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Identification of epigallocatechin-3-gallate in green tea polyphenols as a potent inducer of p53-dependent apoptosis in the human lung cancer cell line A549

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ABSTRACT

The effects of green tea polyphenols on cultured cancer cells have been well characterized, especially the effects of epigallocatechin-3-gallate (EGCg), since EGCg suppresses oncogenic signaling pathways and induces cell cycle arrest or apoptosis by regulating cell cycle-associated proteins. In the present study, we attempted to identify signaling pathways or target molecules regulated by each of or a mixture of green tea polyphenols, including epicatechin (EC), epicatechin-3-gallate (ECg), epigallocatechin (EGC), and EGCg, in the human lung cancer cell line A549. ECg, EGC, and a catechin mixture, in addition to EGCg, significantly decreased cell viability. In contrast, caspase 3/7 activity, an apoptosis indicator, was specifically induced by EGCg. By conducting a series of luciferase-based reporter assays, we revealed that the catechin mixture only up-regulates the p53 reporter. EGCg was a more potent inducer of p53-dependent transcription, and this induction was further supported by the induced level of p53 protein. RNA interference (RNAi)-mediated p53 knockdown completely abolished EGCg-induced apoptosis. Finally, a proteome and western blot analysis using approximately 70 different antibodies failed to detect up-regulated proteins in catechin mixture-treated A549 cells. Taken together, these results indicate that EGCg, among several green tea polyphenols, is a potent apoptosis inducer that functions exclusively through a p53-dependent pathway in A549 cells.

1. Introduction

Catechin ($C_{15}H_{14}O_6$) is a well-known flavonoid that is mainly obtained from tea leaves as a polyphenolic compound. Green tea (*Camellia sinensis L.O. Kuntze*) contains polyphenols such as epicatechin (EC), its hydroxyl derivative epigallocatechin (EGC), and their gallic acid esters epicatechin-3-gallate (ECg) and epigallocatechin-3-gallate (EGCg) (Brown, 1999). The catechin composition in tea leaves is EGCg > EGC > ECg > EC, and EGCg, which accounts for about 60% of the catechin content, is known to possess broad physiological activities such as antioxidative, antiallergic, anticarcinogenic, antibacterial, and antiviral effect (Feng, 2006). Moreover, catechin can reduce blood cholesterol and glucose levels and maintain blood pressure level (Feng, 2006). Indeed, weight loss in subjects taking EGCg supplements has been reported (Chantre and Lairon, 2002). On the other hand, a serious concern exists about possible toxicities in individuals with high EGCg supplement intakes. Recently, EGCg-induced liver damage has been successively reported in studies from France, Spain, and Canada (Gloro et al., 2005; Molinari et al., 2006). Therefore, the precise modes of action of green tea polyphenols should be assessed at the molecular and cellular levels.

Other than toxic effect of EGCg, an anticancer effect of EGCg at a molecular level has been extensively characterized, as EGCg mainly inhibits growth factors/receptors-mediated cellular signal transduction pathways such as NF-κB, phosphatidylinositol-3-kinase (PI3-K)/protein kinase B (AKT), RAS/RAF/mitogen-activated protein kinase (MAPK), and AP-1 in addition to inducing cell cycle arrest and apoptosis mediated by the modulation of cell cycle-associated proteins (Khan et al., 2006; Shankar et al., 2007). In the case of cell cycle arrest and apoptosis induction, p53 is the most prominent protein to be up-regulated by EGCg (Gupta et al., 2003; Hastak et al., 2003; Hofmann and Sonenshein, 2003). In the prostate cancer cell line PC3, p53 downstream targets p21 and BAX have been reported to be required for the induction of p53-dependent apoptosis by EGCg (Hastak et al., 2005). In contrast, the up-regulation of Bax during EGCg-induced apoptosis in p53 null mouse embryonal fibroblasts has been reported (Shankar et al., 2007). These results argue the possibility that p53-dependent and p53-independent pathways of EGCg-induced apoptosis may exist, suggesting that more detailed analyses should be performed on different cell types. In addition, the effects of various catechin constituents other than EGCg and mixtures of these constituents on cancer cell phenotypes

Abbreviations: DMSO, dimethyl sulfoxide; EC, epicatechin; ECg, epicatechin-3-gallate; EGC, epigallocatechin; EGCg, epigallocatechin-3-gallate; MAPK, mitogen-activated protein kinase; PI3-K, phosphatidylinositol-3-kinase; RNAi, RNA interference; siRNA, short interference RNA.

