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Epigallocatechin-3-Gallate Induces Cell Apoptosis of Human Chondrosarcoma Cells Through Apoptosis Signal-Regulating Kinase 1 Pathway

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ABSTRACT

Chondrosarcoma is a malignant primary bone tumor that responds poorly to both chemotherapy and radiation therapy. (-)-Epigallocatechin-3-gallate (EGCG), the major polyphenol in green tea, has been shown to inhibit tumorigenesis and cancer cell growth in animal models. The aim of this study was to elucidate the mechanism of EGCG-induced apoptosis of human chondrosarcoma cells. EGCG induced cell apoptosis in human chondrosarcoma cell lines but not primary chondrocytes. EGCG induced upregulation of Bax and Bak, downregulation of Bcl-2 and Bcl-XL, and dysfunction of mitochondria in chondrosarcoma. We also found that the accumulation of reactive oxygen species (ROS) is a critical mediator in EGCG-induced cell death. EGCG induced apoptosis signal-regulating kinase 1 (ASK1) dephosphorylation and its dissociation from 14-3-3. Treatment of chondrosarcoma cells with EGCG induced p38 and c-jun-NH2-kinase (JNK) phosphorylation. Transfection with ASK1 siRNA or p38 and JNK mutant antagonized the EGCG-induced cell apoptosis. Therefore, EGCG triggered ROS and activated the ASK1-p38/JNK pathway, resulting chondrosarcoma cell death. Importantly, animal studies revealed a dramatic reduction in tumor volume after 24 days of treatment. Thus, EGCG may be a novel anti-cancer agent for the treatment of chondrosarcoma. J. Cell. Biochem. 112: 1601–1611, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: CHONDROSARCOMA; ASK1; EGCG; CHINESE HERB; ROS

Chondrosarcoma is the third most common primary bone malignancy after myeloma and osteosarcoma, accounting for approximately 20% of bone sarcomas and mainly affecting the middle-aged population [Ozaki et al., 1997; Gelderblom et al., 2008]. Chondrosarcoma is a malignant primary bone tumor with a poor response to currently used chemotherapy or radiation treatment, making the management of chondrosarcomas a complicated challenge [Terek et al., 1998]. Due to the absence of an effective adjuvant therapy, this mesenchymal malignancy has a poor prognosis and, therefore, it is important to explore a novel and adequate remedy [Yuan et al., 2005].

It is widely reported that catechin derivatives contained in green tea have anti-neoplastic activity [Khan et al., 2006]. One of these compounds, (-)-epigallocatechin gallate (EGCG), is a major component of green tea and has long been known to inhibit carcinogenesis of diverse tumor types [Adhami et al., 2007; Shankar et al., 2008]. Recent studies have shown potential chemotherapeutic efficacy of EGCG against cancers of the skin, lung, breast, colon, liver, stomach, and prostate [Lu et al., 2002; Witschi et al., 2004]. As with many other natural dietary substances that have been studied in recent years as potential anti-cancer agents, EGCG is attractive as a potential therapeutic due to its lack of significant toxicity in normal cells.

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Reactive oxygen species (ROS) are derived from the metabolism of molecular oxygen. Aerobic respiration coupled to the generation of ATP leads to the formation of the superoxide anion radical (O_2^-). Superoxide anion radicals can then form other ROS, such as hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radicals [Feig et al., 1994; Schumacker, 2006]. Oxidative stress occurs when this critical balance is disrupted because of excess ROS, antioxidant depletion, or both. Accumulating evidence indicates that chemotherapeutic agents may be selectively toxic to tumor cells because they increase oxidant stress and enhance these already stressed cells beyond their limit [Ham et al., 2006; Moungjaroen et al., 2006]. It has been reported that ROS trigger activation of the apoptosis signal-regulating kinase 1 (ASK1)/mitogen activated protein kinase (MAPK) signaling pathway [Ling et al., 2003; Kim et al., 2005; Imoto et al., 2006]. The upstream activator of MAPK signaling is ASK1, a member of the MAPK kinase kinase (MAPKKK) family [Hsieh and Papaconstantinou, 2006; Imoto et al., 2006]. ASK1 is activated in response to various stresses, including tumor necrosis factor, serum withdrawal, endoplasmic reticulum stress, Fas ligation, and H_2O_2 [Hwang et al., 2005; Ouyang and Shen, 2006]. ASK1 activity is regulated at multiple steps, including dimerization, phosphorylation, and protein–protein interactions [Matsuzawa et al., 2002; Hwang et al., 2005]. Phosphorylation of the ASK1 Ser967 residue is required for the formation of the ASK-14-3-3 complex to keep ASK1 inactive [Liu et al., 2006; Chen et al., 2008]. ROS have been reported to trigger the dissociation of 14-3-3 from ASK1 [Liu et al., 2006]. ASK1 can induce cell death by activating several proapoptotic signaling proteins, including c-jun-NH2-kinase (JNK) and p38 MAPK [Saeki et al., 2002; Su et al., 2005].

EGCG has been reported to inhibit a variety of cancer-related pathways including cell proliferation and tumor growth [Adhami et al., 2007; Siddiqui et al., 2008]. Although the effects of EGCG-induced tumor apoptosis have been studied in some cancers, the role of EGCG in the process of cell apoptosis in chondrosarcoma remains largely unknown. To the best of our knowledge, this study is the first to attempt to determine the apoptosis activity of EGCG in human chondrosarcoma cell lines. Our data provide evidence that EGCG reduced cells survival and tumor growth in human chondrosarcoma cells in vitro and in vivo.

MATERIALS AND METHODS

MATERIALS

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for Bax, Bcl-XL, Bcl-2, Bak, p38, p-p38, JNK, p-JNK, ASK1, p-ASK1, 14-3-3, caspase-3, and caspase-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The p38 dominant negative mutant was provided by Dr. J. Han (South-western Medical Center, Dallas, TX). The JNK dominant negative mutant was provided by Dr. M Karin (University of California, San Diego, CA). EGCG and all other chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

CELL CULTURE

The human chondrosarcoma cell line (JJ012) was kindly provided from the laboratory of Dr. Sean P Scully (University of Miami School of Medicine, Miami, FL). The human chondrosarcoma cell line (SW1353) was obtained from the American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium/α-minimum essential medium supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified atmosphere of 5% CO_2.

