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Persimmon Leaf Extract Inhibits the ATM Activity during DNA Damage Response Induced by Doxorubicin in A549 Lung Adenocarcinoma Cells

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Persimmon leaf (PL) has been commonly recognized for its wide variety of health benefits. A previous study has reported that persimmon leaf extract (PLE) contained flavonols with the 2″-galloly moiety (PLEg). Galloylated homologues generically show stronger activity in their biological function, so enhanced functions can be expected for PLEg. We investigated in this present study the effect of PLEg on the cellular DNA damage checkpoint signaling to sensitize cancer chemotherapy. Treatment with PLE and PLEg significantly increased the cytotoxicity of doxorubicin (DOX) in A549 adenocarcinoma cells. PLE and PLEg reduced the phosphorylation of checkpoint proteins such as structural maintenance of chromosomes 1 (SMC1), checkpoint kinase 1 (Chk1), and p53 in DOX-treated cells. Moreover, PLE decreased the phosphorylation of ATM (ataxia telangiectasia mutated) in a dose-dependent manner. PLE, and especially PLEg, abrogated the G2/M checkpoint during DOX-induced DNA damage. These results suggest that PLEg specifically inhibited ATM-dependent checkpoint activation by DOX, and that PLEg might be a useful sensitizer in cancer chemotherapy.

Key words: DNA damage; checkpoint; persimmon leaf; ataxia telangiectasia mutated; galloylated flavonol glycoside

ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad-3-related) protein kinases play important roles in cellular DNA damage response.[1–3] Once the DNA damage has occurred, checkpoint effector proteins such as structural maintenance of chromosomes 1 (SMC1), checkpoint kinase 1 (Chk1), and p53 are phosphorylated and activated by ATM and/or ATR which lead to cell cycle arrest in G1, S, G2, and M phase. Since radio- or chemo-therapy activates the checkpoints, it can be expected that abrogating the DNA damage checkpoints, especially G2/M, by certain agents would sensitize DNA damage leading to the death of cancer cells. Moreover, the G2/M checkpoint, which is usually preserved even in p53-defective or mutated cancer cells, plays quite an important role in the DNA damage checkpoint system.[4] Schisandrin B has been found to specifically inhibit ATR,[5] so many attentions have been paid to finding compounds from natural sources that modulate DNA damage checkpoint activity and provide alternative method to manipulate cancer treatment.

Persimmon (*Diospyros kaki*) leaf is commonly brewed into a beverage because of its wide variety of health benefits. It is recognized that persimmon leaf have an anti-oxidant activity,[6,7] anti-tumor effects,[8,9] inhibition of angiotensin-converting enzyme,[10,11] attenuation of allergic responses[12,13] and α-amylase inhibition.[14] Kameda *et al.* have previously reported that persimmon leaf extract (PLE) contained four flavonols: kaempferol-3-*O*-glucoside, quercetin-3-*O*-glucoside and their corresponding 2″-gallates.[11] We have recently found that PLE also contained two additional 2″-gallates of kaempferol and quercetin 3-*O*-galactosides,[15] and that 2″-galloylation played a critical role in developing the strong anti-oxidative activity of flavonol glycoside in the persimmon leaf.[16] It is well known with tea catechins that the galloylated homologues showed stronger activity in their biological functions such as anti-oxidative and anti-tumor effects than other homologues without galloyl conjugation.[17–19] Therefore, it is important to elucidate the influence of galloylation in the flavonols of PLE. Several reports have revealed that galloylated flavonols had various biological activities with its specificity toward targets, such as inhibitory activities against xanthine oxidase[20] and HIV-1 integrase[21] and inducible nitric oxide synthase (iNOS) expression.[22] Further studies are required to assess their biological activities and how these activities depend on the presence or absence of a galloyl moiety.

In the present study, we investigated whether PLE has the potential to sensitize cancer chemotherapy by inhibiting ATM activity and the subsequent checkpoint signal cascade during the DNA damage response, with special attention to the ability of galloylated and non-galloylated flavonols isolated from PLE to affect the checkpoint signals.

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Abbreviations: PLE, persimmon leaf extract; PLEng, non-galloylated flavonols; PLEg, galloylated flavonols; DOX, doxorubicin; 5FU, 5-fluorouracil; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad-3-related; SMC1, structural maintenance of chromosomes 1; Chk1, checkpoint kinase 1; DAD-HPLC, diode-array detected high-performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide

Materials and Methods

Materials. β-Glucosidase from almond and β-galactosidase from *Aspergillus oryzae* were obtained from Sigma-Aldrich (St. Louis, MO, USA), and tannase (galloyl esterase) from *A. oryzae* was purchased from Kikkoman Co. (Tokyo, Japan). All other reagents were of the highest grade available. The specimen number of dry persimmon leaves (KHM080601) for this study has been recorded and stored for 10 years at Niigata University of Pharmacy and Applied Life Sciences.

