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The Apoptotic Effect of Green Tea (−)-Epigallocatechin Gallate on 3T3-L1 Preadipocytes Depends on the Cdk2 Pathway

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This study was designed to investigate the effect of green tea catechins, especially (−)-epigallocatechin gallate (EGCG), on the apoptosis of 3T3-L1 preadipocytes. Preadipocyte apoptosis as indicated by formation of DNA fragments was induced by EGCG in dose-dependent manners. While EGCG was demonstrated to decrease Cdk2 expression and activity and increase caspase-3 activity, overexpression of Cdk2 and treatment with the caspase-3 inhibitor respectively prevented preadipocytes from induction of DNA fragmentation and caspase-3 activity by doses of 100−400 μM of EGCG. This suggests the Cdk2- and caspase-3-dependent apoptotic effects of EGCG. Moreover, EGCG was more effective than EC, ECG, and EGC in changing the apoptotic signals. Results of this study may relate to the mechanism by which EGCG modulates body weight.

KEYWORDS: Green tea; epigallocatechin gallate; 3T3-L1 preadipocyte; cyclin-dependent kinase; caspase-3; apoptosis

INTRODUCTION

Green tea catechins (GTCs) are polyphenolic flavonoids once called vitamin P (*1*). Since the discoveries that they have unique chemical structures and are major ingredients of unfermented tea (*2*, *3*), they have been found to possess widespread biological functions and health benefits (*3*−*7*). In vivo, GTCs, especially (−)-epigallocatechin gallate (EGCG), lower the incidence of cancers (*3*−*7*), collagen-induced arthritis (*8*), oxidative stress-induced neurodegenerative diseases (*9*), and STZ-induced diabetes (*10*). Also, EGCG can reduce body weight and body fat (*11*). In supporting this antiobese effect of EGCG, other in vivo data have shown that EGCG or EGCG-containing green tea extract reduces food uptake, lipid absorption, and blood triglyceride, cholesterol, and glucose levels, as well as stimulating energy expenditure, fat oxidation, HDL levels, and fecal lipid excretion (*3*, *11*−*13*). These in vivo observations may be explained by in vitro findings that EGCG and caffeine synergistically with norepinephrine stimulate the thermogenesis of brown adipose tissue (*14*), that EGCG regulates various enzymes related to lipid anabolism and catabolism, such as acetyl-CoA carboxylase, fatty acid synthase, pancreatic lipase, gastric lipase, and lipoxygenase (*3*, *15*), that EGCG is a potent prooxidant and antioxidant (*3*, *16*), and that EGCG reduced serum- or insulin-induced increases in cell numbers and the triacylglycerol content during a 9-day period of differentiation (*12*). These in vivo and in vitro observations suggest that green tea EGCG appears to modulate the mitogenic, endocrine, and metabolic functions of fat cells.

Despite the importance of EGCG, the mechanism of its action in regulating the apoptosis of preadipocytes is still unknown. The fact that the EGCG receptor, the so-called laminin receptor, discovered in cancer cells (*18*) has not been identified in fat cells, and the fact that fat cells have different isoforms of laminins (*19*) have also caused much controversy. Accordingly, a thorough examination of the signal element through which EGCG executes its modulation of preadipocyte apoptosis should help clarify these observations.

The mechanisms of the action of EGCG in growth, apoptosis, transformation, and signal transduction of many types of cells have been extensively reported (*3*−*7*, *16*, *17*), but several results are still not well understood. Cdk2 and caspase-3 have been found to serve as mitogenic and apoptotic signal transducers in eukaryotic cells (*20*, *21*), including 3T3-L1 preadipocytes (*22*, *23*), and they have been related to signals of EGCG in modulating the growth and death of cancer cells based on various studies (*3*−*7*, *16*, *17*, *24*). Determining whether any of them are responsible for EGCG signaling in preadipocyte apoptosis requires further studies.

The present study was designed to understand the mechanism of how EGCG acts in reducing the number of 3T3-L1 preadipocytes as they grow. Our specific aim was to investigate whether EGCG is able to induce apoptosis in preadipocytes and whether EGCG-regulated apoptosis of preadipocytes is dependent on the Cdk2 and caspase-3 pathways. The mechanistic results of this study may have possible utility in the treatment of obesity using this compound.