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have not been fully investigated. In the present study, we revealed that EGCg is the most powerful inducer of p53 among green tea polyphenols and that p53 is essential for EGCg-induced apoptosis in the human lung cancer cell line A549.

2. Materials and methods

2.1. Cells and reagents

A549 cells (TKG0184; Cell Research Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University) were cultured in modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 1% non-essential amino acids (Invitrogen), and antibiotics–antimycotics (Invitrogen) in a 5% CO_2 humidified atmosphere at 37 °C. (−)-Epicatechin (#C-0011), (−)-epicatechin gallate (#C-0012), (−)-epigallocatechin (#C-0013), (−)-epigallocatechin gallate (#C-0014), and (+)-catechin (#C-0015) were obtained from Mitsui Norin Co., Ltd. (Fujieda, Shizuoka, Japan). Chemicals were dissolved in dimethyl sulfoxide (DMSO) as 100 mM stock solutions and kept at −20 °C until dilution before use.

2.2. WST-1 assay for cell growth/proliferation

The cells were inoculated into a 24-well plate (Nunc, Rochester, NY) at a concentration of 2×10^3 cells/well 24 h before treatment. The cells were then treated with increasing concentrations of catechins at the indicated concentrations for 24 and 48 h. A WST-1 assay was then performed according to the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN) except for medium renewal to remove residual catechins just before WST-1 solution treatment. Briefly, a WST-1 solution was added to each well, followed by 1 h of incubation at 37 °C, and the absorbance was measured at 450 nm. The reference wavelength was 600 nm. Minus number was considered as zero. Cell viability was calculated as the absorbance in the treated cells over the control (treated with 0.1% DMSO). The data are presented as the mean values ± S.D. (standard deviation). Statistical differences were analyzed using a two-tailed Student *t*-test. A value of $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ ($n = 4$) was considered to indicate a statistically significant difference.

2.3. Caspase 3/7 assay for apoptosis

The induction of apoptosis was assessed using a Caspase-Glo 3/7 assay according to the manufacturer's instructions (Promega, Madison, WI). In brief, the cells were inoculated into a 96-well plate (Nunc) at a concentration of 1×10^4 cells/well 24 h before treatment. The cells were then treated with chemicals for 24 h, followed by 1 h of incubation with Caspase-Glo 3/7 substrate at room temperature, and the resulting activity was measured using a GloMax 20/20n Luminometer (Promega). The relative activity was presented as the activity in the treated cells relative to the control value (treated with 0.1% DMSO), which was regarded as being equal to 1. Blanks were also measured in wells containing 0.1% DMSO or chemicals without cells. The data are presented as the mean values ± S.D. Statistical differences were analyzed using a two-tailed Student *t*-test. A value of $P < 0.05$ ($n = 3$) was considered to indicate a statistically significant difference. In the case of p53 Stealth RNAi pre-treated cells, a value of $P < 0.01$ ($n = 4$) was considered to indicate a statistically significant difference.

