases on the lung [16]. SACC is usually treated with surgery and postoperative radiotherapy because the response rates of SACC to chemotherapy or molecular therapies are generally poor [17]. However, chemotherapy continues to play a role in the treatment of metastasized SACC in the palliative setting. Therefore, it is still clinically required to test potential anticancer agents for SACC to augment the current treatment or prevent SACC metastasis. Although statins have been tested in other tumor cell lines, their proapoptotic effects and underlying molecular mechanisms on SACC cells remain unexplored.

In this study, we explored the proapoptotic effects and the underlying molecular mechanisms of mevastatin, an inhibitor of HMG-CoA reductase, on SACC cells.

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Materials and methods

Cell culture and reagents

Both SACC83 and ACC2 cell lines were established from human SACC in our laboratory [18] and in the Shanghai Second Medical University Department of Oral and Maxillofacial Surgery, respectively [19]. The cells were cultured in RPMI-1640 (GIBCO, Grand Island, New York, USA) supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere of 5% CO_2. For cell treatment, the reagents were added singly or combinatorially into the culture media; the same volume of solvent was added into the culture media as the corresponding controls.

Mevastatin, SP600125, and SB203580 were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Mevastatin was chemically activated by alkaline hydrolysis before use as described earlier [20].

Assessment of cell viability and apoptosis

Viable cells were determined by trypan blue exclusion assay. After treatment, the cells were harvested, resuspended in D-Hanks' solution, and mixed with an equal amount of 0.4% trypan blue. The viable cells (from trypan blue exclusion) were counted using a Fuchs-Rosenthal hemocytometer (Shanghai Precision Instruments Co., Ltd., Shanghai, China) under a microscope and calculated as mean ± SD of at least three independent experiments, and presented as a percentage of controls.

Apoptotic cells were determined by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. The cells were fixed with 10% formaldehyde in phosphate-buffered saline (PBS) for 5 min and incubated with 5 μg/ml DAPI in the dark for 3 min at room temperature. After washing with PBS, the cells were examined under a fluorescence microscope (Nikon Eclipse TE2000-U, Tokyo, Japan). The cells presenting features of nuclear condensation and fragmentation were identified as apoptotic cells and counted within the randomly selected fields. The rate of apoptotic cells was presented as mean ± SD of at least three independent experiments.

Western blot

The primary antibodies used in this assay were anti-cytochrome *c*, anti-ERK, anti-phospho-ERK (Thr202/Tyr204), anti-c-Jun N-terminal kinase (JNK), anti-phospho-JNK (Thr183/Tyr185), anti-p38, anti-phospho-p38 (Thr180/Tyr182) (Cell Signaling Technology, Beverly, Massachusetts, USA), and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, California, USA). After treatment for 28 h, the cells were washed with cold PBS twice and lysed with lysis buffer containing 1% Nonidet P-40, 5% sodium deoxycholate, 1 mmol/l phenylmethylsulfonyl fluoride, 100 mmol/l sodium orthovanadate, and 1 : 100 mixture of protease inhibitors (Sigma-Aldrich). Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, Illinois, USA). Equal amounts of samples were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, Massachusetts, USA). The membrane was blocked with 5% fat-free milk in TBS-T (50 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 0.05% Tween 20) for 1 h. After incubation with primary antibodies diluted 1 : 1000 in TBS-T containing 1% milk overnight at 4°C, the membrane was washed extensively with TBS-T and then incubated with a secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. After extensive washes with TBS-T, the membrane was visualized with enhanced chemiluminescence reagents kit (Applygen Technologies, Inc., Beijing, China). For internal controls of equal loading, the blots were also stripped with stripping buffer (100 mmol/l 2-mercaptoethanol, 2% SDS, 62.5 mmol/l, Tris pH 6.8) and reprobed with anti-ERK1/2, anti-JNK, anti-p38, or β-actin antibodies.

Immunocytochemistry

SACC83 cells cultured on glass coverslips were treated with or without 40 μmol/l mevastatin for 24 h. After fixation with 75% methanol and 25% acetic acid, the cells were permeabilized with 0.1% Triton X-100 and blocked with 10% goat serum at room temperature for 30 min, followed by incubation with anticytochrome *c* antibody in PBS overnight at 4°C. After washing three times with PBS, the cells were incubated with the secondary antibody conjugated with fluorescein isothiocyanate for 30 min at room temperature in the dark. After extensive washes with PBS, the cells were stained with 5 μg/ml DAPI for 3 min at room temperature in the dark, and then imaged under ultraviolet or excitation at 488 nm using confocal laser scanning microscopy (Zeiss LSM 510, Carl Zeiss, Jena, Germany).