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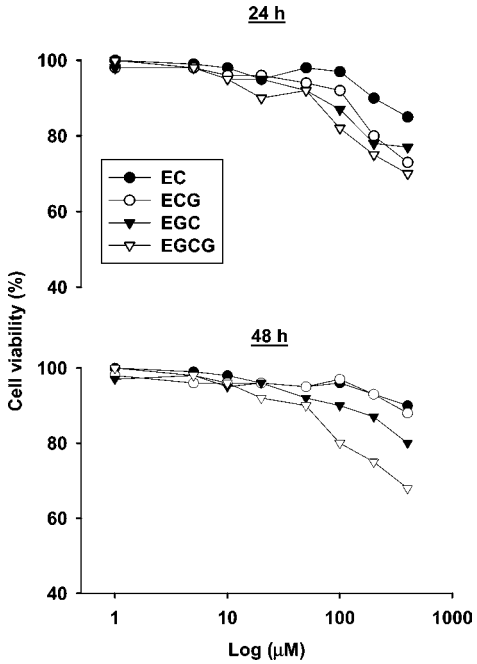

Figure 1. A dose-dependent effect of green tea catechins on cell viability of day 3 3T3-L1 preadipocytes, as examined by the trypan blue exclusion method, was observed after 24 and 48 h of treatment. Data are expressed as the mean ± SEM of triplicate determinations. EC, (–)-epicatechin; EGC, (–)-epigallocatechin; ECG, (–)-epicatechin gallate; EGCG, (–)-epigallocatechin gallate. For clarity, standard error bars and statistical significances are not shown.

MATERIALS AND METHODS

Chemical Reagents. Green tea EGCG and other catechins (>98% pure) were isolated from green tea (*Camellia sinensis*) in our laboratory as described previously (*11*). Catechins were dissolved in 0.1% DMSO and sterile medium for cell treatment. Other materials (i.e., PD98059, etc.) were purchased from Sigma (St. Louis, MO) unless otherwise mentioned. Penicillin-streptomycin, DMEM, FBS, trypsin, agarose, and a 1-kb plus DNA ladder marker were purchased from GibcoBRL of Life Technologies (New York, NY). The 3′-RACE system, Trizol, Taq polymerase, and a BenchMark prestained protein ladder were purchased from Invitrogen Life Science Technologies (Carlsbad, CA). Except for the phospho-histone H1 antibody, which was obtained from Calbiochem, Merck (Darmstadt, Germany), all other antibodies (i.e., Cdk2, anti-rabbit IgG-HRP, etc) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture. According to a published method (*12*), 3T3-L1 cells (American Type Culture Collection, Manassas, VA) were grown in DMEM (pH 7.4) containing 10% FBS, 100 units/mL of penicillin, and 100 μg/mL streptomycin (GibcoBRL) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Medium (10 mL) was replaced every 2 days. Because serum components contain the factors for facilitating 3T3-L1 differentiation from preadipocytes to adipocytes when they are confluent, these cells were subcultured before reaching confluency.

Cell Viability. 3T3-L1 cells (15 000−20 000/cm^2) were plated in triplicate wells of a 12-well plate. Determining whether a dose-dependent effect of GTCs on the cell viability of 3T3-L1 preadipocytes exists, we treated cells (days 1−6 with day 1 being the day of cell inoculum) with epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), or EGCG at various concentrations (0−400 μM) for indicated time periods. After a particular time course of incubation, cells were trypsinized and counted with a hemocytometer using the 0.4% trypan blue exclusion method.

Cdk2 Plasmid Constructs. cDNA encoding wild-type murine Cdk2 ($Cdk2^{+/+}$) and the dominant negative form of murine Cdk2 (dnCdk2; with a mutation of Asp^{145} in Cdk2 to Asn^{145}), as described by Heuvel and Harlow (*21*), was amplified by RT-PCR. Total RNA was isolated from 3T3-L1 preadipocytes with the Trizol kit, and cDNA was then synthesized from equal amounts of RNA using M-MLV RT (Invitrogen, Carlsbad, CA). The forward and reverse primers for obtaining $Cdk2^{+/+}$ cDNA were 5′-ATGGAGAACTTTCAAAAGGTG-3′ and 5′-TCAGAGCCGAAGGTGGGGGC-3′, respectively. To obtain dnCdk2 cDNA, four primers were needed to introduce a site-specific mutation by overlap exclusion (*25*), and they were 5′-ATGGAGAACTTTCAAAAGGTG-3′, 5′-TCAGAGCCGAAGGTGGGGGC-3′, 5′-GTCCAAAGTTGCCAGCTTG-3′, and 5′-CAAGCTGGCAAACTTTGGAC-3′. PCR was performed under the following conditions: an initial denaturing cycle at 94 °C for 3 min, followed by 30 cycles of amplification consisting of denaturation at 94 °C for 45 s, annealing at 53 °C for 30 s, and extension at 72 °C for 90 s. A final extension at 72 °C for 10 min was added after the last cycle. The PCR product was electrophoresed on a 1.5% (w/v) agarose gel using 40 mM Tris-acetate buffer (pH 8.0) containing 1 mM EDTA, and was visualized using 0.5 μg/mL ethidium bromide. The $Cdk2^{+/+}$ and dnCdk2 products predicted to be about 900 bp were cloned to the pTargetT vector (Promega) as described by Sambrook and Russell (*25*). Prior to their transfection into the preadipocytes, they were verified with nucleotide sequencing performed at the Institute of Biomedical Sciences, Academia Sinica, Taiwan. The molecular weights of overexpressed $Cdk2^{+/+}$ and dnCdk2 proteins were verified to be about 34 kDa by Western blot analysis, and their activities were also confirmed to catalyze the phosphorylation of the histone H1 substrate (*26*).