2.4. Luciferase reporter assay

The cells were inoculated into a 24-well plate (Nunc) at a concentration of 3×10^4 cells/well 24 h before treatment. The cells were then transfected with FuGENE6 (Roche, Basel, Switzerland) according to the manufacturer's instructions. Briefly, 200 ng/well of firefly luciferase reporter plasmids were used as follows: pTA-Luc, pE2F-TA-Luc, pMyc-TA-Luc, pp53-TA-Luc, pRb-TA-Luc, pAP1(PMA)-TA-Luc, pNFAT-TA-Luc, pSTAT3-TA-Luc, pISRE-TA-Luc, pGAS-TA-Luc, pTAL-Luc, pAP1-Luc, pCRE-Luc, pGRE-Luc, pHSE-Luc, pNF-κB-Luc, and pSRE-Luc (Clontech, Mountain View, CA), and pLuc-MCS, pp53-Luc, pAP1-Luc, and pCRE-Luc (Stratagene, La Jolla, CA). As an internal transfection control, 0.6 ng/well of *Renilla* luciferase reporter plasmid pRL-TK (Promega) was also used. The cells were lysed 24 h after transfection by applying 100 μL of Passive Lysis Buffer from the Dual Luciferase Reporter Assay Kit (Promega) into each well of the 24-well plate. Five microliters of cell lysate was used for the luciferase reporter assay using the same kit, according to the manufacturer's protocol. The light intensity was quantified using a GloMax 20/20n Luminometer (Promega). As a control for the transfection efficiency, the firefly luciferase activity values were normalized to the *Renilla* luciferase activity values. The relative luciferase activity was calculated based on the activity obtained from control plasmid (pTA-Luc, pTAL-Luc, or pLuc-MCS)-transfected cells prior to DMSO treatment regarded as being equal to 1. Otherwise, the activity obtained from pp53-TA-Luc or pp53-Luc reporter plasmid-transfected cells prior to DMSO treatment was regarded as being equal to 1. The data are presented as the mean values ± S.D. Statistical differences were analyzed using the two-tailed Student *t*-test. A value of $P < 0.05$ ($n = 3$) was considered to indicate a statistically significant difference.

2.5. Western blotting

The cells were harvested after 24 h of DMSO or chemical treatment and lysed in modified RIPA lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, and 1 mM EDTA) containing a protease inhibitor cocktail (Sigma, Saint Louis, MO) and a phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) for 20 min on ice. The cell lysates were centrifuged and the protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, CA). Protein lysate (20–50 μg) were loaded onto each lane of a gel. Before performing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the reaction was stopped by the addition of Laemmli sample buffer containing 100 mM dithiothreitol (DTT). Equal amounts of cellular protein were electrophoresed on NuPAGE 4–12% Bis–Tris gel with MES running buffer (Invitrogen) and transferred to a Hybond-PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membrane was first blocked using phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 5% non-fat dried milk and then incubated with the following antibodies: p53 (FL-393), phospho-p53 (Ser15), p21 (C-19), NOXA (FL-54), p73 (S-20), and MDM2 (SMP14) antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and BAX, PUMA, and cleaved poly (ADP-ribose) polymerase (PARP) (Asp214) antibodies purchased from Cell Signaling Technology (Danvers, MA). GAPDH antibody was purchased from Applied Biosystems (Foster City, CA). Alkaline phosphatase (AP)-labeled secondary antibodies were purchased from Promega. A Western blue stabilized substrate was used to detect the signals according to the manufacturer's protocol (Promega).

2.6. RNA interference

The Validated Stealth RNAi DuoPak for p53 (Invitrogen) and the Stealth RNAi Negative Control Kit with High GC (Invitrogen) was transfected into the cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Briefly, 2×10^5 cells

were inoculated into each well of a 6-well plate prior to the transfection. The cells in each well were transfected with 125 pmol of control or p53-specific Stealth RNA (#1 and #2) on consecutive days. EGCg or DMSO was added 24 h after transfection. The cell lysates were collected 24 h after the EGCg or DMSO treatment. To detect apoptosis, the cells were inoculated into a 96-well plate at a density of 1×10^4 cells/well.

