Free Rad. Res., Vol. 33, pp. 623-633
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Distinct Mechanisms of DNA Damage in Apoptosis Induced by Quercetin and Luteolin

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Accepted for publication by Prof. B. Halliwell

(Received 24 February 2000; In revised form 27 March 2000)

Quercetin has been reported to have carcinogenic effects. However, both quercetin and luteolin have anti-cancer activity. To clarify the mechanism underlying the carcinogenic effects of quercetin, we compared DNA damage occurring during apoptosis induced by quercetin with that occuring during apoptosis induced by luteolin. Both quercetin and luteolin similarly induced DNA cleavage with subsequent DNA ladder formation, characteristics of apoptosis, in HL-60 cells. In HP 100 cells, an H_2O_2-resistant clone of HL-60 cells, the extent of DNA cleavage and DNA ladder formation induced by quercetin was less than that in HL-60 cells, whereas differences between the two cell types were minimal after treatment with luteolin. In addition, quercetin increased the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), an indicator of oxidative DNA damage, in HL-60 cells but not in HP 100 cells. Luteolin did not increase 8-oxodG formation, but inhibited topoisomerase II (topo II) activity of nuclear extract more strongly than quercetin and cleaved DNA by forming a luteolin-topo II-DNA ternary complex. These results suggest that quercetin induces H_2O_2-mediated DNA damage, resulting in apoptosis or mutations, whereas luteolin induces apoptosis via topo II-mediated DNA cleavage. The H_2O_2-mediated DNA damage may be related to the carcinogenic effects of quercetin.

Keywords: DNA damage; apoptosis; hydrogen peroxide; flavonoid; carcinogenesis; topoisomerase II

Abbreviations: ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine (also known as 8-hydroxy-2′-deoxyguanosine); topo II, topoisomerase II; HPLC-ECD, an electrochemical detector coupled to a high-pressure liquid chromatograph; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline

INTRODUCTION

Flavonoids, particularly flavonols and flavones, which are commonly found in many vegetables, may act as chemopreventive agents. Quercetin is the most widely distributed flavonol, and luteolin is one of the most widely distributed flavones in the plant kingdom. Recently, many studies have reported anti-cancer effects of these flavonoids[1], including apoptosis-induction activity [2–4], anti-proliferative effects on tumor cells[5–8], and protective effects against oxidative stress and chemical carcinogenesis[9–13].

DNA damage is one of the most potent inducers of apoptosis, which may be an effective anti-cancer strategy[14–16]. However, DNA damage is also closely related to carcinogenesis. Quercetin has been reported to be carcinoge-

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nic[17–19]. Considerable evidence suggests that quercetin has DNA-damaging ability in cultured cells[20,21]. Quercetin also has pro-oxidant activity and induces oxidative DNA damage[20–25], which can result in carcinogenesis[26]. We previously showed that quercetin induced DNA damage by pro-oxidative effects, but luteolin did not, in a simplified, in vitro model[24].

Some flavonoids, including quercetin and luteolin, are topoisomerase II (topo II) inhibitors and act as anti-tumor agents[27–29]. Topo II inhibitors can induce apoptosis by means of DNA-replication-associated damage involving formation of a stable drug-topo II-DNA ternary complex, called a cleavable complex[30,31].

Because quercetin has both anti-cancer and carcinogenic activities, it is important to evaluate the efficacy and safety of flavonoids when assessing their potential as chemopreventive agents. The properties of flavonoids include anti- and pro-oxidative effects, apoptosis induction, and topo II inhibition[1–4,20–24,27–29,32,33]. The relation among these properties remains largely unclear. To study the carcinogenic mechanism of quercetin, we compared DNA damage occurring during apoptosis induced by quercetin with that occurring during apoptosis induced by luteolin. Specifically, DNA cleavage, DNA ladder formation, and 8-oxodG formation were investigated in a human leukemia cell line, HL-60 cells, and its H_2O_2-resistant clone, HP 100 cells. Moreover, inhibition of topo II activity and formation of a cleavable complex were examined using nuclear extract topo II from HL-60 cells and purified human topo II.

MATERIALS AND METHODS

Materials

Quercetin was obtained from Tokyo Kasei, Co. (Tokyo, Japan), and luteolin was obtained from ExtrasyntheseS.A. (Genay, France). Nuclease P_1 was from Yamasa Shoyu Co. (Chiba, Japan). Proteinase K was from Merck (Darmstadt, Germany). RNase A and bacterial alkaline phosphatase were from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640 was from Nikken (Kyoto, Japan). FBS was from ICN Biochemicals, Inc. (Aurora, OH, USA). ABI lysis buffer was from Applied Biosystems (Foster City, CA, USA). Topo II assay kit were purchased from TopoGEN Inc. (Columbus, OH, USA).