Cell Transfection and Overexpression Experiments. We modified the methods reported by Yang et al. (*27*) to perform our transfection experiments. 3T3-L1 preadipocytes in a 10-cm plate were stably transfected with 15 μg of the pTargetT vector constructed with the $Cdk2^{+/+}$ and dnCdk2 cDNAs. Stable clones were selected with 1 mg/mL of the antibiotic G-418 (BD Biosci Clontech Lab, Palo Alto, CA). Amounts and activities of $Cdk2^{+/+}$ and dnCdk2 proteins expressed in the stable clones were later determined by immunoblotting and immunoprecipitation, respectively. A 45-μL volume of the TransFast transfection reagent was used during the stable and transient transfections and was comprised of the synthetic cationic lipid, (+)-*N*,*N*[bis-(2-hydroxyethyl)-*N*-methyl-*N*-[2,3-di(tetradecanoyloxy)propyl] ammonium iodide, and the neutral lipid, L-dioleoyl phosphatidylethanolamine.

Unless otherwise noted, transfected cells with the vehicle, $Cdk2^{+/+}$ cDNA, and dnCdk2 cDNA were incubated with and without 20−100 μM EGCG for the indicated time periods. After incubation, the cell number of preadipocytes was examined by trypan blue dye exclusion.

DNA Fragmentation. Formation of DNA fragments was analyzed as previously described by Schwartz and Osborne (*28*). In some experiments, day 3 preadipocytes (15 000−20 000/cm^2) were incubated with 10% FBS-supplemented DMEM in the presence and absence of EC, ECG, EGC, or EGCG at 20−400 μM for 48 h of the experiment. In other experiments, transfected cells with the vehicle, $Cdk2^{+/+}$ cDNA, and dnCdk2 cDNA were treated with and without EGCG for 48 h. Harvested cells were washed twice with cold PBS-EDTA and lysed with 50 mM Tris-HCl buffer (pH 8.0, 10 mM EDTA and 0.2% Triton X-100). Their DNAs were extracted with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and then pelleted with an equal volume of 2-propanol and 1/10 volume of sodium acetate at −70 °C for 1 h. After being resuspended in 50 mM Tris-boric acid buffer (pH 8.0) containing 2 mM EDTA (TBE) and incubation at 37 °C for 30 min with 10 μL of 1 mg/mL RNase A (Sigma) to remove the RNA, they were incubated at 50 °C for 1 h with 10 μL of 20 mg/mL Proteinase K to remove the histones. After precipitation with 70% ethanol and drying, DNA pellets were resuspended in 20 μL of TBE buffer, and the concentration was determined at A_{260}. An aliquot of about 30 μg DNA mixed with 3 μL of 10× loading solution (0.05%, w/v; bromophenol blue and 10% (w/v) sucrose; BlueJuice, GibcoBRL) was electrophoresed on a 2% (w/v) agarose gel (Ultrapure, GibcoBRL). DNA fragments were separated at 100 V for 2 h at room temperature in TBE and visualized with 0.5 μg/mL ethidium bromide (Sigma).

Western Blot Analysis. Western immunoblot analysis was performed on supernatant fractions of preadipocytes as described by Kokontis et al. (*26*). An aliquot of 50 μg of supernatant protein was separated by 12% SDS-PAGE with 2× gel-loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 10% β-mercaptoethanol) and then blotted onto Immobilon-NC

transfer membranes (Millipore, Bedford, MA). The immunoblots were blocked for 1 h at room temperature with 10 mM PBS containing 0.1% Tween 20 (PBST) and 5% defatted milk. After washing with PBST, immunoblot analyses were performed. All primary antibodies (β-actin, Cdc2, Cdk2, and phospho-histone H1 antisera) were used at a dilution of 1:1000 ($\sim$0.2 μg/mL). Donkey anti-rabbit IgG, donkey anti-mouse IgG, donkey anti-goat IgG, or goat anti-guinea pig IgG conjugated with horseradish peroxidase were used as the secondary antibodies at a dilution of 1:2000 ($\sim$0.2 μg/mL). The immunoblots were visualized using the Western Lightning chemiluminescence reagent plus kit (Perkin-Elmer Life Science, Boston, MA) for 3 min followed by exposure to Fuji film for 2−3 min. Blots were quantified using the FX Pro Plus Molecular Imager (Bio-Rad Laboratories, Hercules, CA). After normalization to β-actin protein, levels of these intracellular proteins were expressed as a percent of the control, unless noted otherwise.