3. Results

3.1. Comparative analyses of effects of green tea polyphenols on cell growth/proliferation and apoptosis in A549 cells

EGCg is a representative green tea polyphenol used in catechin research. We compared various catechin constituents in green tea to determine whether they have similar or different effects on cell growth/proliferation and apoptosis in the human lung carcinoma cell line A549. Additionally, we examined whether these compounds have any synergistic effects. Towards this end, we initially examined cell growth/proliferation by adding increasing concentrations of catechin (C), EC, ECg, EGC, EGCg, and a mixture of four constituents (EC + ECg + EGC + EGCg) to exponentially growing cells. A WST-1 assay was then performed at 24 and 72 h after chemical treatment. Cell viability (% control) was determined by comparison with that in DMSO-treated cells (100% growth) for each of the time periods. As shown in Fig. 1A, C and EC had no effect on the growth/proliferation of A549 cells, even at a high concentration (100 μM), at a long exposure time (48 h), as assessed by the criteria mentioned in Section 2. In contrast, 100 μM of ECg had a medium suppressive effect on cell viability, beginning at 24 h and ending to 48 h after treatment. Similarly, cell viability was significantly decreased, at 24 and 48 h, by 50 μM of either EGC or EGCg. In the case of the mixture of catechins, 50 and 100 μM concentrations suppressed cell viability at 24 and 48 hours after treatment, whereas a 10 μM concentration required 48 h to suppress cell viability. These results indicate that some constituents of green tea polyphenols, such as ECg, EGC, and EGCg, can inhibit the growth/proliferation of A549 cells in a concentration-dependent manner at 48 h after chemical treatment.

Next, we examined caspase 3/7 activity, which is generally recognized as an apoptosis indicator, to determine whether apoptosis was induced in the chemical-treated cells. A549 cells were treated with 100 μM of each chemical for 24 h. Although all green tea polyphenols except for C and EC showed severe suppressive effects on cell viability at 24 h after treatment at a concentration of 100 μM, caspase 3/7 activity was significantly induced only in EGCg-treated cells (Fig. 1B). The induction of caspase 3/7 activity was also detected at 48 h after treatment with EGCg (data not shown). The induction of caspase 3/7 activity in A549 cells appeared to require an EGCg concentration of 100 μM (Fig. 1C). Taken together, these data suggest that among the green tea polyphenols that were examined, apoptosis is only induced by EGCg.