Statistical analysis

Statistical analysis was performed with SPSS 13.0 for Windows (SPSS, Inc., Chicago, Illinois, USA). All data were presented as mean ± SD. Differences were analyzed by one-way analysis of variance, and a P value of less than 0.05 was regarded as statistically significant.

Results

Induction of cell growth inhibition and apoptosis by mevastatin in SACC cells

To examine the effects of statins on SACC, SACC83 cells were treated with mevastatin at concentrations of 0, 10, 20, and 40 μmol/l for 24 h. Mevastatin treatment significantly induced SACC83 cell growth inhibition and apoptosis. The apoptotic cells showed morphological changes of shrinkage (arrows) under an inverted microscope (Fig. 1a), and nuclear fragmentation and condensation with DAPI staining under a fluorescence microscope (Fig. 1d). The viable cells decreased, whereas the

apoptotic cells increased in a dose-dependent manner (Fig. 1c and e). Cell differentiation as indicated by neurite extension was also observed (Fig. 1a).

To further confirm the effect of mevastatin on SACC cells, ACC2 cells were treated with mevastatin at different concentrations. Mevastatin showed similar cytotoxicity to ACC2 as in SACC83 cells, but in a higher concentration (Fig. 1b).

Release of cytochrome *c* and cleavage of caspase-3 by treatment with mevastatin

To confirm whether mevastatin-induced apoptosis in SACC cells was through the mitochondrial pathway,