Cdk2 and Caspase-3 Activity Assays. After immunoprecipitation (*29*) and protein determination (*30*), Cdk2 activity was determined as modified from the method of Kokontis et al. (*26*). Assays were performed at 37 °C for 30 min in a final volume of 25 μL. The final substrate mixture per tube contained 20 mM ATP, 10 μg histone H1, 20 mM Tris-HCl buffer (pH 7.5), 4 mM $MgCl_2$, 0.8 mM EGTA, and Cdk2 immunoprecipitates. The reaction was terminated by the addition of 50 μL of SDS-PAGE sample buffer as described above. We removed a 20-μL aliquot of the solution to load onto SDS-PAGE and used anti-phospho-histone H1 as the primary antibody to immunoblot the samples as described above. Changes in the amounts of phospho-histone H1, after normalization to the immunoprecipitated Cdk2 protein, indicated alterations in Cdk2 activity.

Caspase-3 activity was essentially assayed using the colorimetric method of the CaspACETM Assay System (Promega, Madison WI). Briefly, day 3 preadipocytes were incubated for 24−48 h with 10% FBS-DMEM containing EC, ECG, EGC, or EGCG (0−400 μM) in the presence and absence of 50 μM of the caspase-3 inhibitor, Z-VAD-FMK. Harvested cell lysates were assayed for caspase-3 activity in a 96-well plate at 37 °C in a final volume of 100 μL per well. The reaction mixture contained 100 mM HEPES buffer (pH 7.5), 10% (w/v) sucrose, 0.1% (w/v) 3-[(3-cholamido-propyl)-dimethylammonio]-1 propane-sulfonate, 20% DMSO, 10 mM DTT, 0.2 mM Ac-DEVD-pNA substrate, and 100 μg enzyme protein. Blank control reactions contained no cell extract. After a 4-h incubation, A_{405} was determined in a microtiter plate reader. One unit of caspase-3 activity was expressed as 1 pmol of pNA formed per hour, and its specific activity was that unit divided by micrograms of protein.

Statistical Analysis. Data are expressed as the mean ± SEM, unless noted otherwise. Unpaired Student's *t*-test was used to examine differences between the control and EGCG-treated groups. One-way ANOVA followed by the Student−Newman−Keuls multiple range test was used to examine differences among multiple groups. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using SigmaStat (Jandel Scientific, Palo Alto, CA).

RESULTS

Effects of Green Tea Catechins on Cell Viability and Apoptosis of 3T3-L1 Preadipocytes. As observed in **Figure 1**, doses of 100−400 μM EGCG decreased the cell viability of day 3 preadipocytes by 15−30% during the 48-h treatment. The possibility that reductions in the cell viability of preadipocytes by EGCG could be due to its induction of cell apoptosis was also examined (**Figure 2**). Using day 3 preadipocytes as the test cells, we found that EC, ECG, EGC, and EGCG at doses of 100 or 400 μM all induced the apoptosis of preadipocytes, as indicated by the formation of DNA fragments. At 100 μM, EGCG had a greater effect than the other three catechins.

Effect of Green Tea Catechins on Caspase-3 Activity. With further use of an in vitro cell culture system, we examined whether green tea catechins affect the activity of caspase-3, one of the downstream enzymes responsible for formation of DNA fragments (**Figure 3A**, **B**). EC, EGC, ECG, and EGCG at doses of 100−400 μM, but not of 20 μM, all stimulated the activity of caspase-3 in day 3 and 6 preadipocytes after 24 and 48 h of treatment. Treatment with the specific inhibitor of caspase-3, Z-VAD-FMK, effectively prevented the EGC- or EGCG-induced increase in the activity of caspase-3. Interestingly, EGCG dose dependently inhibited caspase-3 activity in a cell-free system when the pure human caspase-3 enzyme expressed