Cell Lines and Cell Treatment

Human myelogenous leukemic cell lines HL-60 and HP 100 were grown in RPMI 1640 supplemented with 6% fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere. HP 100 cells were derived from HL-60 cells by repeated exposure to H_2O_2, followed by outgrowth of viable cells, and were approximately 340-fold more resistant to H_2O_2 than HL-60 cells. The catalase activity of HP 100 cells is 18 times higher than that of HL-60 cells[34]. Exponentially growing cells were used and cell viability in trypan blue exclusion was over 90 % in all experiments. Flavonoids were dissolved in 1% DMSO. One percent DMSO served as control.

Detection of Cellular DNA Cleavage

HL-60 cells ($2x10^6$ cells) and HP 100 cells ($2x10^6$ cells) were both incubated in 2 ml of RPMI 1640 supplemented with 6% FBS at 37°C for 3 to 7 h with 30 and 100 μM flavonoids. The control was incubated for 7 h. After incubation, the cell suspension (2 ml) was divided into two aliquots, and used for detection of cellular DNA damage and DNA ladder formation. To determine DNA cleavage, the treated cells ($1x10^6$ cells) were washed twice with cold PBS(-) and resuspended in 50 μl PBS(-). The cell suspension was solidified with agarose, followed by treatment with proteinase K as described previously[35].

Electrophoresis was performed with a CHEF-DRII pulsed field electrophoresis system (Bio-Rad) at 200 V and 14°C. The switch time was 60 s for 15 h followed by 90 s for 9 h. The DNA in the gel was visualized with ethidium bromide.

Detection of Apoptosis

Apoptotic cells were determined by fluorescence microscopy after staining with acridine orange. DNA ladder formation, which is associated with apoptosis, was analyzed as described previously[33].

Analysis of 8-oxodG Formation in Cultured Cells

For analysis of 8-oxodG formation, HL-60 cells ($1x10^7$ cells) and HP 100 cells ($1x10^7$ cells) were both incubated with 30 and 100 μM flavonoids in 10 ml of RPMI 1640 supplemented with 6% FBS at 37°C for 4 h. After incubation, the medium was removed, and the cells were washed twice with cold PBS(-). The cells were then suspended in 0.05 mg/ml RNase, 0.5 mg/ml proteinase K, and 500 μl of ABI lysis buffer, followed by incubation at 60°C for 1 h. After ethanol precipitation, DNA was digested to nucleosides with 8 U nuclease P_1 and 1.2 U bacterial alkaline phosphatase. The amount of 8-oxodG was determined as described previously[36].

Fluorescence Measurement of Quercetin

Cellular accumulation of quercetin in HL-60 and HP 100 cells was determined by a modification of a reported method[2]. Uptake of quercetin was carried out by incubating cells with 100 μM quercetin in Hank's balanced salt solution at 37°C for 1 h. Cells were rinsed with PBS(-) and suspended in PBS(-). Fluorescence of cell suspension was measured at 345 nm for excitation and 540 nm for emission using fluorescence spectrometer (RF5300. Shimazu Co. Japan).

Preparation of Crude Nuclear Extract

HL-60 cells ($1x10^7$ cells) were used to prepare nuclear extract according to a modified method[28]. Briefly, cells were suspended in 5 ml of TEMP buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 4 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride) and centrifuged. Cells were resuspended in 1 ml of TEMP buffer, and the cell suspension was frozen in liquid N_2 and thawed at 37°C. The freeze-thaw procedure was repeated three times. Nuclei were centrifuged, and nuclear pellets were resuspended in a small volume (no more than 4 pellet volumes) of TEP (same as TEMP but lacking $MgCl_2$), and an equal volume of 1 M NaCl was added. Salt extraction of the isolated nuclei was performed by gentle rotations for 1 h. The suspension was centrifuged; the supernatant contained topo II activity. All procedures were performed at 4°C.

Assessment of Topo II Catalytic Activity

The standard reaction mixture contained 50 mM Tris-HCl (pH 8), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, and 30 μg/ml bovine serum albumin. Decatenation of kinetoplast DNA (kDNA) was carried out by incubation of 0.25 μg of kDNA and flavonoids with 1 μl of 10% nuclear extract dilution in a final volume of 20 μl at 37°C for 40 min. Reaction was terminated by sodium *N*-laurylsarcosinate, and electrophoresis was then carried out in a 1% agarose gel containing 0.5 mg/l of ethidium bromide at 100 V for 75 min.