Primary cultures of human chondrocytes were isolated from articular cartilage as previously described [Chiu et al., 2007]. The cells were grown in plastic cell culture dishes in 95% air–5% CO_2 with DMEM which was supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), 10% FBS, 2 mM-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

SULFORHODAMINE B ASSAY

Cells viability by EGCG was measured using the sulforhodamine B (SRB) assay. EGCG was added at a range of concentration for 48 h. Cells were fixed with 50% trichloroacetic acid to terminate reaction and 0.4% SRB in 1% acetic acid was added to each well. After 15-min incubation, the plates were washed, and dyes were dissolved by 10 mM Tris buffer. Then, the 96-well plate was read by enzyme-linked immunosorbent assay reader (515 nm) to get the absorbance density values.

COLONY ASSAY

To determine long-term effects of EGCG, cells (1,000/well) were treated with FTBP at various concentrations for 3 h. After being rinsed with fresh medium, cells were allowed to form colonies for 7 days and then were stained with crystal violet (0.4 g/L). After washing with ddH_2O three times, acetic acid was added to final concentration of 33% (v/v), followed by measuring the absorbance at 550 nm.

QUANTIFICATION OF APOPTOSIS BY FLOW CYTOMETRY

Cells were collected by centrifugation and adjusted to 3×10^6 cells/ml. Pre-chilled methanol was added to 0.5 ml of the cells and incubated at 4°C for 30 min. Methanol was then removed by centrifugation and DNA of the cells was stained with propidium iodide (PI) [100 μg/ml PI, 0.1% Triton-X, and 1 mM EDTA in PBS] in the presence of an equal volume of DNase-free RNase (200 μg/ml) and analyzed immediately by a FACS Calibur flow cytometer (Becton Dickinson and Co., Franklin Lakes, NY). The extent of apoptosis was determined by measuring DNA content of the cells below the G_0/G_1 peak.

DAPI STAINING

4′-6-diamidino-2-phenylindole (DAPI), a DNA-binding fluorescent dye, was used to determine whether the mechanism of growth inhibition after EGCG treatment is through apoptosis. After treatment with EGCG for 48 h, the cells were washed three times with PBS, fixed in a 3.7% formaldehyde solution for 10 min, fixed once in 1 ml of methanol, and then stained with DAPI for 10 min. Results were determined by visual observation of nuclear morphology through fluorescence microscopy.

DETERMINATION OF THE MITOCHONDRIAL MEMBRANE POTENTIAL

Cells were plated at a density of 1×10^4 cells on cover slips. Mitochondrial activity was assessed using the fluorescent probe, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Invitrogen). JC-1 accumulates in mitochondria where its aggregation depends on mitochondrial transmembrane potential ($\Delta\Psi$m). At low $\Delta\Psi$m, JC-1 exists in monomeric form, it is excited at 490 nm and emits at 527 nm. At high $\Delta\Psi$m, JC-1 forms aggregates, resulting in a shift in emission to 585 nm. Cells were incubated with EGCG and then incubated with JC-1 (10 μg/ml) in culture medium for 30 min at 37°C. Cells were then trypsinized, washed twice with PBS, and analyzed by flow cytometry with an argon ion laser at 488 nm.

MEASUREMENTS OF ROS

Levels of intracellular O^{2-} and H_2O_2 were assessed spectrofluorimetrically by oxidation of specific probes: dihydroethidium (DHE) and H_2DCFDA (Molecular Probes). Cells were plated at a density of 5×10^5, allowed to attach overnight, and exposed to EGCG for specified time intervals. The cells were stained with H_2DCFDA (10 μM) and DHE (10 μM) for 10 min at 37°C and the fluorescence intensity in cells was determined using the flow cytometry.

WESTERN BLOT ANALYSIS

The cellular lysates were prepared as described previously [Tang et al., 2009]. Proteins were resolved on SDS–PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against Bax, Bak, Bcl-xL, and Bcl-2 (1:1,000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase conjugated secondary antibody (1:1,000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

CASPASE ACTIVITY

The assay is based on the ability of the active enzyme to cleave the chromophore from the enzymesubstrate LEHD-pNA (for caspase-9) and Ac-DEVD-pNA (for caspase-3). The cell lysates were prepared and incubated with specific anti-caspase-9 and caspase-3 antibodies. Immunocomplexes were incubated with peptide substrate in assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), pH 7.4) for 2 h at 37°C. The release of *p*-nitroaniline was monitored at 405 nm. Results are represented as the percent change of the activity compared to the untreated control.

siRNA TRANSFECTION

The siRNAs against human ASK1 and control siRNA (for experiments using targeted siRNA transfection; each consists of a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA) were purchased commercially from Santa Cruz Biotechnology. Cells were transfected with siRNAs (at a final concentration of 100 nM) using Lipofectamine 2000 (Invitrogen Life Technology) according to the manufacturer's instructions.

IN VIVO TUMOR XENOGRAFT STUDY

Male SCID mice [6-week-old; BALB/cA-nu (nu/nu)] were purchased from the National Science Council Animal Center (Taipei, Taiwan) and maintained in pathogen-free conditions. JJ012 cells were injected subcutaneously into the flanks of these SCID mice (1×10^6 cells in 200 μl), and tumors were allowed to develop for ~14 days until they reached a size of approximately 100 mm^3, when treatment was initiated. The mice were treated with vehicle, 25 or 50 mg/kg (i.p.; total volume 200 μl) EGCG every day for 24 days (10 mice/group). The volume of the implanted tumor in dorsal side of mice was measured twice a week with a caliper, using the formula $V = (LW^2)/2$: where V, is the volume (mm^3); L, the biggest diameter (mm); W, is the smallest diameter (mm). All protocols complied with institutional guidelines and were approved by Animal Care Committee of China Medical Taiwan University.

STATISTICS

The values given are means ± SEM. Statistical analysis between two samples was performed using Student's *t*-test. Statistical comparisons of more than two groups were performed using one-way analysis of variance (ANOVA) with Bonferroni's post hoc test. In all cases, $P < 0.05$ was considered as significant.