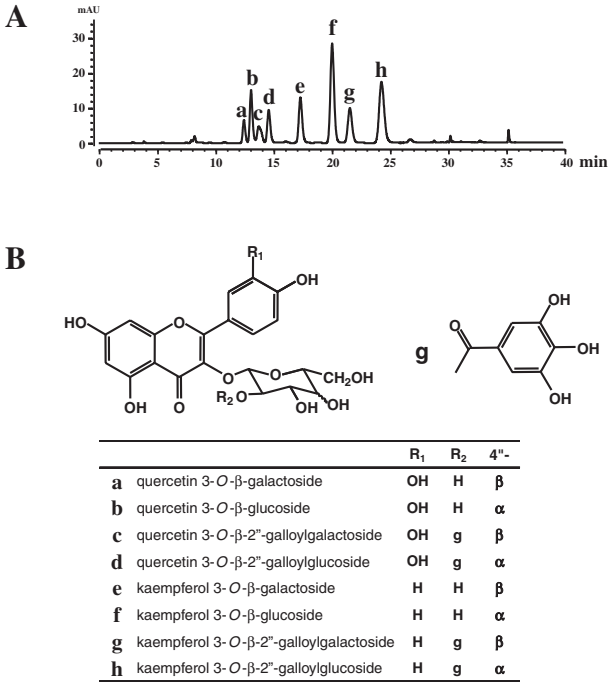
Preparation of persimmon leaf extract. Persimmon leaf extract (PLE) was prepared as described previously.[15] Persimmon leaves were collected from the Niitsu area of Niigata City in Japan. The dried persimmon leaves were treated with boiling water for 30 min, and the soluble extract was subsequently partitioned into the ethyl acetate layer which was used as PLE. Eight flavonol components of PLE consisted of four non-galloylated flavonols (PLEng: kaempferol 3-*O*-glucoside, kaempferol 3-*O*-galactoside, quercetin 3-*O*-glucoside, and quercetin 3-*O*-galactoside) and four galloylated flavonols (PLEg: their 2″-galloylated flavonol glycosides). PLEng and PLEg were successfully separated after treating PLE with tannase, β-glucosidase and β-galactosidase.

HPLC analysis. The eight flavonols in PLE were identified and quantified as described previously.[15] The extract was injected into a DAD-HPLC instrument (SPD-M20A, Shimadzu, Tokyo, Japan) equipped with an ODS column (Inertsil ODS-3, 4.6 × 250 mm; 4 μm, GL Science, Tokyo, Japan), and analyzed by using a mobile phase consisting of (A) water containing 0.5% phosphoric acid and (B) acetonitrile containing 0.5% phosphoric acid under the following gradient conditions: 0–20 min, 20% B; 20–40 min, 20–80% B; at a flow rate of 1.0 mL/min and a column temperature of 40 °C. Elution was monitored at 350 nm. Four non-galloylated flavonols (PLEng: a, b, e, and f) and four galloylated flavonols (PLEg: c, d, g, and h) were detected in PLE (Fig. 1). The respective percentages (% w/w) of PLEg and PLEng in PLE were 60% and 40%. PLEg contained 48% kaempferol 3-*O*-2″-galloylglucoside, 27% kaempferol 3-*O*-2″-galloylgalactoside, 17% quercetin 3-*O*-2″-galloylglucoside, and 7% quercetin 3-*O*-2″-galloylgalactoside. PLEng contained 45% kaempferol 3-*O*-glucoside, 28% kaempferol 3-*O*-galactoside, 18% quercetin 3-*O*-glucoside, and 9% quercetin 3-*O*-galactoside.