Determination of DNA Cleavage by Cleavable Complex Formation

Topo II-mediated DNA cleavage activity was assayed by following the generation of nicked

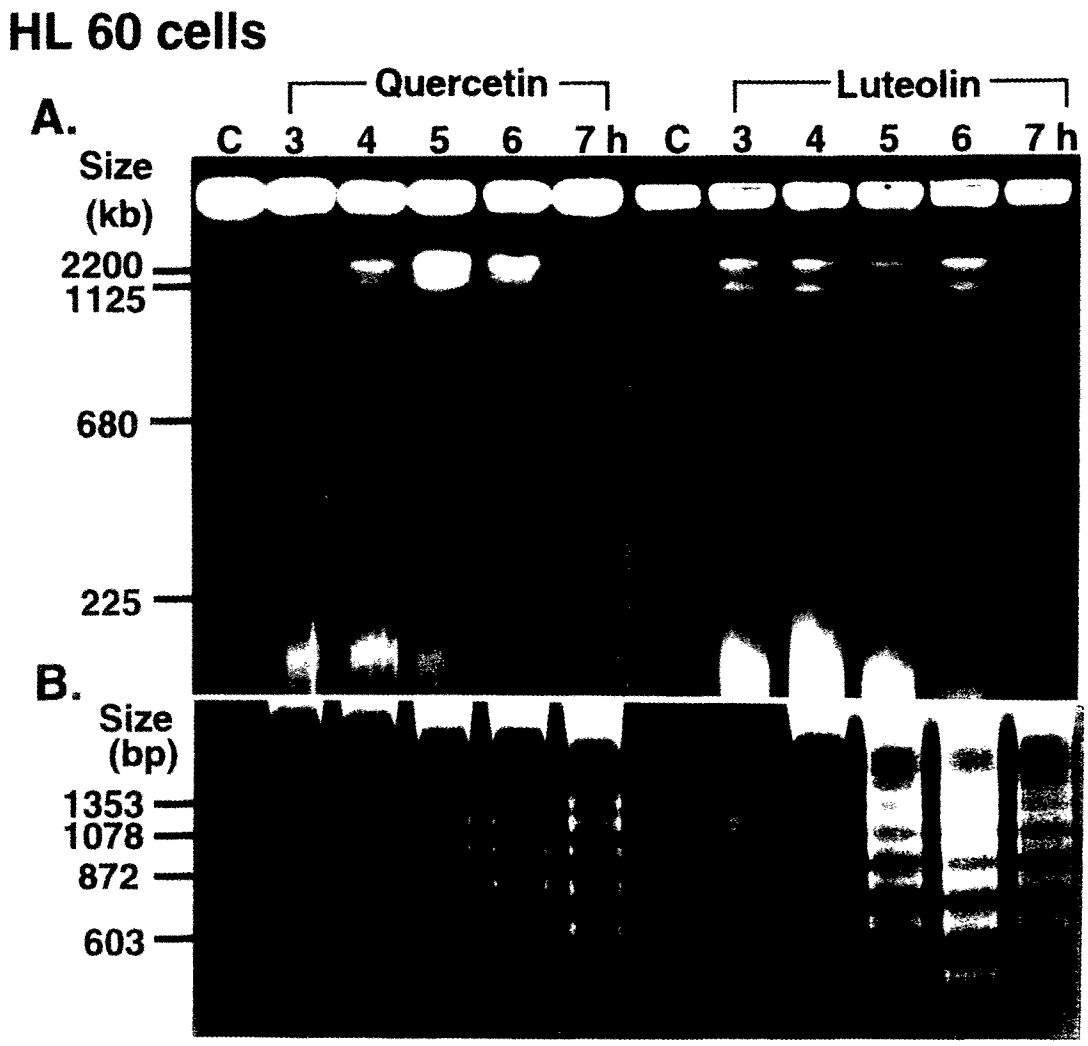

FIGURE 1 Detection of cellular DNA cleavage and DNA ladder formation in HL-60 cells treated with quercetin and luteolin. HL-60 cells were treated with 30 μM flavonoids at 37°C for the indicated times. (A): The cells were prepared as agarose plugs, lysed, and subjected to pulsed field gel electrophoresis as described in Materials and Methods. The gel was stained in ethidium bromide. (B): The cells were lysed, and DNA was extracted and analyzed by conventional electrophoresis as described in Materials and Methods

and linear DNA from supercoiled pBR322 DNA[28]. Supercoiled pBR322 DNA (0.3 μg) and luteolin (3 to 300 μM) in 20 μl of a standard reaction mixture containing 1% DMSO were incubated with 1 μl of crude nuclear extract including topo II at 37°C for 30 min. The reaction was terminated by addition of a mixture of sodium dodecyl sulfate (SDS) and proteinase K. After further incubation for 30 min at 50°C, 5 μl of loading buffer was added, and electrophoresis was then carried out in a 0.7% agarose gel containing 0.5 mg/l of ethidium bromide at 100 V for 1 h.