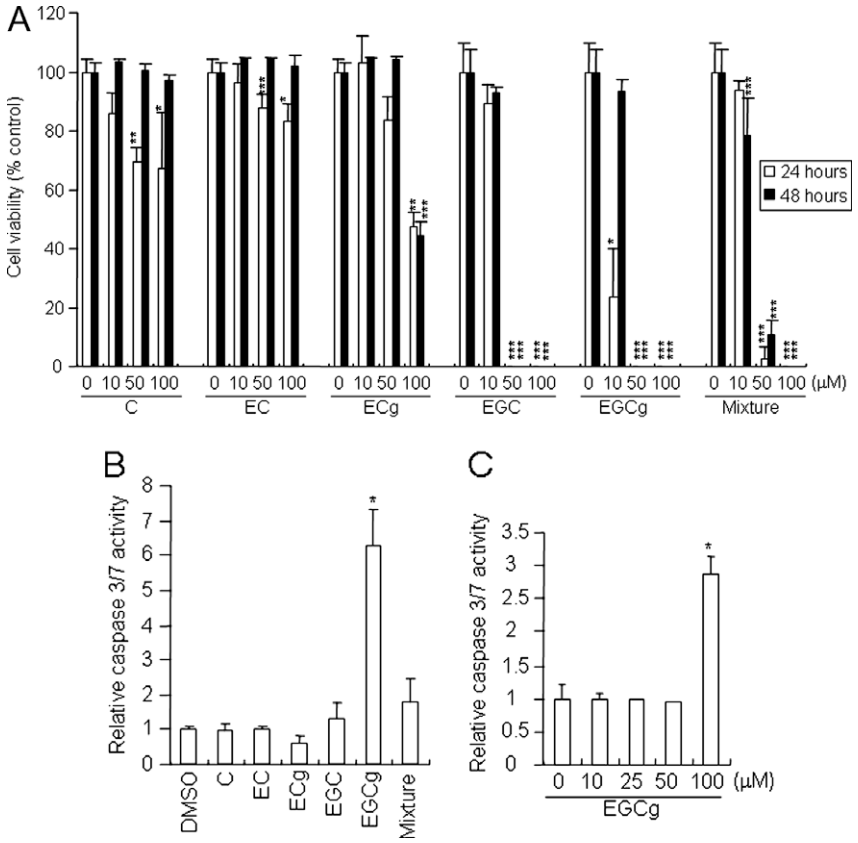

Fig. 1. Effects of green tea polyphenols on cell growth/proliferation and apoptosis in A549 cells. C, EC, ECg, EGC, EGCg, and mixture denote catechin, epicatechin, epicatechin-3-gallate, epigallocatechin, epigallocatechin-3-gallate, and EC + ECg + EGC + EGCg, respectively. (A) Chemicals were added for 24 h (indicated by white box) or 48 h (indicated by black box) at the indicated concentrations. Cell viability (% control) was measured using a WST-1 assay, as detailed in the Section 2. Values are expressed as the means ± S.D. ($n = 4$). Statistical significant differences were indicated by asterisk number as follows; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (compared with DMSO-treated cells at each time point), using a two-tailed Student t-test ($n = 4$). (B) Caspase 3/7 activity detected in A549 cells treated with 100 μM of the indicated chemicals for 24 h. Values are expressed as the means ± S.D. ($n = 3$). $^{*}P < 0.05$ (compared with DMSO-treated cells), using a two-tailed Student t-test. (C) EGCg-induced apoptosis in A549 cells. Cells were treated for the indicated concentrations for 24 h. Values are expressed as the means ± S.D. ($n = 3$). Asterisk, $P < 0.05$ (compared with DMSO-treated cells), using a two-tailed Student t-test.

3.2. p53 pathway is a unique target of green tea polyphenols in A549 cells

Although we found that EGCg is a potent inducer of caspase 3/7 activity in A549 cells, we selected a mixture of four catechins to search for the cellular targets of green tea polyphenols. At this time, we still expected that a mixture of catechins (containing 25 μM of EGCg) might have synergistic effects on cellular targets. We compared the protein profiles of mixture- and DMSO-treated A549 cells using CBB G250-stained 2D-PAGE. After 24 h of exposure to the mixture, several spots where the signals had changed by greater than or less than 3-fold were selected and processed for peptide mass fingerprinting using MALDI–TOF MS AXIMA-CFR (Shimadzu Corporation) and a Mascot search (Matrix Science). We identified mitofilin and hnRNP as candidates and used a western blot analysis to analyze the changes in their expressions in A549 cells after treatment with the catechin mixture but failed to reproduce the same results obtained using 2D-PAGE. At the same time, using the same samples, we searched for any changes in protein expression and modification after treatment with the catechin mixture. We used approximately 70 different antibodies to proteins involved in functional categories of the cell cycle, apoptosis, checkpoints, DNA mismatch repair, MAPK signaling, histone modification, NF-κB signaling, ER stress, heat shock protein/chaperon, and 4E-BP (see Supplementary Table 1); however, no changes were detected. From these results, we assumed that the mixture of green tea polyphenols had no effects on any proteins, though cell viability was severely suppressed.

Next, we used a luciferase-based reporter plasmid assay because this assay is expected to be more sensitive than a western blot approach. Eighteen reporter plasmids, in which fifteen reporters are responsive to transcription factors and cellular signaling pathways, were transiently transfected into A549 cells prior to the addition of the catechin mixture. As shown in Fig. 2A, the p53 reporter was exclusively up-regulated by the catechin mixture at 24 h treatment. Luciferase activity derived from the p53 reporter in other vector systems was reproducibly up-regulated by the catechin mixture (Fig. 2B). Among the green tea polyphenols, EGCg was solely responsible for the induction of p53-dependent reporter activity, as shown by results obtained using two different vector backbones (Fig. 2C). In other words, the p53-dependent luciferase activity induced by the catechin mixture was caused by the 25 μM of EGCg, since the other compounds (100 μM) lacked the ability to induce p53 reporter activity. Together, these results strongly suggest that the p53 pathway is a pivotal target of EGCg and that this mechanism could be solely important for EGCg-dependent apoptosis in A549 cells.

