Grape Seed Extract Prevents H_2O_2-Induced Chromosomal Damage in Human Lymphoblastoid Cells

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We examined the effects of grape seed extract (GSE) on chromosomal damage in two ways; induction on its own and prevention against treatment of reactive oxygen species (ROS). Chromosomal damage was evaluated by cytokinesis-block micronucleus assay (CBMN) in a human lymphoblastoid cell line, WIL2-NS cells. The GSE was composed of 89% proanthocyanidin with a degree of polymerization ranging from 2 to 15. GSE did not induce chromosomal damage in WIL2-NS cells at GSE concentrations up to 5 mg/l. In contrast, pretreatment with GSE dose-dependently prevented H_2O_2-induced chromosomal damage at an effective dose of 0.3 to 1 mg/l. A similar preventive effect of GSE was not detected in *tert*-butyl hydroperoxide-induced damage even at 5 mg/l. In a cell free system, GSE (<5 mg/l) directly scavenged H_2O_2, but produced slight amounts of H_2O_2 at higher concentrations (>50 mg/l). These results suggest that GSE is not genotoxic, but rather has an antigenotoxic effect against H_2O_2 *via* direct scavenging action of H_2O_2.

Key words chromosomal damage; grape seed extract; proanthocyanidin; H_2O_2; human lymphoblastoid cell line; micronucleus assay

Proanthocyanidins are polyphenolic compounds, and derivatives of flavan-3-ol flavonoids. They are mainly composed of dimers, oligomers and polymers of (+)-catechin, (−)-epicatechin and their gallic acid esters. Their degree of polymerization is generally distributed between 2 to 15. Although proanthocyanidins are found in grape, cocoa, chocolate, apple and berries, the abundant source of proanthocyanidins is in grape seeds and skins, which contribute to most of the polyphenols in red wine.[1)]

Proanthocyanidins from grape seed have been reported to show various beneficial properties such as anticarcinogenic, antiatherogenic, cardioprotective, hepatoprotective effects *via* their potent antioxidative activities.[2,3)] In Europe, proanthocyanidins are used for medical therapy for vascular disorders, such as venous insufficiency, and microvascular problems including capillary fragility and retinopathies. Proanthocyanidins are also used as food additives and nutritional supplements in many countries.

Recently the safety of food or food supplements has received great attention from the general public. Safety evaluations on proanthocyanidins from grape seed or grape seed extract (GSE) have been carried out using bacteria, mammalian cells and animals.[4—8)] However, only a few studies have evaluated chromosomal damage using human cells.[9)] Previously, we examined the effects of tea polyphenols on chromosomal damage in two ways; induction by each component on its own and prevention against treatment of reactive oxygen species (ROS) using CBMN assay in a human lymphoblastoid cell line.[10—12)] In these studies, we showed that tea polyphenols did not have a genotoxic effect, but rather an antigenotoxic effect against ROS *via* their antioxidant activity. In this study, we applied the same methods to examine the safety and effectiveness of GSE on chromosomal damage in a human cell line.

MATERIALS AND METHODS

Materials Grape seed extract (GSE, Gravinol-Super™) was kindly provided by Kikkoman Co. (Chiba, Japan). The GSE was composed of 89% proanthocyanidin, 6% monomers, and 5% other materials. The proanthocyanidins in GSE had a degree of polymerization ranging from 2 to 15 (mean degree: 7 to 9). Fetal bovine serum, RPMI 1640 medium, antibiotic solution (5000 U/ml penicillin and 5 g/l streptomycin), L-glutamine, and Hank's balanced salt solution (HBSS) were purchased from GIBCO (Grand Island, NY, U.S.A.). Cytochalasin B was obtained from Sigma (St. Louis, MO, U.S.A.). H_2O_2 was obtained from the Mitsubishi Gas Chemical Co. Inc. (Tokyo, Japan) and *tert*-butyl hydroperoxide (*tert*-BuOOH) from Katayama Chemical (Osaka, Japan). Giemsa's solution was obtained from Merck (Darmstadt, Germany).