RESULTS

Flavonoid-Induced Cellular DNA Cleavage and DNA Ladder Formation

Figure 1 shows the time-course of DNA strand cleavage and DNA ladder formation in HL-60 cells treated with 30 μM flavonoids. Quercetin and luteolin induced DNA strand breakage to produce 1 to 2 Mb and less than 200 kb DNA fragments at 3 to 6 h (Fig. 1A). No fragments were obtained within 2 h (data not shown). The DNA fragments induced by both flavonoids were less obvious at 7 h. DNA ladder formation, a characteristic of apoptosis, appeared at 4 to 7 h in quercetin- and luteolin-treated HL-60 cells (Fig. 1B). Fluorescence microscopic examination revealed that apoptotic cells, identified by chromatin condensation and nuclear fragmentation, were induced in HL-60 cells treated with flavonoids. After treatment with 100 μM flavonoids, similar results were obtained, and quercetin-induced DNA cleavage slightly increased (data not shown).

Figure 2 shows the time-course of DNA cleavage and DNA ladder formation in HP 100 cells, an H_2O_2-resistant clone of HL-60, after treatment with 30 μM flavonoids. Quercetin slightly induced DNA cleavage and DNA ladder formation, but the extent was obviously less than that in HL-60 cells. Densitometric quantitation using a laser densitometer showed that the extent of DNA cleavage in HP 100 cells decreased to approximately 35% of that in HL 60 cells, quantitating the extent of DNA cleavage as a total of the heights of the absorbance peaks in 1–2 Mb DNA fragments. Apoptotic cells were observed less frequently in quercetin-treated HP 100 cells than HL-60 cells by fluorescence microscopy. Luteolin induced DNA cleavage and DNA ladder formation, but the extent was not decreased in HP 100 cells, unlike quercetin. When 100 μM flavonoids were used instead of 30 μM, similar results were obtained (data not shown).

No significant difference was observed in cellular accumulation of quercetin between HL-60 and HP 100 cells using fluorescence spectrometry. This result indicated that the difference of quercetin-induced DNA damage between the two cell types was not due to the extent of the cellular accumulation.

Effects of Flavonoids on 8-oxodG Formation in Cultured Cells

The amount of 8-oxodG was measured in HL-60 cells and HP 100 cells (Fig. 3). Quercetin increased 8-oxodG formation, but luteolin did not in HL-60 cells. The formation of 8-oxodG after treatment with quercetin increased in HL-60 cells, but not in HP 100 cells. These results suggest that quercetin can cause oxidative DNA damage and that H_2O_2 plays a critical role in such damage.

Inhibitory Effects of Flavonoids on Topoisomerase II Activity

The effects of flavonoids on topo II catalytic activity of crude nuclear extract were assayed by following the decatenation of kDNA. Figure 4 shows that the decatenation activity of crude nuclear extract was slightly inhibited by 30 μM quercetin, but completely inhibited by 30 μM luteolin, suggesting that the inhibitory effect of luteolin on topo II activity was stronger than that of quercetin.

DNA Cleavage by Cleavable Complex Formation

Topo II inhibitors interfere with the breakage-rejoining step of the DNA strand-passing reaction by forming a stable drug-topo II-DNA ternary complex called cleavable complex[28,30]. Treatment of this cleavable complex with SDS and proteinase K results in the formation of both single and double DNA strand breaks[28]. To test