RESULTS

EGCG INDUCED CELL APOPTOSIS IN HUMAN CHONDROSARCOMA CELLS

To investigate the potential cell death of EGCG in human chondrosarocoma cells, we first examined the effect of EGCG on cell survival in human chondrosarcoma cells (JJ012). Treatment of JJ012 cells with EGCG-induced cell death in a concentration-dependent manner by using SRB assay (Fig. 1A). Next we investigated the anti-cancer effect of EGCG in other chondrosarcoma cell lines. Figure 1A also shows that EGCG-induced cell death in other chondrosarcoma cell line (SW1353). However, EGCG did not affect the cell viability of normal chondrocytes (Fig. 1A). Colongenic assay correlate very well with in vivo assays of tumorigenicity in nude mice [Freedman and Shin, 1974]. JJ012 cells showed the ability to form clones in the un-treat control wells (Fig. 1B). However, upon addition of EGCG, a dose-dependent inhibition in clonogenicity was observed, with a >70% inhibition at dosages as low as 400 μM EGCG (Fig. 1B). The quantitative data are shown in Figure 1B; lower panel. We next investigated whether EGCG induces cell death through an apoptotic mechanism by DAPI staining and PI staining. Treatment of JJ012 cells with EGCG significantly increased the condensation of chromatin by DAPI staining using immunofluorescence microscopy (Fig. 2A). In addition, the results showed that treating cells with EGCG caused a concentration-dependent increase of cell apoptosis, resulting in an increase in the percentage of cells in the Sub G1 phase (Fig. 2B).

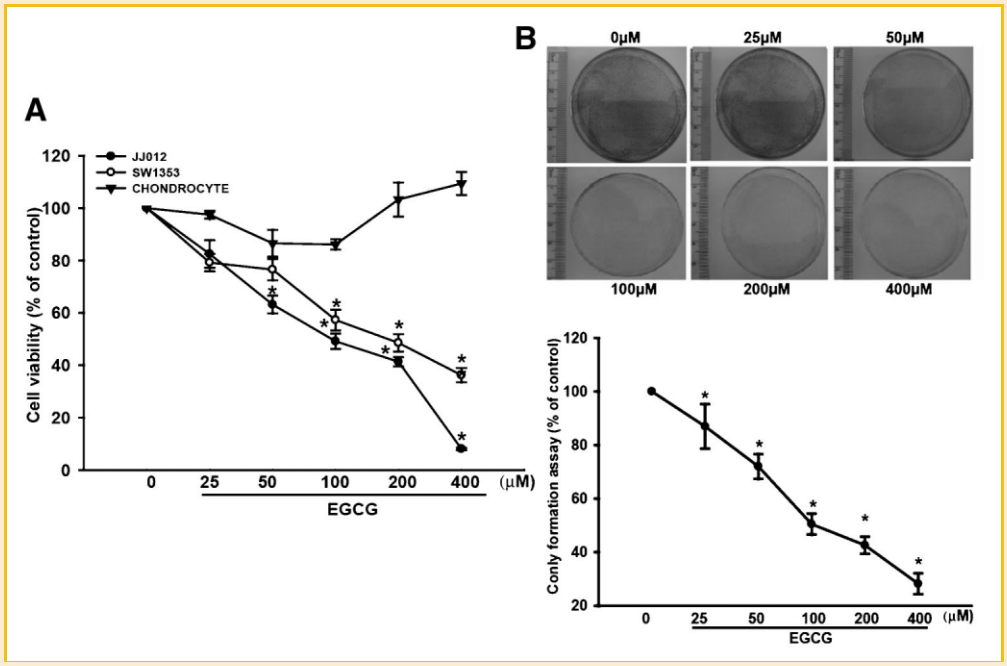

Fig. 1. The effects of EGCG on cell viability and colony formation in human chondrosarcoma cells. A: JJ012, SW1353, and primary chondrocytes were incubated with various concentrations of EGCG for 48 h, and the cell viability was examined by SRB assay. B: For colony-forming assay, the clonogenic assay was performed as described under Materials and Methods Section. The quantitative data are shown in lower panel. Results are expressed as the mean ± SE of four independent experiments. $^{*}P < 0.05$ as compared with control group.

These data indicate that EGCG induced cell death through an apoptosis mechanism.

EGCG CAUSED MITOCHONDRIAL DYSFUNCTION

To explore whether EGCG-induced cell apoptosis is mediated through mitochondrial dysfunction, we determined the mitochondrial membrane potential with the mitochondria-sensitive dye, JC-1. As shown in Figure 3A, treatment of JJ012 cells with EGCG induced the loss of the mitochondrial membrane potential in a dose-dependent manner (Fig. 3A). To further determine whether EGCG induces apoptosis by triggering the mitochondrial apoptotic pathway, we measured the change in the expression of Bcl-2 family proteins. Treatment of JJ012 cells with EGCG induced Bax and Bak protein levels (Fig. 3B). In addition, EGCG decreased the expression of Bcl-XL and Bcl-2, which led to an increase in the proapoptotic/anti-apoptotic Bcl-2 ratio (Fig. 3B). These data suggest that EGCG induced cell apoptosis through mitochondrial dysfunction.

ROS AND ASK1 ARE INVOLVED IN EGCG-INDUCED APOPTOSIS IN HUMAN CHONDROSARCOMA CELLS

It has been reported that ROS generation plays an important role in the proapoptotic activities of various anti-cancer agents [Kim et al., 2005; Hou et al., 2008]. Therefore, we next examined whether the ROS accumulation is involved in EGCG-induced cell death. DHE-based FACS detection revealed that intracellular O^{2-} level was increased in JJ012 cells following treatment with EGCG (Fig. 4A). On the other hand, EGCG also induced an increase in intracellular H_2O_2 levels, as shown by H_2DCFDA-based FACS detection assay (Fig. 4B). Pre-treatment of cells with catalase (H_2O_2 scavenging enzyme), vitamin C (scavenger of oxygen-free radicals), and *N*-acetylcysteine (NAC is a thiol compound that can act as a cysteine source for the repletion of intracellular glutathione and act as a direct scavenger of ROS) reduced EGCG-increased cell apoptosis in human chondrosarcoma cells (Fig. 4C). Therefore, ROS release is involved in EGCG-medicated cell death in human chondrosarcoma cells. In various pathologic conditions, NADPH oxidase is an important enzymatic source for the production of ROS [Chowdhury et al., 2005]. Therefore, the role of NADPH oxidase in EGCG-induced cell death was investigated. As shown in Figure 4C, pre-treatment of cells with NADPH oxidase inhibitor (DPI) abrogated EGCG-induced cell apoptosis. These results indicate that NADPH oxidase activation also involved in EGCG-induced cell death in human chondrosarcoma cells.