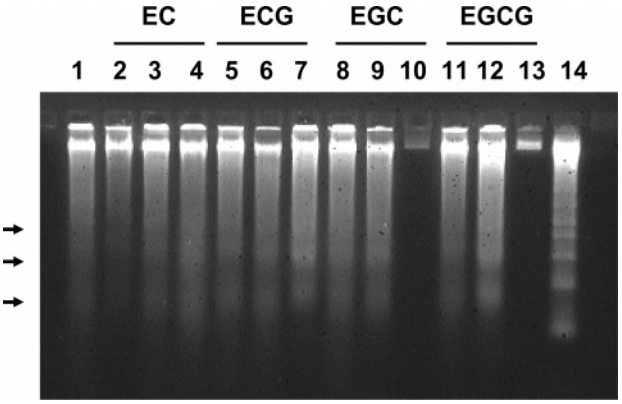

Figure 2. A dose-dependent effect of green tea catechins on formation of DNA fragments from day 3 3T3-L1 preadipocytes was observed after 48 h of treatment. An aliquot of about 30 μg DNA was loaded on 2% agarose gels. Insufficient amounts of DNA were used in lanes 10 and 13. Lanes: 1, 0 μM; 2, 5, 8, and 11, 20 μM; 3, 6, 9, and 12, 100 μM; 4, 7, 10, and 13, 400 μM; 14, marker. EC, (–)-epicatechin; EGC, (–)-epigallocatechin; ECG, (–)-epicatechin gallate; EGCG, (–)-epigallocatechin gallate.

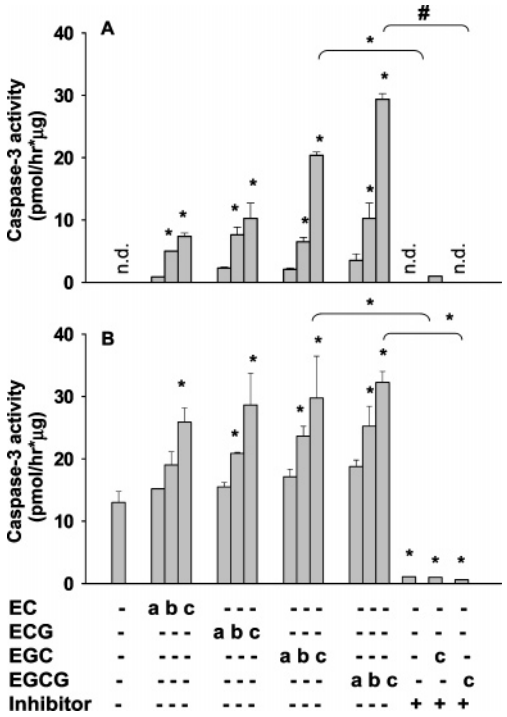

Figure 3. A dose-dependent effect of green tea catechins on increasing caspase-3 activity in the culture system of day 3 preadipocytes was observed after 24 (**A**) and 48 (**B**) h of treatment. I, caspase inhibitor Z-VAD-FMK (50 μM). Values are the mean ± SD from duplicate determinations. EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate. n.d., not detectable. *, $P < 0.05$, versus the control (without EGCG treatment) in each tea catechin; EGC versus EGC+inhibitor (bracket); or EGCG versus EGCG+inhibitor (bracket; #). The letters, such as a, b, and c, shown in each catechin were indicated as concentrations of 20, 100, and 400 μM, respectively.

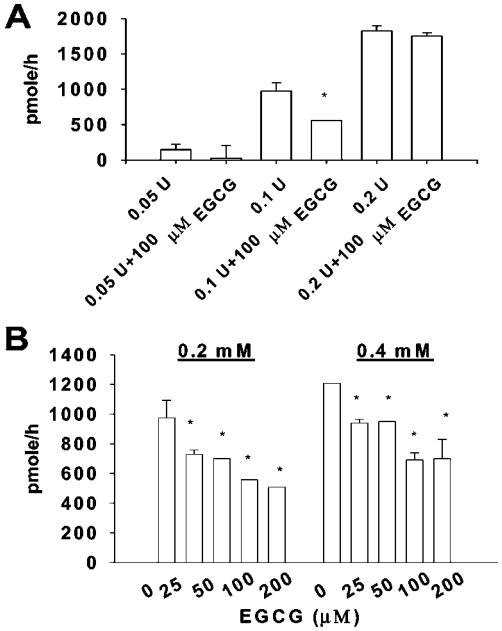

Figure 4. (–)-Epigallocatechin gallate (EGCG) reduced the activity of human caspase-3 enzyme in the cell-free system, depending on the amounts of EGCG (**A**, 100 μM; **B**, 0–200 μM), caspase-3 enzyme (**A**, 0.05–0.2 U; **B**, 0.1 U), and the Ac-DEVD-pNA substrate (**A**, 0.2 mM; **B**, 0.2–0.4 mM) added. Values are the mean ± SD from duplicate determinations. *, $P < 0.05$, versus the control (without EGCG treatment) in the given amounts of enzyme (**A**) and substrate (**B**).