Cell culture. A549 human lung adenocarcinoma cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Determination of cell viability. Cell viability was determined by an MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide) assay. A549 cells were pre-incubated with or without PLE, PLEng or PLEg (0.3, 1, and 30 μg/mL) for 1 h, and the cells were respectively treated with doxorubicin (DOX, 0.01, 0.03, and 0.1 μM) and 5-fluorouracil (5FU, 2.5, 5, and 10 μM). After incubating, the cells were treated with a 0.5% MTT solution (Wako Pure Chemical Industries, Tokyo, Japan) for 4 h at 37 °C. Following incubating, the cells were solubilized in lysis buffer (50% *N*,*N*-dimethyl-formamide and 20% SDS), and the absorbance was measured at 595 nm with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblotting analysis. The phosphorylation level of each checkpoint protein (SMC1, Chk1, p53, and ATM) was determined by western blotting, respectively using phospho-specific antibodies of Ser966, Ser317, Ser15, and Ser 1981. A549 cells were cultured for 24 h before treating with PLE, PLEng or PLEg for 1 h and subsequently exposed to DOX (0.1 μM) for 16 h. After this treatment, the cells were lysed in UTB-buffer (8 M urea, 150 mM 2-mercaptoethanol, and 50 mM Tris–HCl at pH 7.5), and the protein concentrations were determined by using a Bradford protein assay kit (Bio-Rad Laboratories). The prepared proteins were separated by SDS polyacrylamide gel electrophoresis (SDS–PAGE) for the immunoblotting analysis and electrophoretically transferred to a PVDF membrane. The membrane was blocked by 2% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature. Immunoblotting was performed by using primary antibodies for phosphor-SMC1 and phospho-Chk1 (Bethyl, Montgomery, TX, USA), phospho-p53, phospho-ATM, and GAPDH (Cell Signaling Technology, Beverly, MA, USA) for 4 h at room temperature. The bound antibodies were detected with secondary peroxidase-conjugated anti-rabbit or anti-mouse IgG (Cell Signaling Technology). The target proteins were visualized by using an ECL reaction solution (Millipore Co., Bedford, MA, USA) and a chemiluminescence film (Fujifilm Co., Tokyo, Japan).



		R_1	R_2	4"-
a	quercetin 3-*O*-β-galactoside	OH	H	β
b	quercetin 3-*O*-β-glucoside	OH	H	α
c	quercetin 3-*O*-β-2"-galloylgalactoside	OH	g	β
d	quercetin 3-*O*-β-2"-galloylglucoside	OH	g	α
e	kaempferol 3-*O*-β-galactoside	H	H	β
f	kaempferol 3-*O*-β-glucoside	H	H	α
g	kaempferol 3-*O*-β-2"-galloylgalactoside	H	g	β
h	kaempferol 3-*O*-β-2"-galloylglucoside	H	g	α

Fig. 1. HPLC-UV (350 nm) Profile (A) and Chemical Structure (B) of Eight Flavonols in Persimmon Leaf Extract.

Four non-galloylated flavonols (PLEng: a, quercetin 3-*O*-galactoside; b, quercetin 3-*O*-glucoside; e, kaempferol 3-*O*-galactoside; and f, kaempferol 3-*O*-glucoside) respectively correspond to the four 2″-galloylated flavonols (PLEg: c, d, g, and h).

Cell cycle analysis. The cells were treated with PLE, PLEng or PLEg for 24 h and then the DNA content evaluated by flow cytometer (Beckman-Coulter, Fullerton, CA, USA) after propidium iodide (Dojindo, Kumamoto, Japan) staining.

G2/M checkpoint analysis. Phosphorylation of histone H3 at Ser10 was used for monitoring mitosis. A549 cells were cultured for 24 h before treating with PLE, PLEng or PLEg (30 μg/mL) for 1 h and subsequently exposed to DOX (0.1 μM) for 1 h. The A549 cells were suspended in ice-cold 70% ethanol buffered with PBS, and the membrane was rendered permeable with 0.25% Triton X-100 in PBS on ice for 15 min. The cells were then incubated with the polyclonal rabbit phospho-histone H3 (Ser10) antibody (Upstate Biotechnology, Lake Placid, NY, USA) for 4 h and the Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (Invtrogen, Carlsbad, CA, USA) for 1 h at room temperature. Cellular DNA was stained by 50 μg/mL of propidium iodide (Dojindo) for 30 min at room temperature, the stained cells then being analyzed by flow cytometer (Beckman-Coulter).

Statistics. Data are expressed as mean ± SD. A statistical analysis of the data was performed by ANOVA and a subsequent Tukey test to identify differences among groups. Differences are considered significant at $p < 0.05$.