ASK1 activation is a pivotal mechanism in a broad range of cell death paradigms [Hsieh and Papaconstantinou, 2006]. ASK1 activation (phosphorylation at the activation loop Thr845) was assessed by Western blot. Treatment of JJ012 cells with EGCG increased ASK1 activation (Thr845 phosphorylation; Fig. 5B). Dissociation of ASK1 from 14-3-3 (an inhibitory protein) leads to ASK1 activation. Phosphorylation of the ASK1 Ser967 residue is required for ASK1 binding to 14-3-3 [Zhang et al., 1999]. To explore whether ASK1 activation contributes to EGCG-induced JJ012 death, the ASK1 dephosphorylation at Ser967 was examined. We found that EGCG induced ASK1 Ser967 dephosphorylation in a time-dependent manner (Fig. 5B). Coimmunoprecipitation was then used to confirm the hypothesis that EGCG-induced ASK1 dephosphor-

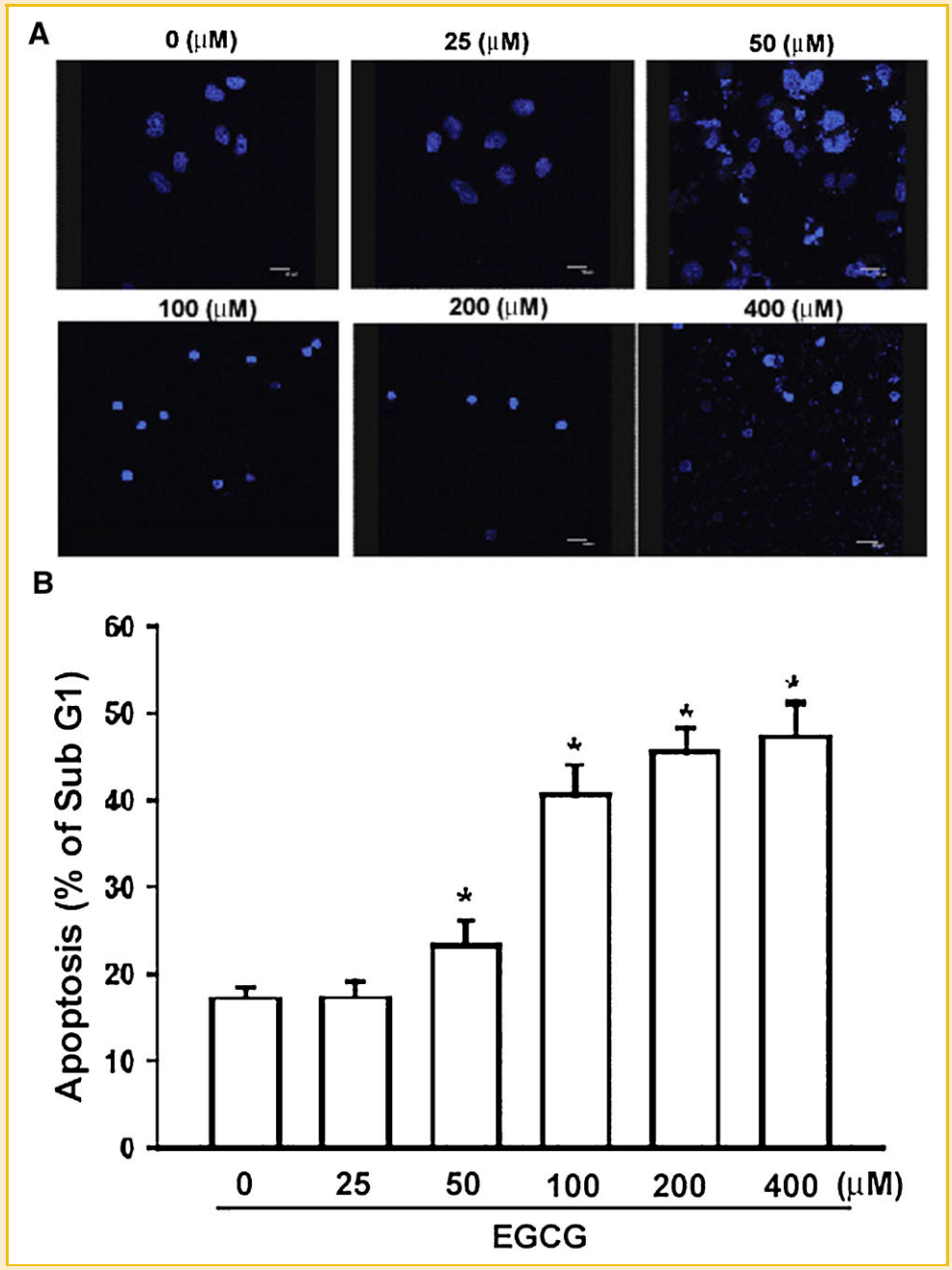

Fig. 2. EGCG induced the apoptosis of human chondrosarcoma cells. A: JJ012 cells were incubated with EGCG for 48 h, and DAPI staining was performed as described under Materials and Methods Section. B: JJ012 cells were incubated with EGCG (200 μM) for 48 h, the percentage of apoptotic cells was analyzed by flow cytometric analysis of PI-stained cells. Results are expressed as the mean ± SE $^{*}P < 0.05$ compared with control.

ylation is accompanied by the dissociation of the ASK1-14-3-3 complex. As shown in Figure 5A, EGCG caused ASK1 dissociation from 14-3-3. These findings suggest that ASK1 Ser967 dephosphorylation and subsequent ASK1 dissociation from 14-3-3 are involved in EGCG-induced chondrosarcoma apoptosis. To further investigate whether EGCG can induce cell apoptosis through the ASK1 pathway, JJ012 cells were transfected for 24 h with ASK1 siRNA, which inhibited the expression of ASK1 and EGCG-induced cell death, respectively (Fig. 5B). These results indicated that ASK1 activation plays a role in EGCG-induced cell death in human chondrosarcoma cells.