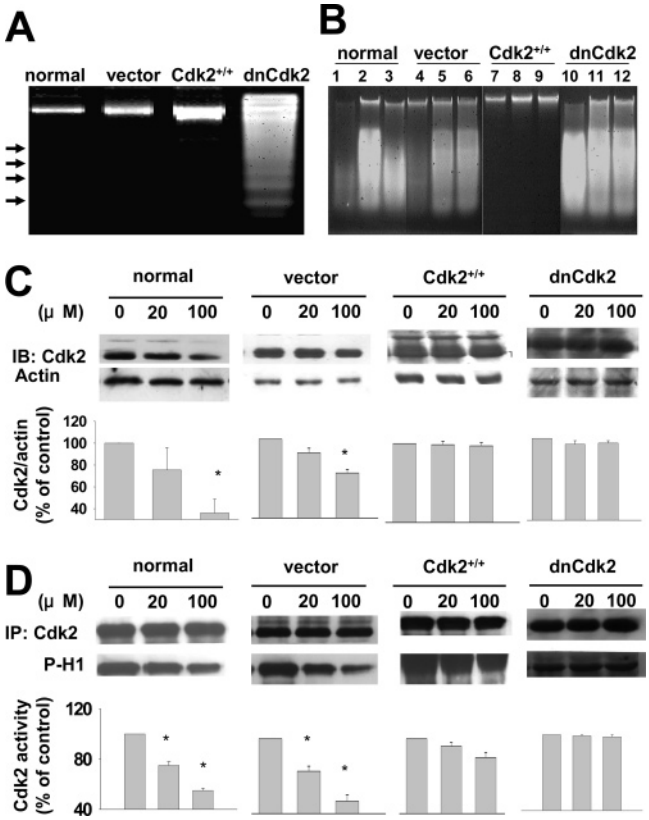

Figure 5. Overexpression of Cdk2$^{+/+}$ and dnCdk2 affected EGCG-induced formation of DNA fragments from 3T3-L1 preadipocytes. In (**A**), cells were treated without EGCG. In (**B**): lanes 1, 4, 7, and 10, 0 μM EGCG; lanes 2, 5, 8, and 11, 20 μM EGCG; lanes 3, 6, 9, and 12, 100 μM EGCG. Protein expression (**C**) and activity (**D**) of Cdk2 were confirmed by Western blot analysis (or IB, immunoblotting) and by the immunoprecipitation (IP) method, and were then expressed after normalization to actin and to Cdk2, respectively. Values are the mean ± SD from two of 10-cm plates. *, $P < 0.05$, versus the control (without EGCG treatment) in groups of cells. EGCG, epigallocatechin gallate.

from *E. coli* was used (**Figure 4A**, **B**). The IC_{50} of EGCG was about 100 μM when it was incubated with 0.1 U of enzyme and 0.2 mM of the Ac-DEVD-pNA substrate. The inhibitory effect of EGCG on human caspase-3 activity either disappeared when an increased enzyme concentration was used (**Figure 4A**) or appeared to a lesser extent when increasing concentrations of Ac-DEVD-pNA were added (**Figure 4B**).

Overexpression of Cdk2$^{+/+}$ Prevented EGCG-Induced Apoptosis in Preadipocytes. A further examination of whether overexpression of Cdk2$^{+/+}$ prevents the EGCG-induced apoptosis of preadipocytes was made by measuring the disappearance and appearance of DNA fragmentation (**Figure 5**). As indicated in **Figure 5A** and **B**, overexpression of Cdk2$^{+/+}$ did not cause any formation of DNA fragments in the absence of EGCG, but prevented EGCG-induced formation of DNA fragments. However, overexpression of dnCdk2 with a mutation of Asp^{145} in Cdk2 to Asn^{145} stimulated formation of DNA fragments after a 48-h incubation no matter the presence and absence of EGCG. There were similar findings that EGCG induction of the sub-G0/G1 phase, also known as an apoptotic index, appeared in the cell cycle of vehicle-transfected preadipocytes, but was not observed in Cdk2$^{+/+}$-overexpressing preadipocytes treated with EGCG (data not shown). The amounts of Cdk2 protein and its activity, as indicated with the amounts of phosphorylated histone H1, in Cdk2$^{+/+}$- and dnCdk2-transfected preadipocytes were respectively confirmed as indicated in **Figure 5C** and **D**.