Results

Effect of persimmon leaf extract on the viability of A549 cells after their treatment by chemotherapeutic agents

We investigated the effect of persimmon leaf extract (PLE) on cellular DNA damage response induced by

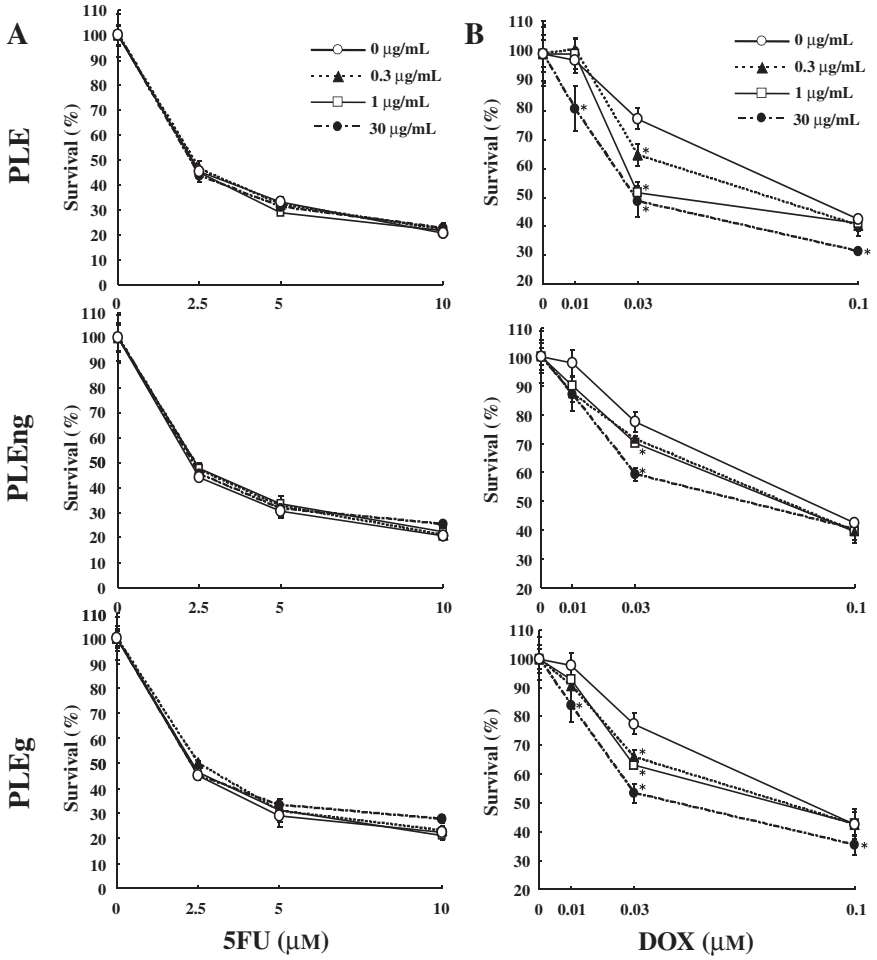


Fig. 2. Dose-Dependent Effects of 5FU (A) and DOX (B) on Cell Viability.
After pre-incubating for 1 h with or without various concentrations of PLE, PLEng or PLEg, cells were treated with different concentrations of 5FU or DOX and incubated for 72 h. Each value is presented as mean ± SD. obtained from triplicate determinations (*$p < 0.05$ *vs.* untreated control).

two anti-tumor agents, 5FU and DOX. The viability of A549 cells after 5FU or DOX treatment was determined by the MTT assay. Both chemotherapeutic agents induced cell death in A549 cells in a dose-dependent manner (Fig. 2). PLE, PLEng or PLEg did not sensitize the cytotoxicity induced by 5FU when the cells had been pretreated (Fig. 2A). In contrast, the toxicity induced by DOX was significantly sensitized by PLE and the isolated PLEng and PLEg compounds (Fig. 2B). PLE, PLEng, and PLEg themselves did not induce significant cell death or inhibit cell cycle progression (data not shown). The cells treated with 30 μg/mL PLE and 0.1 μM DOX resulted in the cell viability declining to approximately 11% compared to DOX control (Fig. 3). The sensitizing effect of PLEg on DOX-induced cell death was greater than that of PLEng.

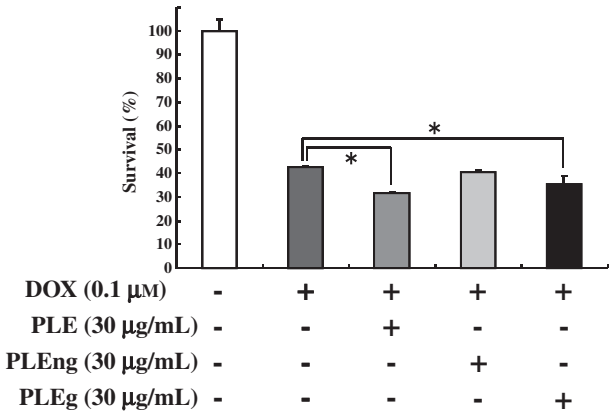


Fig. 3. Effect of Persimmon Leaf Extract on Viability of A549 Cells.
After pre-incubating for 1 h with PLE, PLEng or PLEg (30 μg/mL), cells were treated with DOX (0.1 μM) for 72 h. Each value is presented as mean ± SD obtained from triplicate determinations (*$p < 0.05$ *vs.* DOX).