As expected, EGCg (100 μM) increased the p53 protein and its Ser15 phosphorylation levels in A549 cells 24 h after addition; however, the catechin mixture had slight or no effects on the p53 protein and its Ser15 phosphorylation levels (Fig. 3A). To identify the EGCg target proteins downstream of p53, we examined the

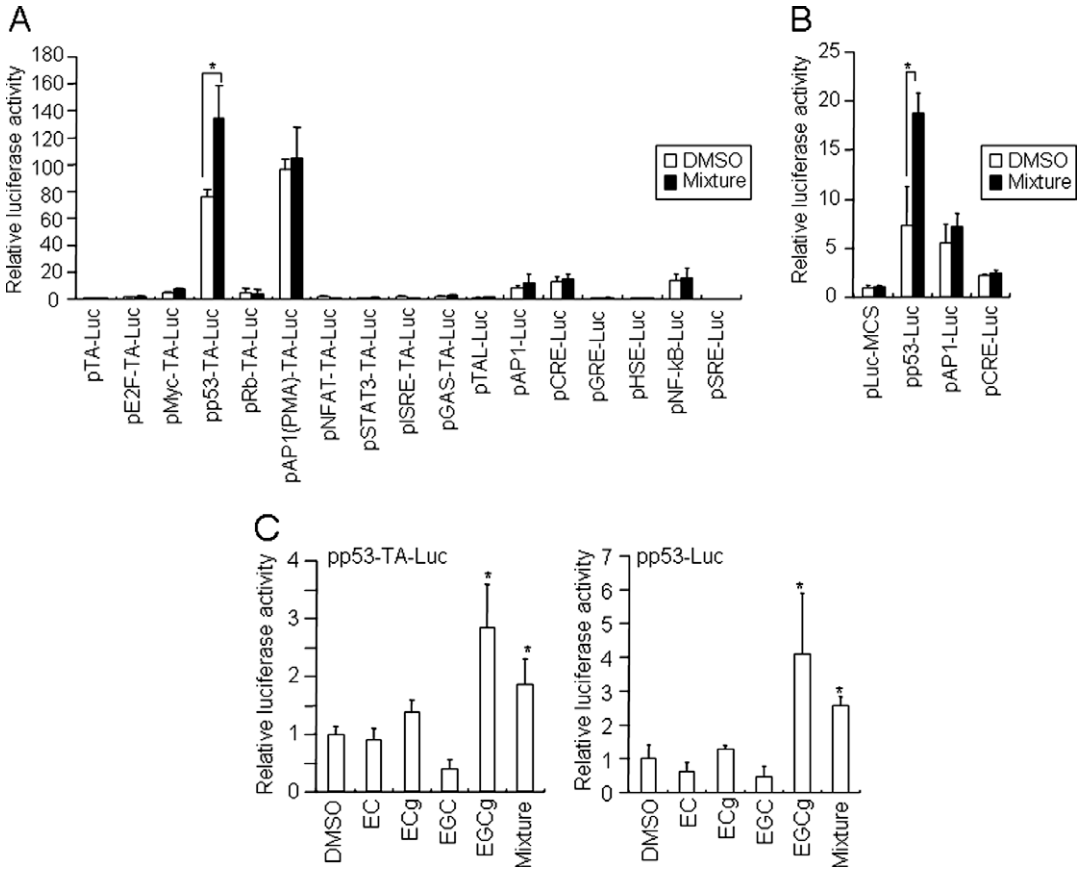

Fig. 2. Effects of green tea polyphenols on cellular signaling pathways. Luciferase expression of pTA-Luc and pTAL-Luc (A) and pLuc-MCS (B) reporter systems. Each reporter bears unique responsive elements for the indicated transcription factors and signaling pathways upstream of the luciferase genes. A reporter assay was performed as described in the Section 2. The relative luciferase activity is shown as the fold induction relative to the pTA-Luc, pTAL-Luc, or pLuc-MCS vector, which was designated as having a value of 1 (DMSO-treated A549 cells). The white and black boxes indicate the DMSO- and mixture-treated cells, respectively. Values are expressed as the means ± S.D. ($n = 3$). Asterisk, $P < 0.05$ (compared with DMSO-treated cells), based on a two-tailed Student *t*-test. (C) Luciferase expression of pp53-TA-Luc (*left*) or pp53-Luc (*right*) treated with the indicated chemicals in A549 cells. A reporter assay was performed as described above. The relative luciferase activity is shown as the fold induction relative to the activity in the DMSO-treated cells, which was designated as having a value of 1. Values are expressed as the means ± S.D. ($n = 3$). $^{*}P < 0.05$ (compared with DMSO-treated cells), using a two-tailed Student *t*-test.