Fig. 1

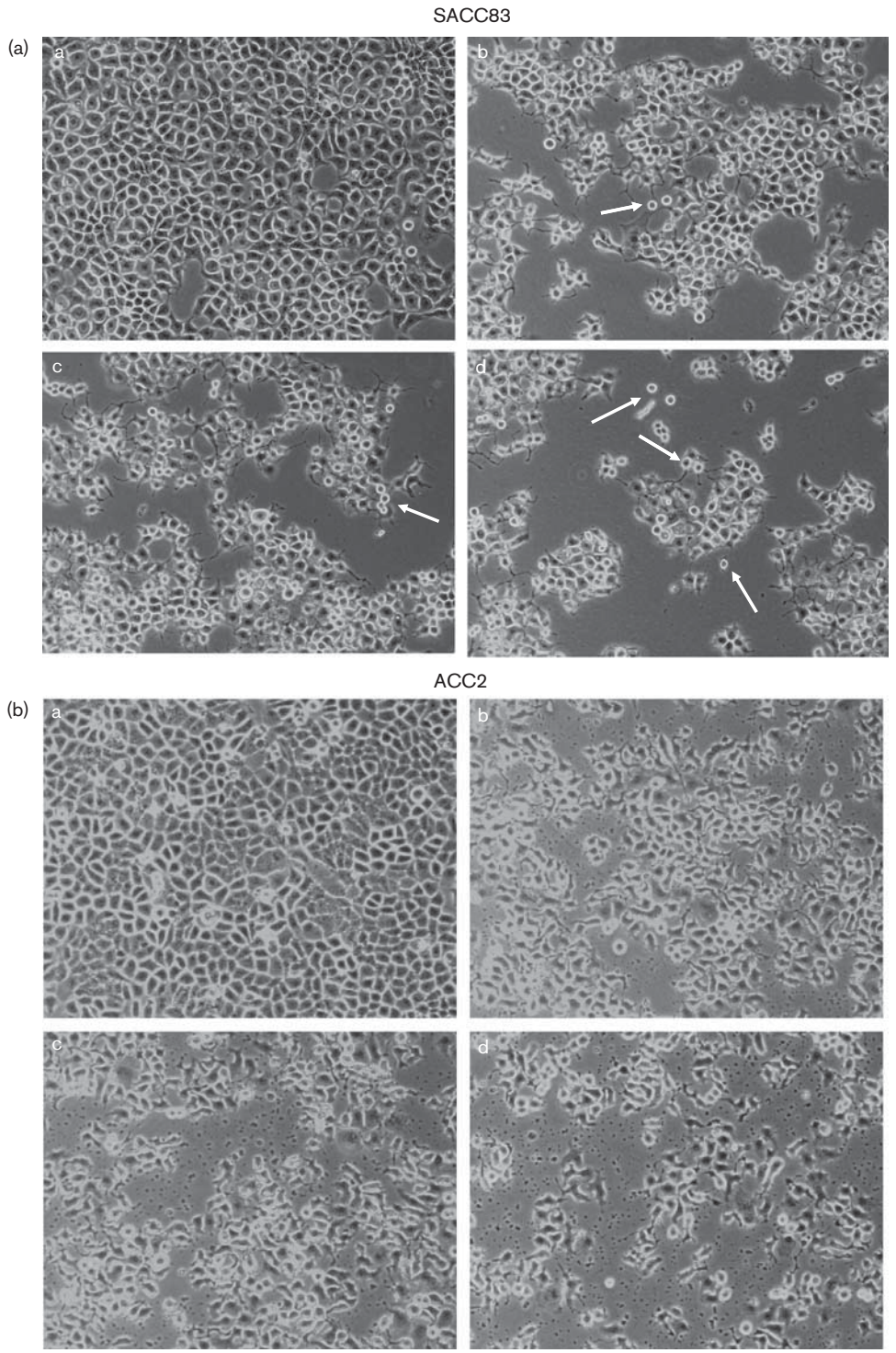
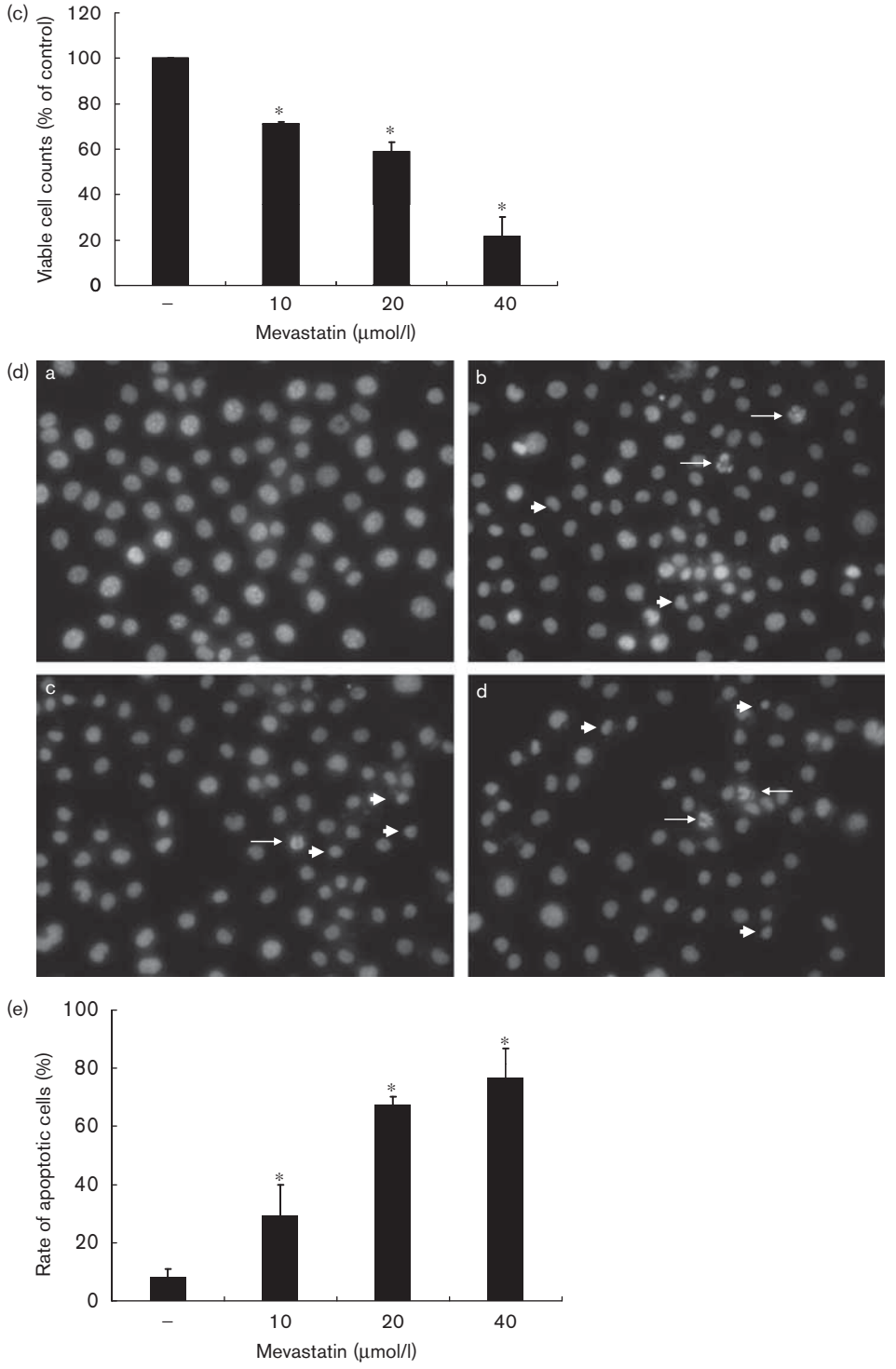
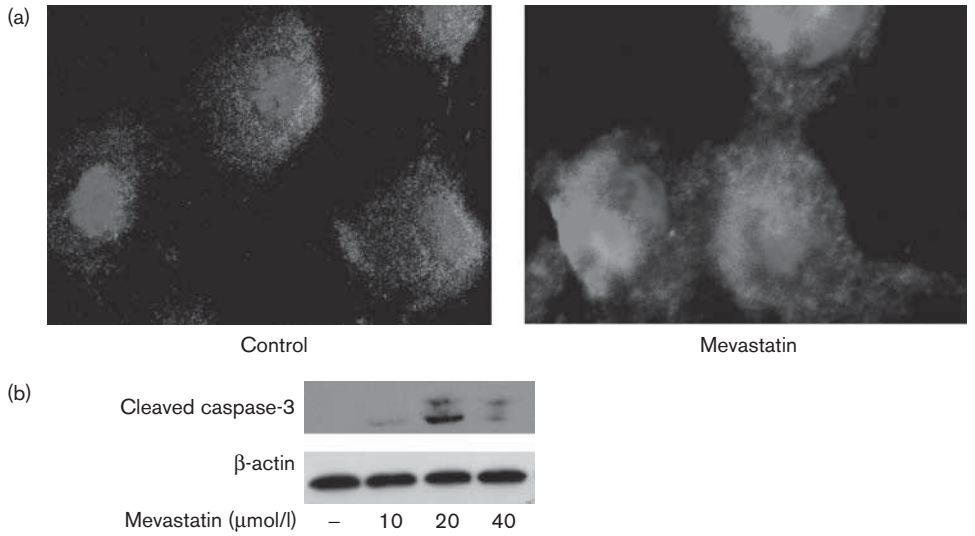