Effect of persimmon leaf extract on the phosphorylation of checkpoint proteins

We further examined whether PLE affected the signal transduction of DNA damage checkpoint pathways. The DOX treatment increased the phosphorylation levels of checkpoint proteins such as SMC1, Chk1, and p53 at Ser966, Ser345, and Ser15, respectively (Fig. 4, lane 2). On the other hand, PLE pre-treatment dose-dependently prevented the DOX-induced phosphorylation of SMC1 and Chk1 (Fig. 4A). PLE at 30 μg/mL decreased the level of p53 phosphorylation though 1 and 10 μg/mL slightly increased the phosphorylation of p53. Although PLEng decreased the phosphorylation of Chk1, it increased the phosphorylation of SMC1 (Fig. 4B). The phosphorylation of checkpoint proteins in the DOX-

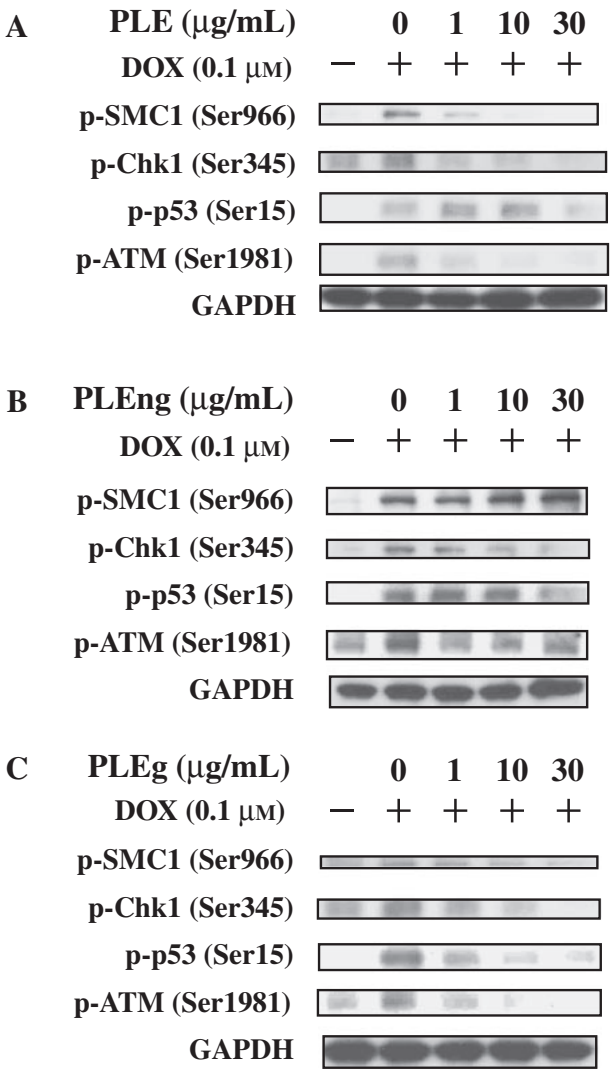


Fig. 4. Effect of Persimmon Leaf Extract (PLE, A), the Non-Galloylated Flavonols (PLEng, B), and the Galloylated Flavonols (PLEg, C) on the Phosphorylation Level of Checkpoint Kinase.

After preincubating for 1 h with the indicated concentrations of PLE, PLEng, or PLEg, A549 cells were treated with or without 0.1 μM DOX for 16 h. The total cellular protein was then subjected to western blotting, using anti-phospho-antibody. The intracellular levels of phosphorylation in each checkpoint kinase (SMC1, Chk1, p53, and ATM) were determined by western blotting, respectively using phospho-specific antibodies of Ser966, Ser345, Ser15, and Ser1981.