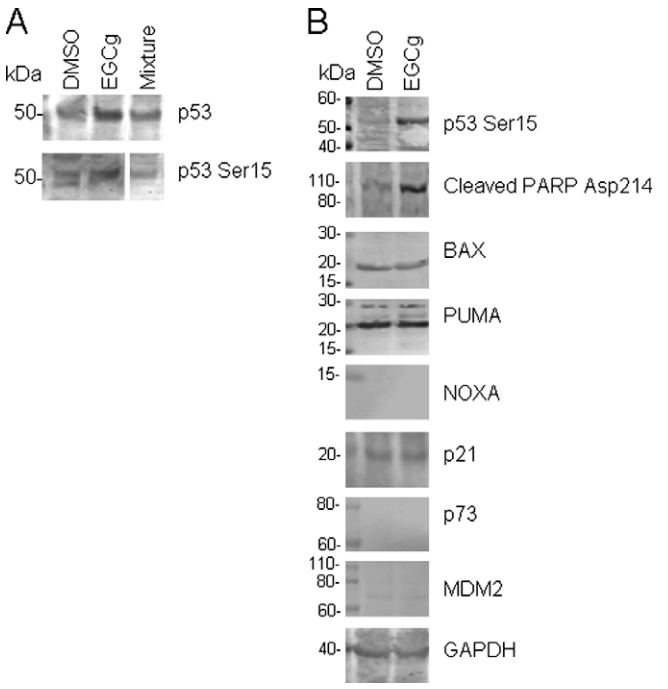

Fig. 3. Changes in protein and Ser 15 phosphorylation levels of p53 (A) and its related cell cycle and apoptotic proteins (B). Protein lysates were prepared from A549 cells that had been pre-treated with DMSO or the indicated chemicals (100 μM) for 24 h. The indicated proteins shown on the right side were probed using corresponding specific antibodies and examined using a western blot analysis. The molecular weight marker is shown on the left side.

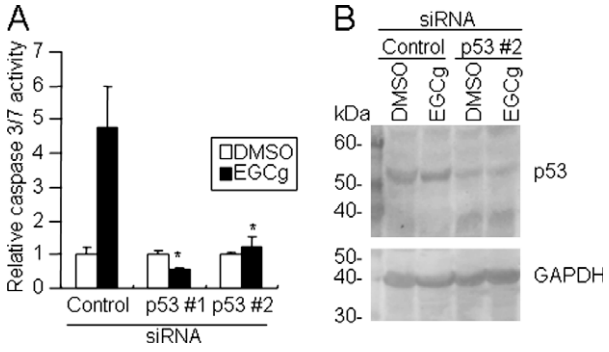

Fig. 4. Effect of p53 RNAi on EGCg-induced apoptosis in A549 cells. (A) A549 cells were transfected with control or p53-specific stealth RNAs (#1 and #2), followed by DMSO (white box) or 100 μM of EGCg (black box) for 24 h. Relative caspase 3/7 activity was measured similar to the method described in (B) of Fig. 1. Values are expressed as the means ± S.D. ($n = 4$). Asterisk, $P < 0.01$ (compared with control siRNA + EGCg-treated cells), using a two-tailed Student t-test. (B) Western blot analysis of knockdown of p53 protein. The cells were prepared as in (A) of this Fig. GAPDH is shown as a loading control. The molecular weight marker is shown on the left side.

protein levels of BAX, PUMA, NOXA, p21, p73, and MDM2. In addition to the GAPDH protein loading control, the levels of all of these proteins remained unchanged in the cell lysates, whereas the p53 Ser15 phosphorylation and cleaved PARP Asp214 levels were significantly up-regulated by EGCg (Fig. 3B).