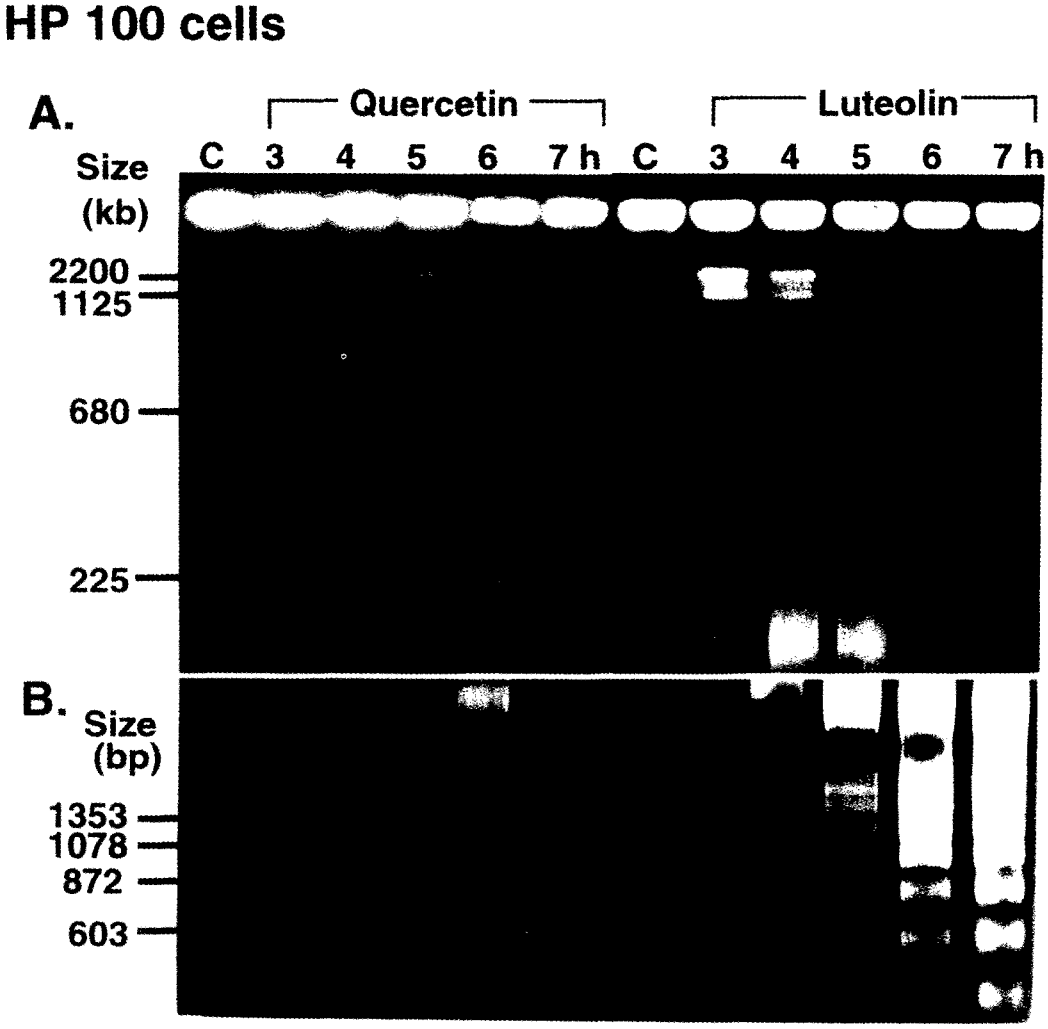

FIGURE 2 Detection of cellular DNA cleavage and DNA ladder formation in HP 100 cells treated with quercetin and luteolin. HP 100 cells were treated with 30 μM flavonoids at 37°C for the indicated times. (A): The cells were prepared as agarose plugs, lysed, and subjected to pulsed field gel electrophoresis as described in Materials and Methods. The gel was stained in ethidium bromide. (B): The cells were lysed, and DNA was extracted and analyzed by conventional electrophoresis as described in Materials and Methods

whether luteolin can induce DNA strand breaks, supercoiled pBR322 DNA was used as a substrate with crude nuclear extract from HL-60 cells. Owing to ethidium bromide intercalation, the covalent circular DNA relaxed by topo II became much more positively supercoiled (more compact) than the original negatively supercoiled DNA; consequently, the relaxed DNA migrated faster than the original DNA. Figure 5 shows that luteolin decreased the conversion of original DNA to relaxed DNA by inhibiting topo II activity, and induced the generation of linear and nicked DNA at concentrations of 10 μM or higher. Inhibition of topo II activity was closely