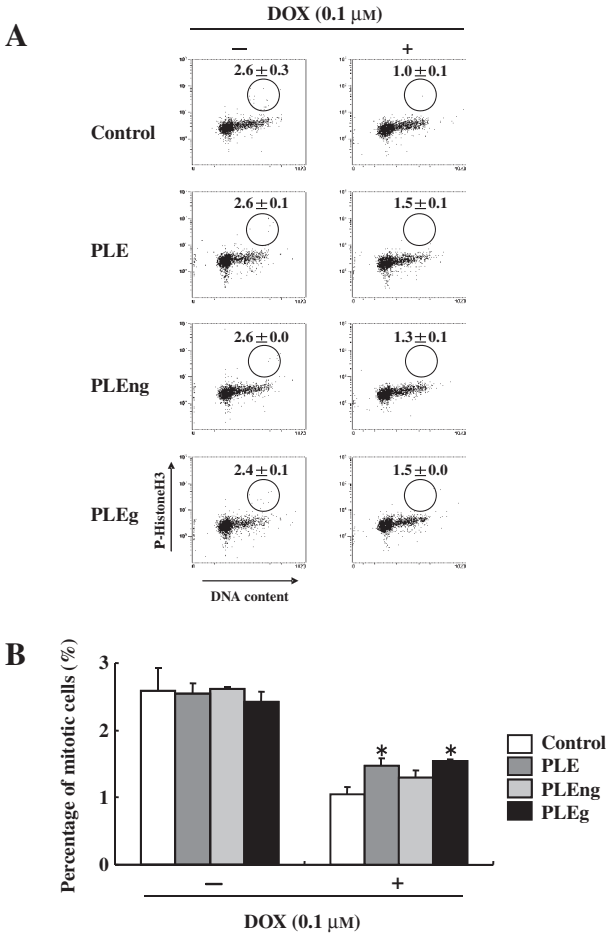


Fig. 5. Effect of Persimmon Leaf Extract on G2/M Checkpoint Activity.

A, A dot plot analysis was performed by FACS. The percentage of mitotic cells was estimated by the phosphorylated histone H3 (Ser10) positive cells. B, Data are expressed as the percentage of mitotic cells with respect to the total number of cells. Each value is presented as mean ± SD (n = 4). $^{*}p < 0.01$ *vs.* control.

treated cells was clearly decreased by PLEg (Fig. 4C). PLE- and PLEg-induced inhibition of the phosphorylation of checkpoint proteins was only apparent in the DOX-treated cells, and not in the 5FU- or hydroxyurea (HU)-treated cells (data not shown).

We further tested the effect of PLE and the isolated components, PLEg and PLEng, on the phosphorylation of ATM (Ser1981), a key molecule in DNA damage checkpoint signaling. Both PLE (Fig. 4A, fourth line) and PLEg (Fig. 4C, fourth line) dose-dependently decreased the phosphorylation of ATM at Ser1981. PLEng also decreased the phosphorylation of ATM after the DOX treatment though it was not dose-dependent (Fig. 4B, fourth line). It seemed that the dose-dependent reduction of phosphorylation in checkpoint effector proteins SMC1, Chk1, and p53 was due to the inhibitory potential of PLE and PLEg toward ATM.

Effect of persimmon leaf extract on the G2/M checkpoint

We further examined the mitotic transition by a flow-cytometric analysis to check the specificity of the inhibiting effect of PLE on cellular DNA damage response (Fig. 5A). The percentage of mitotic cells was estimated by the level of histone H3 phosphorylation at Ser10. The cells treated for 24 h with PLE, PLEng or PLEg did not change the mitotic percentage in the untreated cells, keeping cell cycle distribution unchanged by the treatment (data not shown). The percentage of phospho-histone H3-positive cells had clearly decreased after DOX treatment (Fig. 5A). However, treatment of cells with PLE plus DOX significantly increased the percentage of mitotic cells in comparison with only DOX treatment (Fig. 5B). We noted that the percentage of mitotic cells in the PLEg-pretreated cells was larger than that by PLEng pretreatment. These data indicate that PLE, especially PLEg, abrogated the G2/M checkpoint in DOX-induced DNA damage.

Discussion

Persimmon leaves have a long history of being used as a folk medicine or medicinal herb in traditional

oriental medicine because of its variety health benefits such as anti-oxidant activity,[6,7] prevention of cancer,[8,9] hypertension,[10,11] and inflammation.[12,13] It has also been reported that the leaves of *Diospyros kaki* are used as health food (persimmon leaf tea) in Japan and Korea.

The radio- and/or chemo-therapy for cancer are methods to induce severe DNA damage in tumor cells, leading to cell death or tumor growth inhibition.[23] However, the efficacy of anti-tumor therapy is occasionally attenuated by DNA damage checkpoint activation and the subsequent repair system. ATM plays a key role in checkpoint signaling and critically modulates a whole series of events in the cell cycle through the phosphorylation of effector proteins.[1,2] It is thus recognized that regulating ATM activity is vital for sensitizing cancer therapy.