Fig. 1 ***(continued)***



Induction of cell growth inhibition and apoptosis by mevastatin treatment in SACC cells. (a) Inverted photomicrographs of SACC83 cells treated with mevastatin (a–d: 0, 10, 20, and 40 μmol/l) for 24 h. Apoptotic cells were shrunk and floating (arrows). Neurite extension was also observed. (b) Inverted photomicrographs of ACC2 cells treated with mevastatin (a–d: 0, 40, 80, and 160 μmol/l) for 24 h. (c) Cell viability in SACC83. Viable cells were identified with trypan blue exclusion assay after treatment with mevastatin for 24 h and presented as a percentage of controls. (d) Fluorescence photomicrographs of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining of SACC83 cells treated with mevastatin (a–d: 0, 10, 20, and 40 μmol/l) for 28 h. Apoptotic cells showed nuclear fragmentation (arrows) and condensation (arrowheads). (e) Apoptotic rate of SACC83. Apoptotic cells were identified with DAPI staining under a fluorescence microscope after treatment with mevastatin for 28 h. Data are mean ± SD of at least three independent experiments. *$P < 0.01$ versus controls.

Fig. 2



Induction of mitochondrial-dependent apoptosis by mevastatin treatment. (a) Release of cytochrome *c* from the mitochondria after treatment with mevastatin. Immunofluorescence staining of cytochrome *c* was punctuated (located in the mitochondria) in the cytoplasm of cells without treatment, and diffused (released from the mitochondria) in the cytoplasm of cells treated with 40 μmol/l mevastatin for 24 h. (b) Activation of caspase-3 by treatment with mevastatin. Immunoblotting showed that caspase-3 was cleaved in a dose-dependent manner in SACC83 cells after treatment with mevastatin for 28 h.

we examined the release of cytochrome *c* from the mitochondria and the cleavage of caspase-3 in SACC83 cells after treatment with mevastatin. Figure 2a shows that immunofluorescence staining of cytochrome *c* was punctuated (located in the mitochondria) in the cytoplasm before treatment with mevastatin, and diffused (released from the mitochondria) in the cytoplasm after treatment with 40 μmol/l mevastatin for 24 h. Immunoblotting showed that cleaved caspase-3 was induced by treatment with mevastatin for 28 h in a dose-dependent manner (Fig. 2b).