P38 AND JNK ARE INVOLVED IN EGCG-INDUCED APOPTOSIS IN HUMAN CHONDROSARCOMA CELLS

ASK1 belongs to the MAPKKK family and activates the p38 and JNK pathways via MKK3/6 and MKK4/7, respectively [Ichijo et al., 1997]. As shown in Figure 6A, treatment of JJ012 cells with EGCG resulted

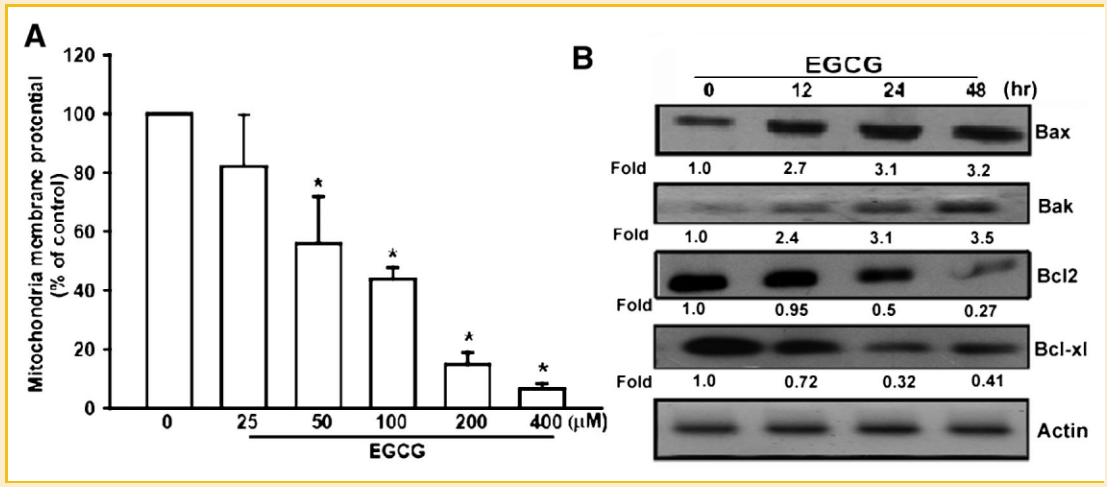

Fig. 3. EGCG induced mitochondrial dysfunction in human chondrosarcoma cells. A: Cells were incubated with EGCG (200 μM) for 48 h, mitochondrial membrane potential was satiated with JC-1 and examined by flow cytometry (n = 4). B: JJ012 cells were incubated with EGCG (200 μM) for different time intervals, the Bax, Bak, Bcl-2, and Bcl-XL expressions were examined by Western blot analysis. Results are expressed as the mean ± SE of four independent experiments. $^{*}P < 0.05$ compared with control; $\#P < 0.05$ compared with EGCG-treated group.

in time-dependent phosphorylation of p38 and JNK. Pre-treatment of cells with p38 inhibitor (SB203580) and JNK inhibitor (SP600125) or transfection with p38 and JNK mutant antagonized the EGCG-induced cells death (Fig. 6B,C). Taken together, these findings suggest that the ASK1-p38/JNK pathway is involved in EGCG-induced chondrosarcoma death.

One of the hallmarks of the apoptotic process is the activation of cysteine proteases (caspases), which represent both initiators and

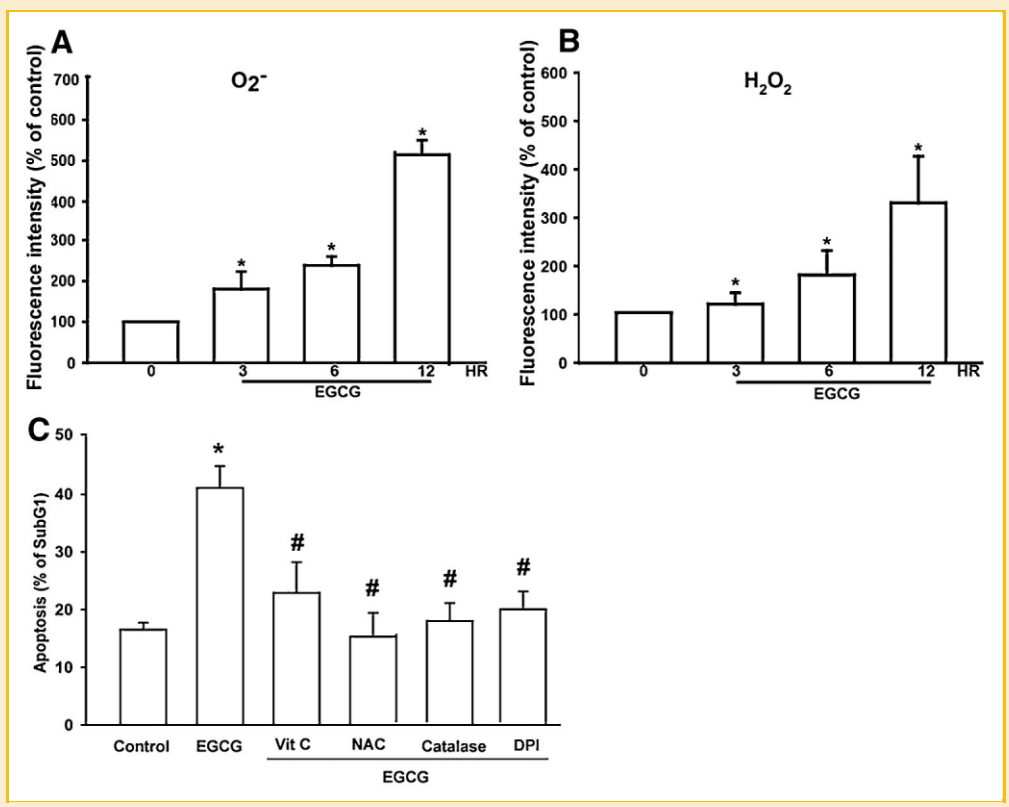

Fig. 4. EGCG induced ROS production in human chondrosarcoma cells. A,B: JJ012 cells were incubated with EGCG (200 μM) for different time intervals, the production of O^{2-} and H_2O_2 were examined by flow cytometry (n = 4). C,D: JJ012 cells were pre-treated for 30 min with catalase (10,000 U/ml), NAC (4 μM), vitamin C (100 μM), and DPI (10 μM) followed by stimulation with EGCG (200 μM) for 48 h, the percentage of apoptotic cells was then analyzed by flow cytometic analysis of PI-stained cells. Results are expressed as the mean ± SE of four independent experiments. $^{*}P < 0.05$ compared with control; $\#P < 0.05$ compared with EGCG-treated group.