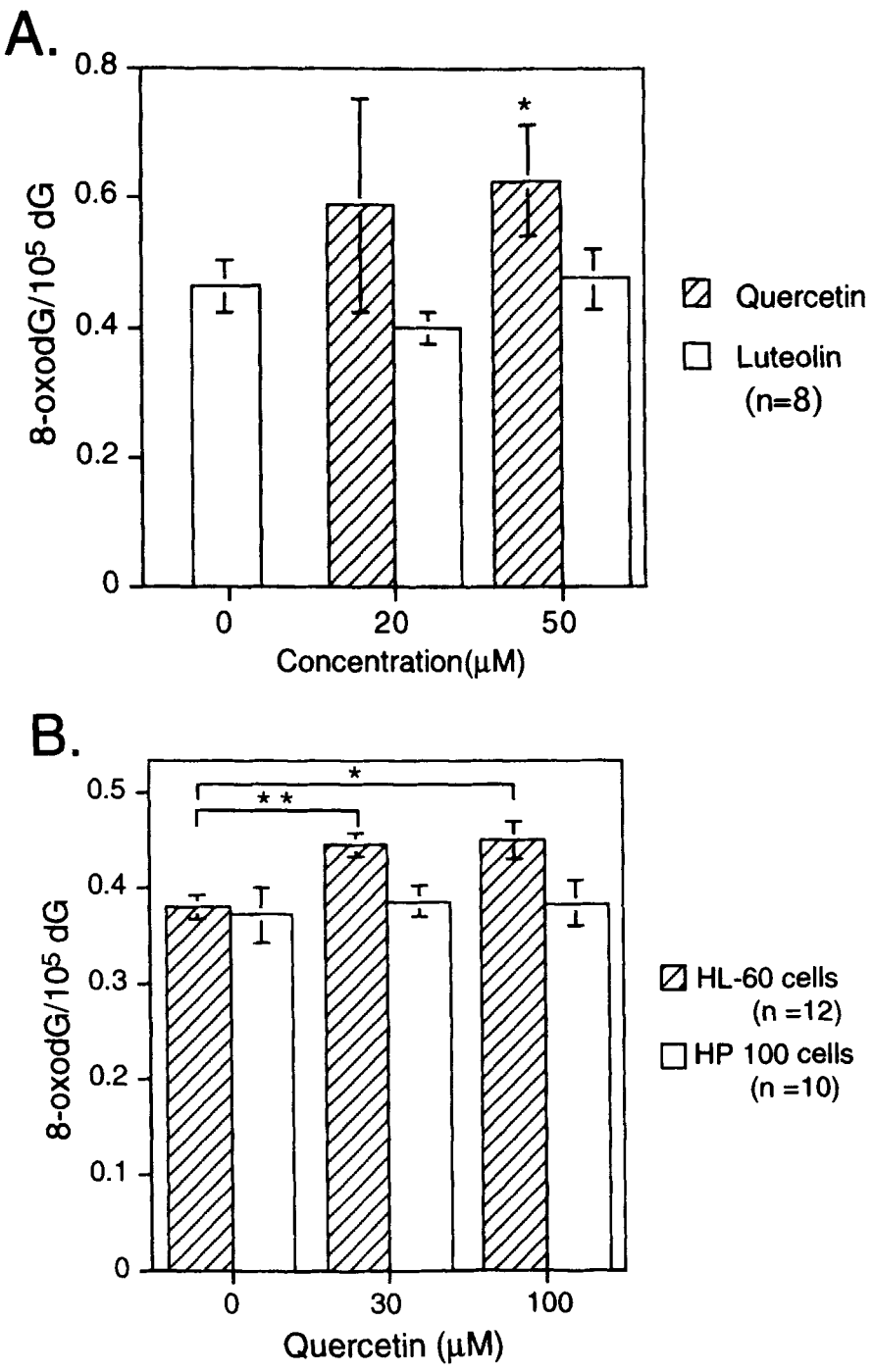

FIGURE 3 Effects of flavonoids on 8-oxodG formation in HL-60 and HP 100 cells. (A) HL-60 cells were incubated with the indicated concentrations of flavonoids for 4 h. (B) HL-60 and HP 100 cells were incubated with the indicated concentrations of quercetin for 4 h. The extracted DNA was subjected to enzyme digestion and analyzed as described in Materials and Methods. Results are expressed as means ± SE of values obtained from 4, 5 and 6 independent experiments. Asterisks indicate significant differences as compared with control by t-test ($^{*}P<0.05$, $^{**}P<0.01$)