Activation of JNK and p38 and inhibition of ERK1/2 by treatment with mevastatin

To explore which MAPKs were involved in mevastatin-induced apoptosis of SACC cells, we examined the phosphorylation of JNK, p38, and ERK1/2 in SACC83 cells treated with mevastatin at concentrations of 0, 10, 20, and 40 μmol/l for 28 h. Figure 3 shows that phosphorylation of JNK and p38 increased, whereas phosphorylation of ERK1/2 decreased by treatment with mevastatin in a dose-dependent manner.

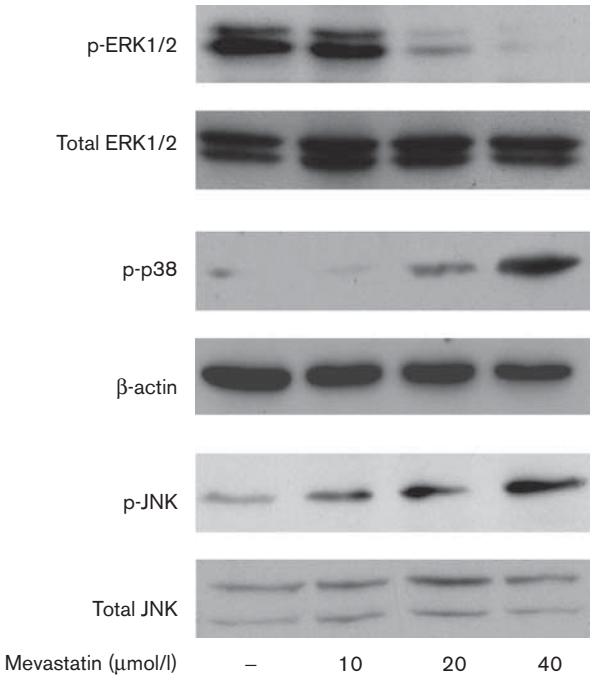
Inhibition of JNK, but not p38, blocked mevastatin-induced cell growth inhibition and apoptosis

To explore whether the activation of JNK or p38 was required for mevastatin-induced inhibition of cell growth and apoptosis, we treated SACC83 cells with 40 μmol/l mevastatin in the absence or presence of the JNK-specific inhibitor SP600125 (10 μmol/l) or the p38-specific inhibitor SB203580 (10 μmol/l) for 28 h. Figure 4a and c show that the JNK inhibitor abolished mevastatin-induced cell growth inhibition and apoptosis. The viable cell count significantly decreased in the group treated with mevastatin compared with that in the other three groups treated with the vehicle, mevastatin and JNK inhibitor combinatorially, and the JNK inhibitor, respectively ($P < 0.01$). No difference in the viable cell count among the other three groups was observed ($P > 0.05$). The shape of the cells combinatorially treated with mevastatin and the JNK inhibitor became round, but without neurite extension compared with that of the mevastatin-treated group, indicating that mevastatin-induced cell differentiation was also blocked by the JNK inhibitor. However, p38 inhibitor SB203580 neither blocked mevastatin-induced cell growth inhibition and apoptosis nor enhanced proliferation in SACC83 cells (data not shown).

To confirm the inhibition of JNK by SP600125 and to examine whether ERK1/2 and p38 were influenced by SP600125, we examined the phosphorylation of JNK, ERK1/2, and p38 in SACC83 cells treated with the same combinations as described above for 28 h. Figure 4e shows that mevastatin-induced JNK phosphorylation, but not p38 phosphorylation, was almost blocked by SP600125; mevastatin-induced downregulation of ERK1/2 phosphorylation was further decreased by SP600125. Interestingly, SP600125 alone also induced p38 phosphorylation and downregulated ERK1/2 phosphorylation.

To further confirm that mevastatin-induced cell death of SACC cells was JNK dependent, ACC2 cells were treated with the same combinations as described above.

Fig. 3



Activation of c-Jun N-terminal kinase (JNK) and p38 and inactivation of ERK1/2 by mevastatin treatment. Representative immunoblottings are shown for phosphorylated ERK1/2 (p-ERK1/2), phosphorylated p38 (p-p38), and phosphorylated JNK (p-ERK) in SACC83 cells after treatment with mevastatin for 28 h. Phosphorylation of JNK and p38 increased, whereas phosphorylation of ERK1/2 decreased in a dose-dependent manner. Total ERK1/2, JNK, and β-actin served as internal controls of equal loading.

Consistently, the JNK inhibitor abolished mevastatin-induced apoptosis in ACC2 cells (Fig. 4b and d) and blocked mevastatin-induced JNK phosphorylation (Fig. 4f).