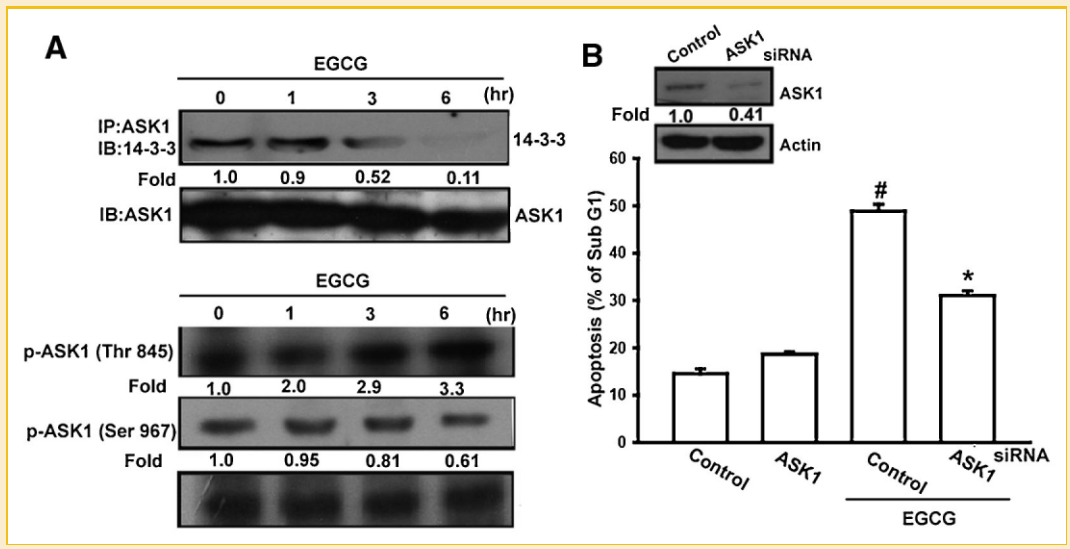

Fig. 5. The ASK1 signaling pathway is involved in EGCG-mediated apoptosis in chondrosarcoma cells. (A; upper panel) JJ012 cells were incubated with EGCG (200 μM) for different times, and the phosphorylation of ASK1 at Ser967 and Thr845 was examined by Western blotting. (A; lower panel) JJ012 cells were incubated with EGCG (200 μM) for different times and then immunoprecipitated (IP) with anti-ASK1. The IP complexes were subjected to immunoblotting (IB) with anti-14-3-3. B: Cells were transfected with ASK1 siRNA or control siRNA for 24 h, and ASK1 expression was examined by Western blotting (upper panel). Cells were transfected with ASK1 siRNA or control siRNA for 24 h and then stimulated with EGCG (200 μM) for 48 h and the percentage of apoptotic cells was analyzed by flow cytometry of PI-stained cells (lower panel). Results are expressed as the mean ± SE of four independent experiments. $^{*}P < 0.05$ compared with control; $\#P < 0.05$ compared with EGCG-treated group.

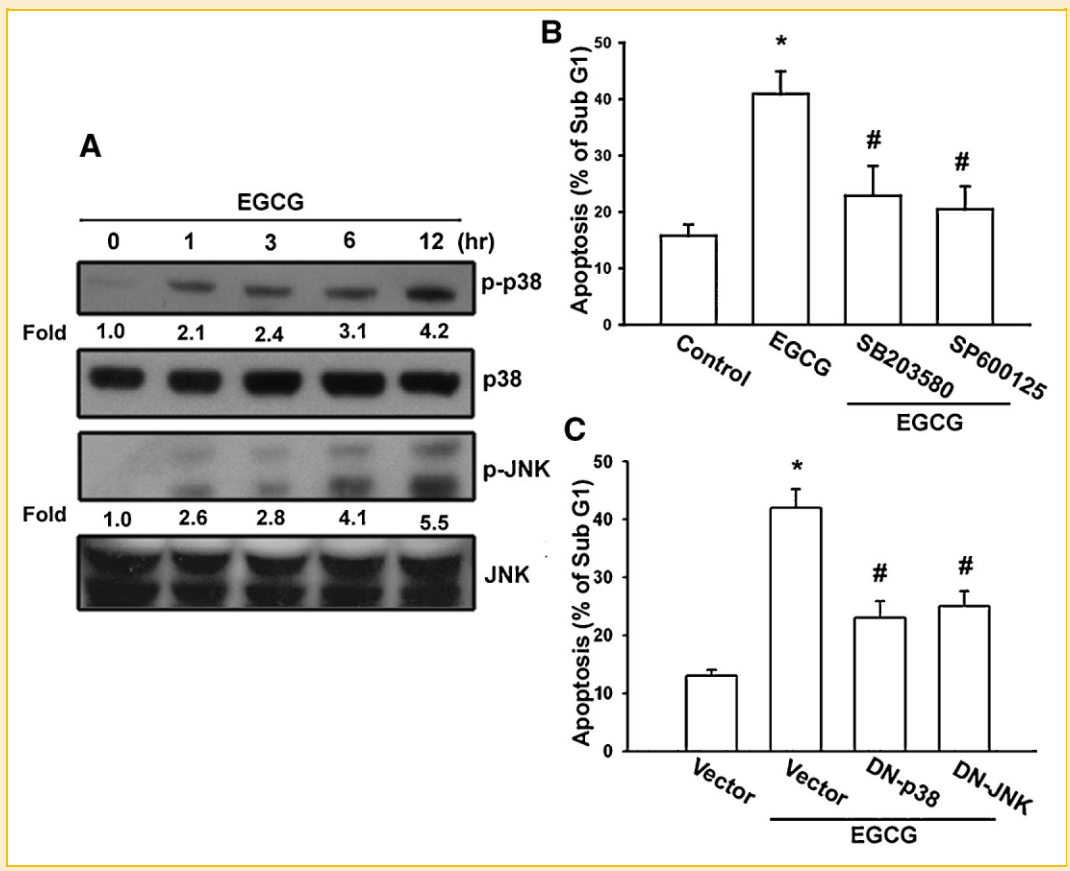

Fig. 6. P38 and JNK are involved in EGCG-mediated cell apoptosis in chondrosarcoma cells. A: JJ012 cells were incubated with EGCG (200 μM) for different times, and expression of p-p38, and p-JNK was examined by Western blotting. B,C: JJ012 cells were pre-treated for 30 min with SB203580 (10 μM) and SP600125 (3 μM) or transfected with p38 and JNK mutant followed by stimulation with EGCG (200 μM) for 48 h, and the percentage of apoptotic cells was analyzed by flow cytometry of PI-stained cells. Results are expressed as the mean ± SE of four independent experiments. $^{*}P < 0.05$ compared with control; $\#P < 0.05$ compared with EGCG-treated group.

executors of death signals. EGCG increased the expression and activation of caspase-3 in JJ012 cells (Fig. 7A,B). Pre-treatment of cells with the specific caspase-3 inhibitor z-DEVD-FMK reduced the EGCG-induced cell death (Fig. 7D). Upstream caspase-9 activities increased significantly upon treatment with EGCG in JJ012 cells (Fig. 7A,C). Pre-treatment of cells with caspase-9 inhibitor z-LEHD-FMK reduced EGCG-mediated cell apoptosis (Fig. 7D).