We have demonstrated that DOX-induced cytotoxicity was significantly amplified by PLE treatment (Fig. 2B), but it was not apparent with 5FU- induced cell damage (Fig. 2A). Since PLE itself did not have any effect on cell cycle distribution, including apoptosis induction (data not shown), these results suggest that PLE had the potential to enhance DOX-induced DNA damage. In another word, PLE, PLEng, and PLEg could have the potential to sensitize tumor cell death by regulating the DNA damage checkpoint activity, although the cytotoxic effect was more obvious in PLE-treated cells than in those treated by PLEng or PLEg. We therefore examined how PLE, PLEng, and PLEg regulated cellular DNA damage checkpoint signaling. PLEg dose-dependently inhibited phosphorylation of checkpoint effector proteins such as Chk1, SMC1, and p53 (Fig. 4). In contrast, PLEng sensitized the cytotoxicity induced by DOX, although PLEng pretreatment increased the phosphorylation level of SMC1. As shown in Fig. 1, PLE contains eight flavonols consisting of two groups of flavonol glycosides, PLEg and PLEng. PLEg is the 2″-galloylated homologue of flavonol glycoside (PLEng).[15] It has also been reported that catechins with a galloyl moiety, epigallocatechin gallate (EGCg), had stronger anti-tumor activity than non-galloyl catechins.[17–19] PLEng might inhibit not only the DNA damage checkpoint activity but also other types of intracellular signaling such as phosphoinositide 3-kinases (PI3Ks), leading to cell viability; for example, caffeine as a global inhibitor of PI3Ks.[24] The cytotoxicity observed in PLE was possibly combined with the effects of PLEng and PLEg. It is also thought that the inhibition of DNA damage checkpoint signaling by PLEg was more specific than by PLEng due to the galloyl moiety.

On the other hand, PLE, PLEng, and PLEg did not inhibit the phosphorylation of checkpoint effector proteins when activated by HU or 5FU (data not shown). DOX mainly activates ATM through the induction of double-strand break in cellular DNA,[1,25,26] whereas HU or 5FU preferentially activates ATR that is sensitive to single-strand break.[27–30] Therefore we further examined whether PLE could inhibit the phosphorylation of ATM. As expected, ATM phosphorylation was dose-dependently inhibited in the cells pre-incubated with PLE (Fig. 4A).

It has also been reported that DOX induced the generation of intracellular reactive oxygen species (ROS) followed by ATM activation.[25] PLEg might have some scavenging activity toward DOX-treated cells in terms of ROS elimination.[15] However, the concentration of PLEg required for ROS elimination was comparatively higher (mg/mL order) than that for ATM inhibition (μg/mL order). Our results suggest that μg/mL of PLEg did not contribute to ROS scavenging in this case.

It is well recognized that the mutation or deletion of p53 and lack of G1/S checkpoint frequently occur in the most cancer cells.[31] The G2/M checkpoint is therefore an important target for cancer therapy.[32–35] Thus we further studied whether PLE influenced the G2/M checkpoint. PLE, and particularly PLEg, abolished the G2/M checkpoint after DOX treatment (Fig. 5B). It was confirmed that PLE inhibited the checkpoint activity not only of G1/S and S phase but also of G2/M in the cell cycle. These observations indicate that PLEg abrogated the cellular DNA damage checkpoint activated by DOX through the inhibition of the ATM kinase activity.

In conclusion, PLE inhibited ATM-dependent checkpoint activity in DNA damage response, and it seemed that this inhibitory activity was mainly due to the function of galloylated flavonol glycosides (PLEg). Further study will be required for the clinical application of PLEg as a sensitizing agent in anti-cancer therapy, because in the effect of combining PLE and chemotherapy on normal cells demonstrating regular DNA damage responses such as G1 and S-phase checkpoints may not be ignorable. A carefully considered protocol should be constructed before any clinical use.