Discussion

In this study, we showed that mevastatin induced cell growth inhibition and apoptosis in SACC cells. Although SACC does not clinically respond well to current anticancer agents, our results suggest that the proapoptotic effects of statins on SACC cells may be potentially exploited for the chemoprevention of SACC metastasis or chemotherapy of metastasized SACC, considering the safety and relatively low cost of statins. Therefore, statins alone or in combination with other conventional agents may represent a novel therapy for advanced or metastasized SACC. They may also be applied to augment postoperative radiotherapy for SACC.

Mevastatin induced apoptosis of SACC cells through the mitochondrial (intrinsic) apoptotic pathway. Given that cytochrome *c* was released from the mitochondria and caspase-3 was activated in the SACC83 cells by treatment with mevastatin, the mitochondrial apoptotic pathway was activated in the cells. The results also confirmed that SACC cells treated with mevastatin, presenting morphological shrinkage and nuclear condensation and fragmentation, were undergoing apoptosis.

ERK1/2, p38, and JNK were all probably involved in the proapoptotic effects of mevastatin on SACC cells. ERK1/2, p38, and JNK are the three major MAPKs and seem to be differentially involved in the proapoptotic effects of statins in different cell types [7,15,21,22]. ERK1/2 is expressed widely and is involved in the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells [23]. Inactivation of ERK1/2 by dephosphorylation leads to a decrease in the proliferation rate of tumor cells [23]. For this reason, inhibitors of the ERK pathways are perceived as potential anticancer agents and have already been used in clinical trials [24]. ERK1/2 phosphorylation decreased in a dose-dependent manner in the SACC83 cells treated with mevastatin; thus mevastatin-induced inhibition of cell growth in SACC cells could occur, at least in part, through the ERK1/2 pathway. The decrease in ERK1/2 phosphorylation in SACC cells is consistent with the results of earlier studies in HL60 cells treated with mevastatin, in smooth muscle cells with cerivastatin, and in squamous cell carcinoma cells with simvastatin [11,15,25]. In contrast, some studies also show that lovastatin increases ERK1/2 phosphorylation in glioblastoma cells [21] and cerivastatin even activates ERK1/2 at an early stage in smooth muscle cells [26]. These discrepancies may be because of