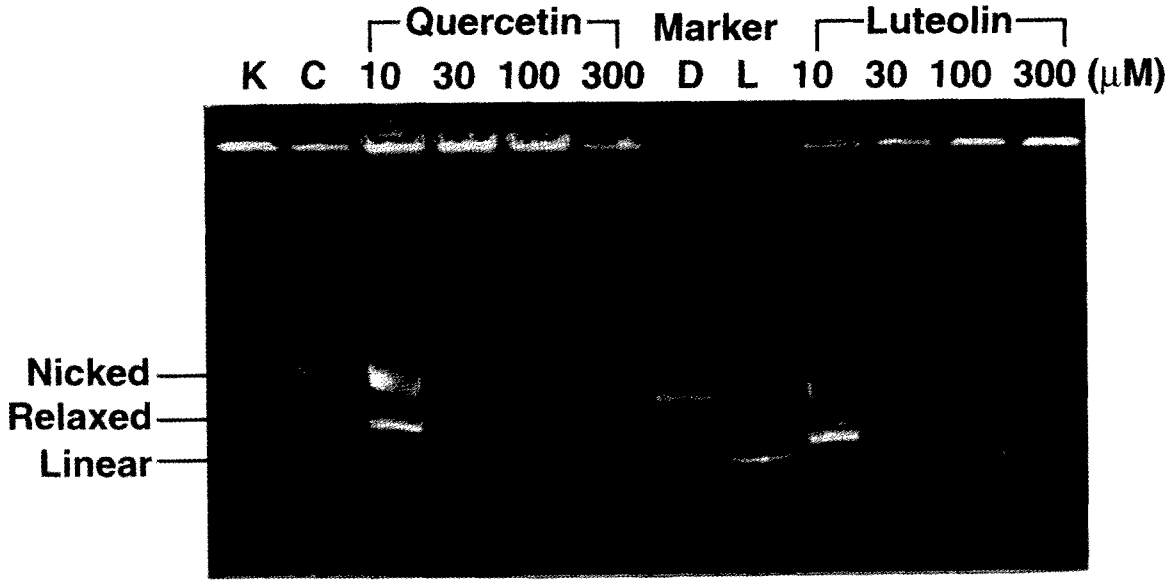

FIGURE 4 Inhibition of human topo II-mediated decatenation by flavonoids. The decatenation reaction and electrophoresis were performed as described in Materials and Methods. The enzymatic activity was assayed by incubation for 40 min in the presence of the indicated concentration of flavonoids. Note that topo II-decatenated DNA product will be nicked DNA and some relaxed DNA, but will not be linear DNA, which indicates nuclease effects in topo II assay using kDNA. K, substrate kDNA alone; C, kDNA reacted with topo II and 1% DMSO; D, decatenated kDNA; L, linear DNA

related to induction of DNA strand-breaks. Linear and nicked DNA respectively represented the generation of double and single strand-breaks by formation of cleavable complex.

DISCUSSION

The relations among DNA damage, apoptosis, and carcinogenesis have attracted considerable interest. Cells that incur DNA damage and undergo apoptosis lose the potential to produce cancer cells. However, failure of cells to undergo apoptotic cell death might be involved in the pathogenesis of many human diseases, including cancer[14].

In this study, both quercetin and luteolin induced DNA cleavage to 1 to 2 Mb and less than 200 Kb DNA fragments, followed by DNA

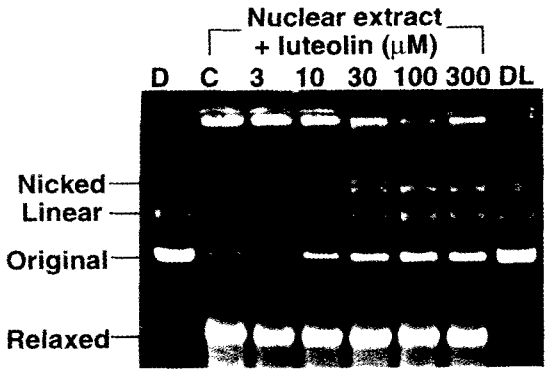

FIGURE 5 Topo II-mediated DNA cleavage induced by luteolin. Cleavage of DNA was analyzed by the agarose gel assay described in Materials and Methods. Supercoiled pBR322 DNA was incubated with nuclear extract topo II from HL-60 cells in the presence of the indicated concentration of luteolin for 30 min at 37 °C. The formation of linear and nicked DNA indicates topo II-mediated DNA cleavage. D, original pBR322 DNA alone; C, control; DL, original pBR322 DNA + 300 μM luteolin

ladder formation in HL-60 cells. DNA cleavage produced large DNA fragments (1–2 Mb and <200 Kb), which were digested into internucleosomal DNA fragments. DNA cleavage preceding apoptosis also occurs in DNA alkylation-induced apoptosis[37]. A mechanism for the digestion of cleaved DNA has been previously proposed[33,37].