EGCG INHIBITED TUMOR GROWTH IN A MOUSE XENOGRAFT MODEL USING JJ012 CELLS

Based on the EGCG-induced apoptotic effect exhibited in vitro, we examined whether EGCG showed anti-tumor activity in vivo using xenografts of JJ012 cells in SCID mice. When tumors reached 100 mm^3 in size, the mice were divided into three groups and treated with either vehicle or EGCG (25 or 50 mg/kg). EGCG induced a dose-dependent inhibition of tumor growth (Fig. 7E). Body weight was not significantly affected by EGCG (Fig. 7F). Finally, ex vivo analysis of tumors by Western blotting showed significant increases in p-ASK1(Thr845), p-p38, or p-JNK expression and decreases in p-ASK1(Ser967) in the EGCG-treated group compared with the control group (Fig. 7G). Taken together, these results suggest that EGCG inhibits tumor growth by inducing JJ012 cell apoptosis in vivo.

DISCUSSION

Unlike other mesenchymal malignancies, such as osteosarcoma and Ewing's sarcoma, which are dramatic increase in long-term survival with the advent of systemic chemotherapy, chondrosarcoma continue to have a poor prognosis due to absence of an effective adjuvant therapy [Fong et al., 2007]. Therefore, development of novel therapeutic agents targeting the malignant behavior of chondrosarcoma cells is important to improve the prognosis of patients. The anti-cancer and cancer-preventive effects of green tea and its main constituent EGCG are well documented in literature including cell culture, animal, epidemiological, and clinical studies [Adhami et al., 2007; Shankar et al., 2008]. However, the anti-tumor effects of EGCG on chondrosarcoma cells are mostly unknown. In this study, we identified EGCG as a potential lead base on anti-tumor activity in human chondrosarcoma cells with good pharmacological properties. We further examined the selectivity of EGCG in chondrosarcoma cells and primary chondrocytes. We found that EGCG did not affect the ROS production, mitochondrial dysfunction, and activation of ASK in primary chondrocytes (Supplementary Fig. S1). It appears that these molecules have greater resistant to apoptosis compounds and that EGCG shows greater anti-cancer potential in human chondrosarcoma cells. Moreover, the metabolic rate is different between primary chondrocytes and chondrosarcoma may can explanation the different sensitive after EGCG treatment.

It has been reported that the enhancement of oxidative stress is associated with the apoptotic response induced by several anti-cancer agents [Haga et al., 2005; Kallio et al., 2005]. ROS can cause apoptotic cell death via a variety of mechanism, among which is the activation of stress kinase. High levels of ROS can also induce apoptosis by triggering mitochondrial permeability transitionpore opening, release of proapoptotic factors, and activation of caspase-9 [Zu et al., 2005; Iwamaru et al., 2007]. O_2^- also has been shown to regulate Bcl-XL expression, and inhibition of O_2^- by O_2^- scavenger *p*-benzoquinone prevents camptothecin-induced apoptosis [Wenzel et al., 2004]. In this study, we found that EGCG mediated oxidative stress by increasing the production of O_2^- and H_2O_2. Treatment of cells with vitamin C (scavengers of oxygen-free radicals), catalase (H_2O_2 scavenging enzyme), and NAC (ROS scavenger) reduced the EGCG-induced cell death. Our data suggest that ROS accumulation contributes to EGCG-induced cell death in human chondrosarcoma cells.

ASK1 is a ubiquitously expressed MAPKKK that is activated by various stressors (including oxidative stress, calcium overload, tumor necrosis factor, Fas ligand, and lipopolysaccharide), and selectively activates the JNK and p38 MAPK pathways [Ichijo et al., 1997; Saitoh et al., 1998; Nishitoh et al., 2002]. The activity of ASK1 is regulated by phosphorylation at different sites. Phosphorylation at Ser967 is essential for ASK1 association with 14-3-3 protein, which attenuates ASK1 activity. ROS induces dephosphorylation of Ser967 as well as phosphorylation of Thr845 in the ASK1 activation loop, both of which are correlated with ASK1 activity and ASK1-dependent apoptosis [Valko et al., 2006]. Here, we found that EGCG enhanced dephosphorylation at Ser967 and phosphorylation at Thr845 of ASK1. Furthermore, ASK1 siRNA inhibited EGCG-induced cell death. Therefore, ASK1 activation is required for EGCG-induced apoptosis of chondrosarcoma cells. ASK1 is an upstream molecule of JNK and p38, which have been shown to be involved in the regulation of cell cycle and cell death [Valko et al., 2006]. We showed in present study that EGCG increased the p38 and JNK phosphorylation. Pre-treatment of cells with p38 and JNK inhibitor antagonized the EGCG-induced cell apoptosis. This was further confirmed by the result that p38 and JNK mutant inhibited the enhancement of cell apoptosis by EGCG. These results suggest that EGCG-induced cell death may be critical for activating ROS-ASK1-p38/JNK apoptotic signaling cascade in human chondrosarcoma cells.

The mitochondrial apoptotic pathway has been described as an important downstream signal of ROS in apoptotic cell death [Zu et al., 2005; Iwamaru et al., 2007]. Here, we found that EGCG reduced mitochondria membrane potential. Pre-treatment of cells with NADPH and ROS inhibitor reduced EGCG-mediated mitochondrial dysfunction (Supplementary Fig. S2). Therefore, NADPH and ROS are upstream molecules of mitochondrial dysfunction. Furthermore, these results suggest that NADPH oxidase is indeed one of the primary targets of EGCG and ROS produced by this enzyme trigger stress kinase activation. Bcl-2 family proteins regulate mitochondria-dependent apoptosis with the balance of anti- and pro-apoptotic members arbitrating life-and-death decisions [Adams and Cory, 2001]. On the other hand, EGCG treatment results in a significant increase of Bax and Bak expression, and a decrease of Bcl-XL and Bcl-2, suggesting that changes in the ratio of pro-apoptotic and anti-apoptotic Bcl-2 family proteins might contribute to apoptosis-promotion activity of EGCG. In agreement of these observations, we noted that the mitochondrial dysfunction may be involved in EGCG-induced cell apoptosis of human chondrosarcoma cells.