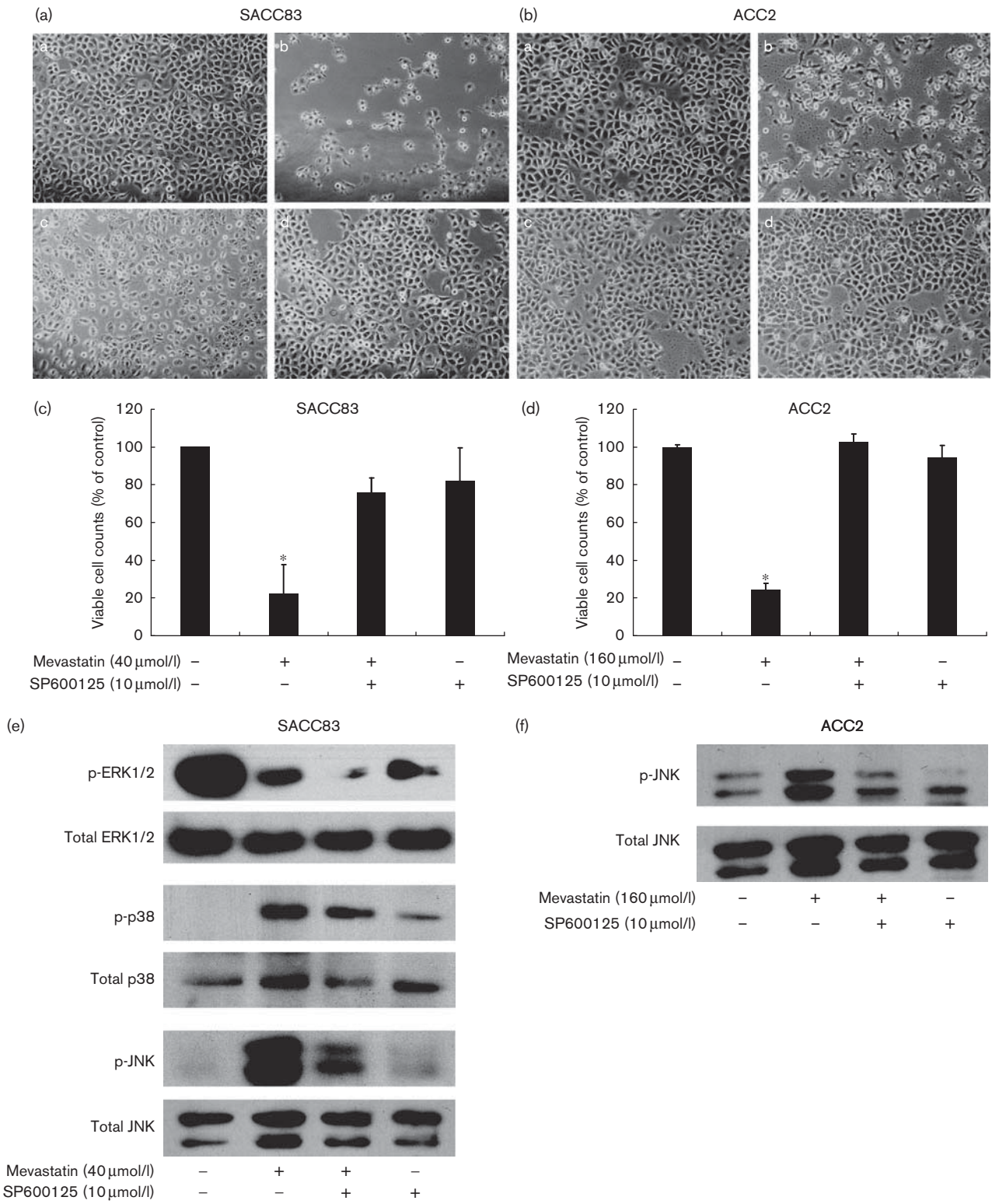
Fig. 4

Blockage of mevastatin-induced cell growth inhibition and apoptosis by c-Jun N-terminal kinase (JNK)-specific inhibitor in SACC cells. (a) Inverted photomicrographs of SACC83 cells exposed to vehicle [a], 40 μmol/l mevastatin [b], 40 μmol/l mevastatin and 10 μmol/l JNK inhibitor SP600125 [c], and 10 μmol/l SP600125 [d] for 28 h. The JNK inhibitor abolished mevastatin-induced cell growth inhibition and apoptosis. The shape of the cells combinatorially treated with mevastatin and the JNK inhibitor became round, but without neurite extension. [b] Inverted photomicrographs of ACC2 cells exposed to vehicle [a], 160 μmol/l mevastatin [b], 160 μmol/l mevastatin and 10 μmol/l JNK inhibitor SP600125 [c], and 10 μmol/l SP600125 [d] for 28 h. (c) and (d) Cell viability of SACC83 and ACC2 cells after treatment with mevastatin in the absence or presence of the JNK inhibitor. The viable cell count significantly decreased in the group treated with mevastatin, whereas no difference in the viable cell count among the other three groups was observed ($P > 0.05$). Viable cells were identified with trypan blue exclusion assay after treatment with reagents as indicated for 28 h and presented as a percentage of controls. Data are mean ± SD of at least three independent experiments. $^*P < 0.01$ versus other groups. (e) Immunoblotting of ERK1/2, JNK, and p38 in SACC83 cells treated as indicated for 28 h. Mevastatin-induced JNK phosphorylation, but not p38 phosphorylation, was blocked by SP600125. Mevastatin-downregulated ERK1/2 phosphorylation was further decreased by SP600125. However, SP600125 also induced p38 phosphorylation and downregulated ERK1/2 phosphorylation. (f) Immunoblotting of JNK in ACC2 cells treated as indicated for 28 h. Mevastatin-induced JNK phosphorylation was also blocked by SP600125.

the different cell types responding differently to statins. Nevertheless, the sustained decrease in ERK1/2 phosphorylation in SACC cells by mevastatin treatment would contribute to the inhibition of cell growth.

Inactivation of ERK1/2 may also help decrease lung metastasis of SACC. ERK1/2 plays a key role in the regulation of tumor metastatic processes [27,28]. Considering that ERK1/2 is more activated in the high lung-metastatic

Fig. 4



SACC cells than in the low lung-metastatic SACC cells [29], the result of inactivation of ERK1/2 in SACC cells by mevastatin treatment would imply that statins may even exhibit chemoprevention of SACC metastasis. Further experiments are required to examine the assumption of chemoprevention of SACC metastasis by statins *in vivo*, given that SACC has a special biological feature of high rate of distant metastasis on the lung.