Analytical Methods Chromosomal damage was evaluated by the CBMN assay using WIL2-NS cells (ATCC No. CRL-8155), a non-immunoglobulin secreting human B lymphocyte line. Cell culture and treatment with GSE and oxidants were performed as described elsewhere.[10)] Briefly, WIL2-NS cells in HBSS (0.95 ml, 0.5×10^6 cells/ml) were incubated at 37 °C for 60 min with various concentrations of GSE (in 50 μl) dissolved in HBSS. Then, the cells were washed with HBSS to eliminate GSE, subjected to CBMN assay for the determination of GSE-induced chromosomal damage. To determine the protective effect of GSE on ROS-induced chromosomal damage, the cell suspensions with or without GSE were exposed to either H_2O_2 (20 μM) or *tert*-BuOOH (0.1 mM) for 30 min, washed with HBSS, and then subjected to CBMN assay, which was performed as described elsewhere.[13)] Briefly, WIL2-NS cells were resuspended in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, 1% (v/v) antibiotic solution, 2 mM glutamine, and 4.44 μg/ml cytochalasin B at a cell density of 0.5×10^6 cells/ml. After 42 h of culture, the cells were harvested. Slides were prepared using a cytocentrifuge (Shandon Southern Products, Cheshire, U.K.), air dried, and fixed with absolute methanol, and then stained with 4% (v/v) Giemsa's solution in water for 30 min. Chromosomal damage rates

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were expressed as the number of micronucleated binucleate cells (MNed BN cells) per 1000 binucleated cells (BN cells).

Production and/or elimination of H_2O_2 in GSE solution was measured by the phenol red method with H_2O_2 as a standard in the cell-free study.[14)]

Statistical Analysis The data are presented as means±S.E. for triplicate experiments. Statistical analyses of the data were carried out using ANOVA followed by a post hoc test of Fisher's protected least significant difference. A p-value <0.05 was considered to be significant. The statistical analyses were performed using a computer program (StatView ver. 5.0, Abacus Concepts, CA, U.S.A.).

RESULTS

WIL2-NS cells were treated with various concentrations of GSE for 60 min to evaluate chromosomal damage by GSE. GSE did not induce chromosomal damage in WIL2-NS cells at concentrations up to 5 mg/l. MNed BN cells/1000 BN cells (mean±S.E.), which is indicative of chromosomal damage, were 6.2±1.4 for vehicle, 8.1±0.9 for 0.05 mg/l, 5.8±1.4 for 0.5 mg/l and 5.9±1.0 for 5 mg/l, respectively. Treatment of GSE at >50 mg/l produced necrotic cells in the CBMN assay. In the next study, WIL2-NS cells were preincubated with various concentrations of GSE for 60 min, then challenged to either 20 μM of H_2O_2 or 0.1 mM of *tert*-BuOOH for 30 min to examine the preventive effects of GSE against ROS-induced chromosomal damage. GSE dose-dependently inhibited H_2O_2-induced chromosomal damage, and a significant effect was detected at concentrations over 0.3 mg/l (Fig. 1A). In contrast, GSE did not significantly prevent *tert*-BuOOH-induced chromosomal damage even at concentrations of 5 mg/l (Fig. 1B).

Direct interaction of GSE with H_2O_2 was examined in a condition where H_2O_2 (10 μM) was added to various concentrations of GSE (up to 500 mg/l) in the cell-free study. As shown in Fig. 2A, GSE reduced H_2O_2 concentration at 5 mg/l, but the reduction was attenuated at the concentration of 50 and 500 mg/l. Production of H_2O_2 by GSE on its own was also evaluated. GSE did not produce H_2O_2 at a dose of up to 0.5 mg/l, but H_2O_2 was produced at a concentration of 50 and 500 mg/l (Fig. 2B). At a high GSE concentration, decrease in H_2O_2 scavenging activity and an increase in H_2O_2 production corresponded well.

DISCUSSION

CBMN assay using WIL2-NS cells is sensitive to detection of ROS-induced chromosomal damage.[13)] In this study, we examined the genotoxic and antigenotoxic effect of GSE using the CBMN assay system. The GSE used were composed of 89% proanthocyanidin, thus the effect of GSE would represent that of proantocyanidin.

GSE dose-dependently prevented the chromosomal damage induced by H_2O_2, and a significant effect was detected at 0.3 and 1 mg/l (Fig.1A). When direct interaction between GSE and H_2O_2 was examined, GSE reduced the concentration of H_2O_2 (Fig.2A). It has already been reported that GSE has hydroxyl radical scavenging activity.[15,16)] According to these facts, the protective effect of GSE on H_2O_2-induced chromosomal damage is thought to be due to direct elimination of H_2O_2 by GSE. Other researchers have reported the preventive effect of GSE against H_2O_2-induced oxidative stress with effective GSE concentration at 10 to 50 mg/l.[6,17,18)] In this study, the effective concentration of GSE was slightly lower than that in the previous reports. Proanthocyanidin is the major component of GSE and seems to be responsible for the antioxidant activities of GSE. Thus it is speculated that the different amount of proanthocyanidin in GSE may be related to the different effective dose of GSE among the studies.