Acknowledgments

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References

1) Shiloh Y, *Curr. Opin. Genet. Dev.*, **11**, 71–77 (2001).
2) Abraham RT, *Genes Dev.*, **15**, 2177–2196 (2001).
3) Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER 3rd, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, Shiloh Y, Gygi SP, and Elledge SJ, *Science*, **316**, 1160–1166 (2007).
4) Powell SN, DeFrank JS, Connell P, Eogan M, Preffer F, Dombkowski D, Tang W, and Friend S, *Cancer Res.*, **55**, 1643–1648 (1995).
5) Nishida H, Tatewaki N, Nakajima Y, Magara T, Ko KM, Hamamori Y, and Konishi T, *Nucleic Acids Res.*, **37**, 5678–5689 (2009).
6) Sakanaka S, Tachibana Y, and Okada Y, *Food Chem.*, **89**, 569–575 (2005).
7) Bei W, Peng W, Ma Y, and Xu A, *Life Sci.*, **76**, 1975–1988 (2005).
8) Khanal P, Oh WK, Thuong PT, Cho SD, and Choi HS, *Planta Med.*, **76**, 689–693 (2010).
9) Chen G, Xue J, Xu SX, and Zhang RQ, *J. Asian Nat. Prod. Res.*, **9**, 347–353 (2007).
10) Funayama S and Hikino H, *Chem. Pharm. Bull.* (Tokyo), **27**, 2865–2868 (1979).
11) Kameda K, Takaku T, Okuda H, Kimura Y, Okuda T, Hatano T, Agata I, and Arichi S, *J. Nat. Prod.*, **50**, 680–683 (1987).
12) Matsumoto M, Kotani M, Fujita A, Higa S, Kishimoto T, Suemura M, and Tanaka T, *Br. J. Dermatol.*, **146**, 221–227 (2002).

13) Kotani M, Matsumoto M, Fujita A, Higa S, Wang W, Suemura M, Kishimoto T, and Tanaka T, *J. Allergy Clin. Immunol.*, **106**, 159–166 (2000).
14) Kawakami K, Aketa S, Nakanami M, Iizuka S, and Hirayama M, *Biosci. Biotechnol. Biochem.*, **74**, 1380–1385 (2010).
15) Kawakami K, Shibukura Y, Kanno T, Furuki T, Aketa S, and Hirayama M, *Phytochem. Anal.*, in press.
16) Masuda T, Iritani K, Yonemori S, Oyama Y, and Takeda Y, *Biosci. Biotechnol. Biochem.*, **65**, 1302–1309 (2001).
17) Yamauchi R, Sasaki K, and Yoshida K, *Toxicol. In Vitro*, **23**, 834–839 (2009).
18) Ravindranath MH, Ramasamy V, Moon S, Ruiz C, and Muthugounder S, *Evid. Based Complement. Alternat. Med.*, **6**, 523–530 (2009).
19) Kawai Y, Matsui Y, Kondo H, Morinaga H, Uchida K, Miyoshi N, Nakamura Y, and Osawa T, *Chem. Res. Toxicol.*, **21**, 1407–1414 (2008).
20) Hatano T, Yasuhara T, Yoshihara R, Ikegami Y, Matsuda M, Yazaki K, Agata I, Nishibe S, Noro T, Yoshizaki M, and Okuda T, *Planta Med.*, **57**, 83–84 (1991).
21) Ahn MJ, Kim CY, Lee JS, Kim TG, Kim SH, Lee CK, Lee BB, Shin CG, Huh H, and Kim J, *Planta Med.*, **68**, 457–459 (2002).
22) Jo HY, Kim Y, Nam SY, Lee BJ, Kim YB, Yun YW, and Ahn B, *J. Vet. Sci.*, **9**, 267–272 (2008).
23) Collis SJ, Swartz MJ, Nelson WG, and DeWeese TL, *Cancer Res.*, **63**, 1550–1554 (2003).
24) Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM, and Abraham RT, *Cancer Res.*, **59**, 4375–4382 (1999).
25) Kurz EU, Douglas P, and Lees-Miller SP, *J. Biol. Chem.*, **279**, 53272–53281 (2004).
26) Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y, and Ziv Y, *Science*, **281**, 1674–1677 (1998).
27) Zhou BB and Bartek J, *Nat. Rev. Cancer*, **4**, 216–225 (2004).
28) Liu L, Choi JH, Yim H, Choi JS, Park BD, Cho SJ, and Lee SK, *Int. J. Biochem. Cell Biol.*, **41**, 1410–1420 (2009).
29) Jardim MJ, Wang Q, Furumai R, Wakeman T, Goodman BK, and Wang XF, *Mol. Biol. Cell*, **20**, 3801–3809 (2009).
30) Ward IM and Chen J, *J. Biol. Chem.*, **276**, 47759–47762 (2001).
31) Levine AJ, *Cell*, **88**, 323–331 (1997).
32) Leach SD, Scatena CD, Keefer CJ, Goodman HA, Song SY, Yang L, and Pietenpol JA, *Cancer Res.*, **58**, 3231–3236 (1998).
33) Kawabe T, *Mol. Cancer Ther.*, **3**, 513–519 (2004).
34) Liu DP, Song H, and Xu Y, *Oncogene*, **29**, 949–956 (2010).
35) Wang Y, Li J, Booher RN, Kraker A, Lawrence T, Leopold WR, and Sun Y, *Cancer Res.*, **61**, 8211–8217 (2001).