DISCUSSION

Effects of Green Tea Catechins on Cell Viability, Apoptosis, and Caspase-3 Activity of 3T3-L1 Preadipocytes. Green tea EGCG has been proposed as an obesity chemopreventative and a fat cell modulator based on various laboratory studies (*3*). Unlike preliminary reports (*12*, *29*), which have shown that the observed decrease in the number of preadipocytes by EGCG could be attributable to its inhibition of cell mitogenesis, this study provides certain in-depth understanding of the mechanism of the action of EGCG in the regulation of apoptosis of preadipocytes. This is evident by the observations that EGCG at doses of 100−400 μM reduced the cell viability of preadipocytes by 15−30%, induced the appearance of DNA fragmentation, and increased the activity of the caspase-3 protein, an apoptotic enzyme. The resulting decreases in the cell viability may be attributable to the formation of DNA fragments. Fragmented DNA by EGCG may be caused by its increase in caspase-3 activity; because this enzyme which exhibited significant induced activity increases by 100−400 μM, but not 20 μM, EGCG is a downstream apoptotic enzyme able to activate DNase (*20*). When the inhibitor of caspase-3, Z-VAD-FMK, was used, it stopped the increase in caspase-3 activity by EGCG. Taken together, these observations suggest that activation of the caspase-3 protein may be related to the mechanism by which EGCG carries out its apoptotic effect on preadipocytes.

A contradictory observation is that EGCG inhibited the activity of human caspase-3 in an in vitro cell-free system, suggesting that the effect of EGCG in altering caspase-3 activity might be species-specific or that an increase in caspase-3 activity induced by EGCG in the cell culture system might be indirectly mediated through activation of upstream caspase-3 activators. The latter conclusion is supported by the facts that EGCG enables binding with the Fas death receptor in vitro, and then

triggers the activity of caspase-3 either by activating caspase-8 protein, an upstream activator of caspase-3 (*33*, *34*), or by inducing the release of cytochrome *c* (*35*), and that the Fas receptor is present in fat cells (*36*).

Overexpression of Cdk2$^{+/+}$ Prevented EGCG-Induced Apoptosis in Preadipocytes. Other various antiapoptotic and apoptotic factors, including Bcl2, Bad, Bax, and p53, have been reported (*20*), and some of them can be regulated by EGCG (*3*−*7*). More studies are required to determine whether any of them are responsible for the apoptotic effect of EGCG on preadipocytes. However, the induction of preadipocyte apoptosis by EGCG in this study was also dependent on Cdk2 and is likely mediated by inactivation of the Cdk2 protein. This is based on the observations that inactivation of the Cdk2 protein via transfection of dnCdk2 cDNA to 3T3-L1 preadipocytes caused the formation of DNA fragments and that overexpression of Cdk2 via the transfection of Cdk2$^{+/+}$ cDNA to 3T3-L1 preadipocytes prevented 48-h decreases in Cdk2 activity and protein levels and formation of DNA fragments by 100 μM EGCG. Because EGCG reduces MEK1 activity, as indicated with decreased amounts of phospho-Erk proteins (*29*), and because the Cdk2 activity is regulated at phosphorylation-dephosphorylation levels via the Erk pathway, the alternative possibility still remains that EGCG induces the apoptosis of preadipocytes via the inhibition of Erk MAPK activity as reported for human chondrocytes (*37*).

Unfortunately, the Cdk2 pathway required for the mechanism of apoptotic action of EGCG is still not clear in this study. However, it was evident that Cdk2 controls the G1 checkpoint in the cell cycle via regulating the formation of retinoblastoma (Rb) protein and E2F, a transcriptional factor being able to interact either with p53 or with caspase 3 to mediate apoptosis, complex (*20*). Accordingly, decreases in Cdk2 expression and activity of 3T3-L1 preadipocytes by EGCG may cause an increase in cell death via promoting the accumulation of the Rb and E2F complex and the sequent p53- or caspase-3-dependent pathway. This assumption may be indirectly supported by the fact that increases in the p21 and p27, two of p53-upregulated and caspase-3-related Cdk inhibitors, expression were shown when EGCG was added to 3T3-L1 preadipocytes (*29*). However, another possibility still remains that decreases in Cdk2 expression and activity of 3T3-L1 preadipocytes by EGCG may link to the activation of caspase-3 via changing the mitochondrial transmembrane potential and cytochrome *c* release and the caspase-9 activity (an activator of caspase-3) as reported for the apoptotic effect of the tea polyphenol theasinensin A, an EGCG dimer, on human lymphoma cells (*38*). Yet, this needs further identification.