Finally, we confirmed whether p53 is really required for EGCg-induced apoptosis in A549 cells using the RNAi-mediated attenuation of p53 expression. We introduced two different p53-specific short interference RNAs (siRNAs) (#1 and #2) separately into the cells on two consecutive days (at a 24-hour interval), and then 100 μM of EGCg or DMSO was added directly to the cell culture for 24 h. As shown in Fig. 4A, the EGCg-induced caspase 3/7 activity detected in the cells treated with the control siRNA was completely inhibited in the cells treated with the p53-specific siRNA. Similarly, the induction of endogenous p53 protein by EGCg was detected in cells treated with control siRNA but disappeared in cells treated with p53-specific siRNA. Collectively, p53 is indispensable for EGCg-induced apoptosis in A549 cells.

4. Discussion

To search for novel biomarkers that are regulated by green tea polyphenols, first of all, we performed comparative experiments to identify which constituents are responsible for given cellular phenotypes, such as cell growth/proliferation suppression and apoptosis induction. From our experiments, even though other constituents not examined in the present study may also suppress cell viability, EGCg appears to be uniquely involved in apoptosis induction in A549 cells. We initially intended to identify novel biomarkers that are up-regulated by a mixture of green tea polyphenols in A549 cells. For this, we performed a proteome analysis. Unfortunately, the proteins identified by a Mascot search were false positive when we re-examined them using western blotting.

We next performed a luciferase-based screening to see if any reporter could be regulated by a mixture of green tea polyphenols and found that the p53 pathway was the only pathway to be up-regulated among the compounds that were examined. This effect was caused exclusively by EGCg, as was the induction of caspases 3/7 activity. Recent studies favor p53-dependent mechanism during EGCg-induced apoptosis in a variety of cancer cells (Hastak et al., 2005; Roy et al., 2005; Manna et al., 2006; Qin et al., 2008). In human prostate carcinoma LNCaP cells, the involvement of EGCg in NF-κB and p53 pathways has been shown to induce apoptosis by modulating the balance between BAX and BCL-2 proteins (Hastak et al., 2003). In EGCg-treated A549 cells, we detected EGCg-induced apoptosis in the absence of changes to the BAX protein level. Moreover, the p21 level, a p53 target that has also been reported to be an essential factor for EGCg-mediated apoptosis (Hastak et al., 2005), was unchanged in the A549 cells. Under certain stress conditions, p53 ubiquitin ligase MDM2 is known to be inhibited, thereby stabilizing the p53 level (Bond et al., 2005). As well as MDM2, the p53 homologue p73 (Irwin et al., 2000) was also unchanged by EGCg, even in the presence of apoptosis. The levels of other p53 targets that are reportedly important for apoptosis, such as PUMA, NOXA, and BCL-2 homology 3 (BH3) proteins (Villunger et al., 2003), were also unchanged and were not induced by EGCg in A549 cells.

In summary, our data clearly demonstrated that p53 is an essential factor for EGCg-induced apoptosis in A549 cells. Using comparative experiments, we demonstrated that EGCg, among the green tea polyphenols examined in this study, is crucial for apoptosis in A549 cells. To limit toxic side effects of chemopreventive agents, one possible hope is to expect synergistic effects of a combination of green tea polyphenols and chemopreventive agents. EGCg was shown to suppress the growth of breast cancer cells when combined with tamoxifen (Scandlyn et al., 2008). Our findings strongly indicate that EGCg is the most promising constituent among green tea polyphenols for the development of synergistic combinations with commercially used anti-tumor chemicals to enhance the efficacy of cancer therapy and also to minimize potential adverse effects of synthesized anti-neoplastic agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tiv.2009.04.011.

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