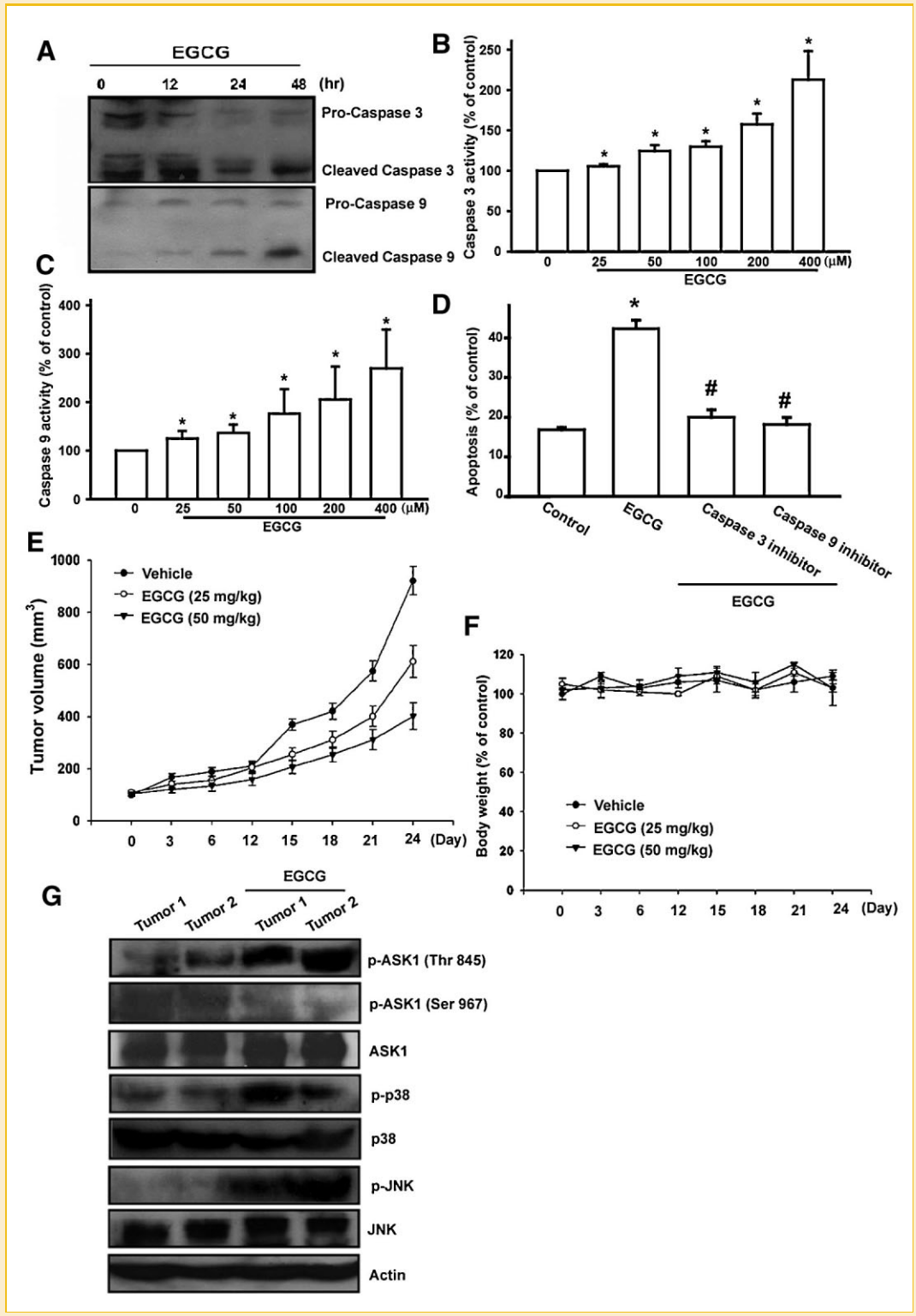

Fig. 7. Effects of EGCG on tumorigenicity and in vivo growth of xenografts in SCID mice. A: JJ012 cells were incubated with EGCG (200 μM) for different time intervals, the caspase-3 and caspase-9 expressions were examined by Western blot analysis. B,C: JJ012 cells were incubated with EGCG (200 μM) for 24 h, and caspase-3 and caspase-9 activities were examined by caspase ELISA kit ($n = 5$). D: Cells were pre-treated for 30 min with z-DEVD-FMK (caspase-3 inhibitor) or z-LEHD-FMK (caspase-9 inhibitor) followed by stimulation with EGCG (200 μM) for 24 h, the percentage of apoptotic cells were then analyzed by flow cytometric analysis of PI-stained cells. E: JJ012 cells (1×10^6) were injected subcutaneously into 6-week-old SCID mice. After the tumors reached 100 mm^3 in size, the animals were treated with an intraperitoneal injection of EGCG (25 or 50 mg/kg), or vehicle daily. The mean tumor volume was measured at the indicated number of days after implantation ($n = 8–10$). F: Mean body weight was measured at the indicated number of days after EGCG treatment. G: Western blot analysis of the levels of p-ASK1, p-p38, and p-JNK expression in tumors with and without EGCG treatment.

Natural product drugs have been suggested to play a dominant role in pharmaceutical care. Natural products are one of the important sources of potential cancer chemotherapeutic and chemopreventive agents. Tea is one of the most popular beverages in the world and has been studied extensively as a health-promoting beverage that may act to prevent a number of chronic diseases and cancers. However, the precise molecular mechanism of exhibited anti-tumor activity by EGCG in human chondrosarcoma is not well understood. Thus, the results of this study provide evidences for the anti-tumor activity of EGCG in chondrosarcoma cells, and more importantly, the molecular basis for its effect. The present study has demonstrated that EGCG causes apoptosis in chondrosarocma cells in vitro and in vivo. EGCG-induced apoptosis in chondrosarcoma cells involves the activation of the ROS-ASK1-p38/JNK signaling cascade. The proposed working models for the molecular basis would provide valuable insights for approaches to the development of effective chemotherapy by targeting appropriate signal transducers.

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