Differences of EC, ECG, EGC, and EGCG in the Regulation of the Apoptotic Signals. Green tea catechins have numerous biological activities that can possibly provide various health benefits (*3*−*7*). In most cases, but not all, gallated catechins, especially EGCG, are more active than other catechins. This contention is supported by our findings in 3T3-L1 preadipocytes that at the same dose and duration of treatment, EGCG was generally more effective than EC, ECG, and EGC in changing the number of viable cells, activities of Cdk2 and caspase-3, and levels of Cdk2 and DNA fragments. The observed catechin-specific effects of green tea suggest that EGCG may act differently from EC, EGC, and ECG in regulating preadipocyte death. According to the nature of the unique structures of these catechins tested (*2*, *3*), EGCG contains the largest number of hydroxyl groups on its three aromatic rings among the tea catechins, and these hydroxyl groups may

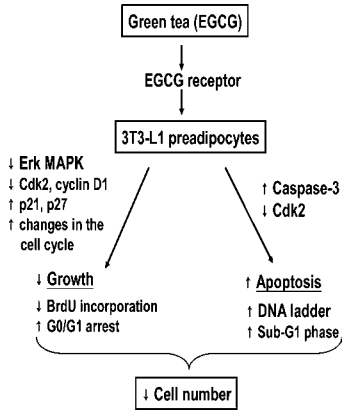

Figure 6. A proposed mechanism of green tea epigallocatechin gallate (EGCG)'s action on mitogenesis (data not shown) and apoptosis in 3T3-L1 preadipocytes. While EGCG signaling to inhibit preadipocyte proliferation was dependent on the Erk MAPKK and Cdk2 pathways (data not shown), EGCG induced apoptosis via caspase-3- and Cdk2-dependent pathways. Antimitogenic and apoptotic signals of preadipocytes induced by EGCG may be related to the mechanism by which it exerts its antiobese effect and adipocyte-regulatory activity.

be important for hydrogen bonding. Also, EGCG has both gallyl and galloyl groups, which have some conformational flexibility, that may also be important for interactions with other molecules. Further exploration of the chemical basis of the apoptotic activities on preadipocytes by EGCG is needed to understand differences in the mechanism of EGCG's action as compared to those of EC, EGC, and ECG on these processes.

To our knowledge, while green tea catechins are excellent antioxidants that act as radical scavengers and protect cell components from radical damage, these antioxidants can also be prooxidants under certain conditions (*3*). This contention is also supported by the reported dose-dependent effect of EGCG on neuroblastoma cells (*16*), in which at 50 μM of EGCG induced oxidative stress and radical formation and upregulated pro-apoptotic genes, while at 20 μM it acted as an antioxidant and upregulated anti-apoptotic genes. In a parallel to this, EGCG at 50 μM altered glucose metabolism in rat hepatoma cells via modifying the redox state of the cell. Despite the rise in reactive oxygen species, the cell viability of hepatoma was not adversely affected by 1 mM EGCG. In our study through the use of trypan blue dye exclusion method, concentrations of EGCG below 50 μM remained the cell viability of day 3 preadipocytes at 90−100% during the 48-h treatment, while doses of 100−400 μM EGCG decreased the cell viability of preadipocytes by 15−30% and induced their apoptosis. As examined the number of viable cells in proliferation or cytotoxicity assays by a CellTiter 96 Aqueous One Solution Reagent (Promega Corp., Madison) containing a tetrazolium compound, the cell viability of day 3 preadipocytes was decreased by 10%, 20%, 25%, 35%, and 50%, respectively, after 10, 20, 50, 100, and 400 μM of EGCG treatment (data not shown). Taken together, at 100−400 μM, EGCG may have some cytotoxic effects on 3T3-L1 preadipocytes. Further study is needed to determine whether EGCG acts at 100−400 μM to induce apoptosis or cytotoxicity of 3T3-L1 preadipocytes via oxidative stress or prooxidant activity as reported for hepatoma (*31*) and neuroblastoma cells (*16*, *17*, *32*).

Conclusions. We conclude that the apoptotic effect of EGCG on 3T3-L1 preadipocytes is dependent on the Cdk2 and caspase-3 pathways and is likely mediated through alterations in their activities (**Figure 6**). Decreases in Cdk2 activity by EGCG may be due to its effect on this particular member of

the CKI family (*29*). In general, EGCG is more effective than other structurally related green tea catechins in changing apoptotic signals. Changes in the endogenous signals of preadipocytes induced by EGCG may help increase our understanding of the modulatory mechanism of green tea EGCG on body weight and fat cells (*3*, *11*, *12*). Future studies on discovering the EGCG receptor in fat cells and on characterizing its oxidative stress are needed to elucidate the mechanisms of how EGCG signals reduce the Cdk2 activity and enhance the caspase-3 activity.

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