Activation of JNK was required for mevastatin to induce apoptosis in SACC cells. Although JNK plays a critical role in controlling apoptosis, JNK is also involved in survival signaling [30,31]. JNK can be activated by cytokines and many environmental stresses. On activation, the phosphorylated JNK translocates to the nucleus where it phosphorylates and transactivates c-Jun, a component of AP-1 transcriptional complex. Whether the activation of JNK leads to apoptosis or cell survival is dependent on stimuli and cell types [30,31]. Given that only the JNK inhibitor SP600125, but not the p38 inhibitor SB203580, could abolish mevastatin-induced cell growth inhibition and apoptosis in both the SACC cell lines (Fig. 4a–d), the activation of JNK was required for mevastatin to induce apoptosis in SACC cells. Moreover, JNK inhibitor SP600125 efficiently blocked mevastatin-induced JNK phosphorylation, but not p38 phosphorylation. Although the JNK inhibitor further decreased mevastatin-induced downregulation of ERK1/2 phosphorylation to some extent, the JNK inhibitor still completely blocked mevastatin-induced cell growth inhibition and apoptosis, further supporting the hypothesis that mevastatin cytotoxicity to SACC cells was dependent on the JNK pathway. Our results regarding JNK requirement in mevastatin-induced apoptosis in SACC cells are consistent with earlier studies of simvastatin in C6 glioma and breast cancer cells, and lovastatin in macrophages and neural blasts [7,22,32,33]. JNK plays a critical role in the apoptosis of tumor cells; thus, agents that can activate the JNK pathway are receiving growing attention in the development of anticancer drugs [30,31]. Our results may also imply that the JNK pathway could be a possible target for future development of anticancer drugs for SACC.

Activation of p38 was not required for mevastatin-induced inhibition of cell growth and apoptosis in SACC cells. As one of the three major MAPKs, the p38 pathway is also activated in response to a wide range of cellular stresses, including inflammatory cytokines, hormones, ligands for G protein-coupled receptors, and stresses, such as osmotic and heat shock. Activation of the p38 pathway by ultraviolet, irradiation, and chemotherapeutic agents leads to apoptosis [34,35]. However, p38 can also mediate cell survival in specific situations, such as in response to agents of DNA damage [36]. Although p38 was dose-dependently activated in the SACC cells by treatment with mevastatin, the p38 inhibitor SB203580 failed to block mevastatin-induced cell growth inhibition and apoptosis in the cells, suggesting that the activation of the p38 pathway, unlike the activation of the JNK pathway, was not required for the proapototic effects of mevastatin in SACC cells. Our result regarding the activation of p38 by mevastatin in SACC is similar to that of the study on lovastatin in glioblastoma [21], but in contrast to that of the study on lovastatin in brain neuroblasts in which p38 was not affected [22]. Therefore, of the three major MAPKs, only the JNK pathway seemed to be required for mevastatin-induced apoptosis in SACC, whereas the activation of p38 and inactivation of ERK1/2 by mevastatin would simultaneously enhance this process.

Statins as potential anticancer agents may be a little more suitable for chemotherapy of SACC. Earlier studies show that statins either activate JNK but without the activation of p38 or without the inactivation of ERK1/2 [7,22], or inactivate ERK1/2 but without the activation of JNK or p38 [11,15,37]. Our results show that mevastatin simultaneously activated JNK and p38, and inactivated ERK1/2 in SACC cells. To the best of our knowledge, this is the first report to show that the three major MAPKs were all involved in statin-induced cell growth inhibition and apoptosis in a single cell type. There is crosstalk among the three major MAPKs. Activation of JNK or p38 can negatively regulate ERK1/2 [38]. However, mevastatin-induced inactivation of ERK1/2 in SACC did not seem to be dependent on the activation of JNK, as the JNK inhibitor further decreased mevastatin-induced downregulation of ERK1/2 phosphorylation (Fig. 4e). Interestingly, JNK inhibitor SP600125 alone simultaneously activated p38 and inactivated ERK1/2 in SACC, but without influence on the proliferation of SACC cells (Fig. 4c and e). However, earlier studies show similar results in which the JNK inhibitor SP600125 induces p38 in MIN6 cells (a mouse β-line) and decreases ERK1/2 phosphorylation in the experimental model of cerulein-induced pancreatitis [39,40]. Nevertheless, these observations further reflect a complicated regulation between JNK and p38 MAPK or ERK1/2. In the future, testing whether statins could play a role in anticarcinogenesis or chemoprevention of SACC metastasis *in vivo* will be clinically interesting.

In conclusion, this study showed that of the three major MAPKs involved, only JNK was required for mevastatin-induced cell growth inhibition and apoptosis in SACC cells. Statins could be potential anticancer agents for chemotherapy of SACC.

Acknowledgements

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