DNA cleavage and DNA ladder formation were also examined in an H_2O_2-resistant clone, HP 100, to clarify the role of pro-oxidative effects of flavonoids. The characteristics of enzymes catabolizing reactive oxygen in HP 100 cells were as follows: activities of catalase and SOD were 18 and 2 times higher than in HL-60 cells, respectively, whereas myeloperoxidase activity was lacking[34]. In HP 100 cells, the extent of DNA cleavage and DNA ladder formation induced by quercetin was considerably lower than in HL-60 cells. These differences between the two cell types in DNA cleavage and DNA ladder formation were mainly due to different abilities to metabolize H_2O_2. In addition, the significant increase in 8-oxodG formation in HL-60 cells and no increase in HP 100 cells after treatment with quercetin showed that quercetin induced oxidative DNA damage and that H_2O_2 was the main mediator of such damage. A comparison of DNA damage between HL-60 and HP 100 cells suggests that H_2O_2-mediated DNA damage is the main pathway in quercetin-induced apoptosis.

The quercetin-induced intracellular increase in H_2O_2, resulting in cellular DNA damage, might be caused by autoxidation of quercetin. We recently studied the mechanism underlying the H_2O_2-mediated damage of isolated DNA by autoxidation of quercetin[24]. Briefly, quercetin forms a ternary complex with DNA and Cu(II), which physiologically maintains DNA structure[38]. The ternary complex produces H_2O_2 and DNA-Cu(I) by autoxidation of quercetin; consequently, H_2O_2 reacts with DNA-Cu(I), causing oxidative DNA damage. Similar pro-oxidative effects of quercetin might be responsible for cellular DNA damage. However, it is difficult to rule out the possibility that glutathione depletion and mitochondrial dysfunction might be involved in intracellular H_2O_2 generation[21,22,39,40]. Moreover, myeloperoxidase, which catalyzes the formation of hypochlorous acid (HOCl) from H_2O_2 and chloride ions, might participate in H_2O_2-mediated DNA damage caused by quercetin[41], because myeloperoxidase activity was high in HL-60 cells but absent in HP 100 cells.

Luteolin inhibited topo II activity more strongly than quercetin in crude nuclear extract. Inhibition of topo II activity by luteolin was associated with DNA cleavage by cleavable complex formation. Luteolin-induced DNA cleavage and DNA ladder formation in HP 100 cells were similar to those in HL-60 cells. However, the considerable decrease in quercetin-induced DNA cleavage in HP 100 cells indicated that topo II-mediated DNA cleavage in HL-60 cells was inappreciable at 30 μM quercetin and moderate at 100 μM quercetin. These results suggest that topo II-mediated DNA cleavage could be a main pathway in luteolin-induced apoptosis, but a minor pathway in quercetin-induced apoptosis.

Quercetin has not only anti-cancer but also carcinogenic effects, whereas it has been reported that luteolin does not have mutagenicity and carcinogenicity[17–19]. Our results indicate that H_2O_2-mediated DNA damage is the main pathway in quercetin-induced apoptosis and topo II-mediated DNA damage is the main pathway in luteolin-induced apoptosis. The carcinogenic effects of quercetin can be explained as follows: quercetin-induced DNA damage, including DNA cleavage and 8-oxodG formation, precedes endonuclease-catalyzed internucleosomal DNA fragmentation, a characteristic of apoptosis. However, it is unclear whether such DNA damage is consistently followed by apoptosis. The fates of cells exposed to quercetin, mutations, or apoptosis, might depend on the concentration of quercetin and the intensity of DNA damage.

It has been reported that vitamin C and E, so-called "anti-oxidants", act as anti-oxidants under some circumstances, and pro-oxidants under other circumstances [42,43]. Caffeic acid, which has anti-oxidant and carcinogenic properties, has pro-oxidative effects and causes oxidative DNA damage[44]. If "anti-oxidants" have pro-oxidant properties, they may be carcinogenic. A recent study reported that anti-oxidants such as vitamin A and *N*-acetylcysteine can act as potent oxidative DNA-damaging agents[45, 46]. Virtually all putative chemopreventive anti-oxidants may have carcinogenic potential.

Quercetin is the main component of flavonoid intake, and luteolin is a minor constituent[47,48]. In daily life, after eating foods containing large quantities of quercetin, the plasma quercetin concentration reaches about 0.4 μM[49]. Regular intake of flavonoids via supplements is assumed to maintain higher plasma concentrations of quercetin, as elimination of quercetin is apparently slow[50]. Our study shows that quercetin has the potential to induce H_2O_2-mediated DNA damage related to carcinogenesis. Since there is general concern about the linear dose-response to chemical carcinogens, much consideration to safety should be given when using quercetin as a medication for chemoprevention or as a nutritional supplement.

Acknowledgements

This work was supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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