For safety evaluation of GSE, the genotoxic effects of GSE have been examined in a chromosomal aberration test using Chinese hamster lung cells[5)] and sister chromatid exchange test using human lymphocytes.[9)] These studies reported that GSE did not show any genotoxicity. Consistent with these reports, we could not detect any genotoxic effects of GSE in CBMN assay using WIL2-NS cells up to the dose of 5 mg/l in this study. We have reported that unphysiological high concentration of tea catechins or some flavonoids induced chromosomal damage *via* the formation of H_2O_2 by the flavonoids themselves.[11,12)] Similar to our previous studies, GSE at high doses produced H_2O_2 and reduced its H_2O_2-scavenging ability as shown Fig. 2A. GSE at >50 mg/l induced cell necrosis. However, such high concentration of GSE would not be expected under physiological condition. When 2 g of GSE contained 18 mg of procyanidin B1 (procyanidin dimer, an available standard) was orally administered to humans, plasma concentration of procyanidin B1 was reported to be approximately 6 ng/l.[19)] Thus, the concen-

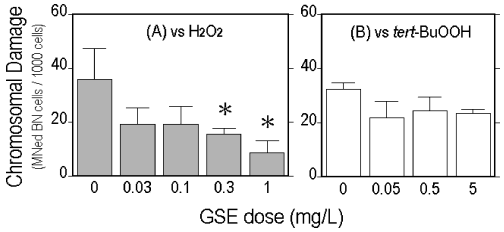

Fig. 1. Preventive Effects of GSE on H_2O_2- and *tert*-BuOOH-Induced Chromosomal Damage in WIL2-NS Cells

Cells were incubated with GSE (A: up to 1 mg/l, B: up to 5 mg/l) at 37 °C, for 60 min and they were exposed to ROS (A: 20 μM of H_2O_2, B: 0.1 mM of *tert*-BuOOH) for 30 min. The extent of chromosomal damage was evaluated by CBMN assay. Each point and vertical bar indicates the mean and SE for triplicate determination from separate culture. * Significance *versus* vehicle treatment (GSE 0 mg/l), $p<0.05$.

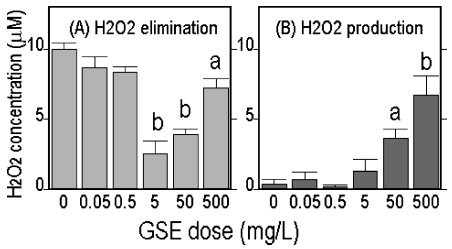

Fig. 2. Direct Interaction of GSE with H_2O_2, and Production of H_2O_2 by GSE

For evaluation of the direct interaction of GSE with H_2O_2 (A), various concentrations of GSE (up to 500 mg/l) were added to H_2O_2 (10 μM), and the H_2O_2 concentration was measured in the cell-free system. For the evaluation of the production of H_2O_2 by GSE (B), H_2O_2 concentration of GSE solutions (up to 500 mg/l) was similarly measured. Each point and vertical bar indicate the mean and S.E. for triplicate determination. Columns with letters are significantly different from untreated control (GSE 0 mg/l), and the columns with different letters are significantly different each other ($p<0.05$).

tration of proanthocyanidine in plasma seems to be extremely lower than that caused adverse reaction, such as production of H_2O_2. Even if GSE produces H_2O_2 in our body, chromosomal damage would not be induced due to lower concentrations of the H_2O_2 produced and the presence of enzymes such as catalase and/or glutathione peroxidase, which effectively remove H_2O_2. In fact, Deprez *et al.*[7] have shown that proanthocyanidin did not induce any toxicity to Caco-2 cells up to the concentration of 1 mM (578 mg/l; calculated as a dimer). These points suggest that GSE is safe in terms of its genotoxic effect under physiological condition.

GSE did not show clear protection against *tert*-BuOOH-induced chromosomal damage (Fig. 1B). In contrast, we have previously reported that monomers (such as (+)-catechin and (−)-epicatechin) showed a weak protection against *tert*-BuOOH-induced chromosomal damage in the same assay system.[11] In this study, 74.8% of the polymers in the GSE used are larger than pentamers. It has been reported that antioxidative properties of GSE depend on the proanthocyanidine composition, especially the degree of polymerization of the catechin unit.[20] Therefore, it is reasonable to speculate that different polymerization of flavanol alters the preventive effect of GSE against *tert*-BuOOH-induced oxidative damage.

Absorption, distribution and metabolism of proanthocyanidine are quite complex, but pharmacokinetic studies on proanthocyanidine have gradually accumulated.[7,19,21,22] For the practical use of proanthocyanidine and/or GSE, further evaluation of their chemical structures under physiological condition will be required.

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