Our previous study indicated that a diet containing a high dose (1%) of green tea polyphenols (GTPs) disrupted liver and kidney function via a reduction in antioxidant enzyme and heat shock protein (HSP) levels in both colitis and non-treated ICR mice. In the present study, we assessed the effects of 0.01%, 0.1%, and 1% dietary GTPs on liver and kidney physiological functioning in dextran sulfate sodium (DSS)-exposed and normal mice. GTPs at 0.01% and 0.1% significantly suppressed DSS-increased serum aspartate 2-oxoglutarate aminotransferase (AST) and alanine aminotransferase (ALT) levels. In contrast, GTPs at 1% increased kidney weight, serum creatinine levels, and thiobarbituric acid-reactive substances (TBARs) in both the kidney and the liver in normal mice, as compared with DSS-exposed mice. GTPs at 0.01% and 0.1% remarkably upregulated the expression of heme oxygenase-1 (HO-1) and heat shock protein 70 (HSP70) mRNA in the liver and kidney of mice exposed to DSS, whereas GTPs at 1% abolished it. Our results indicate that low and medium doses of GTPs have beneficial effects on DSS-induced hepatotoxicity and nephrotoxicity via upregulation of self-protective enzymes, while these effects disappeared at a high dose.

Key words: green tea polyphenols; colitis; hepatotoxicity; nephrotoxicity; self-protective enzymes

The inflammatory bowel disease (IBD), which includes the major chronic diseases ulcerative colitis (UC) and Crohn’s disease (CD), is a chronic disorder of the intestinal tract characterized by excessive production of reactive oxygen species (ROS) and cytokines. As an experimental model, dextran sulfate sodium (DSS)-induced colitis in rodents is used in many laboratories including ours, due to convenient induction of intestinal inflammation. In addition, excess ROS generation in the gut microenvironment disrupts intestinal anti-oxidant systems, contributing to intestinal oxidative injury and initiating pro-inflammatory signaling.

The cellular stress response is an evolutionarily conserved defense mechanism characterized by transcriptionally controlled induction of the synthesis and accumulation of stress proteins, including heat shock proteins (HSPs), following exposure of cells to high temperatures and other environmental challenges including oxidative stress. In particular, a highly stress-inducible member of the 70-kDa family of HSP (HSP70) in cytosol functions as a predominant molecular chaperone. HSP70 has been found to be downregulated in actively inflamed mucosa of individuals with UC and CD. It is also important to note that the production of pro-inflammatory cytokines tumor necrosis factor-α and interferon-γ, which appears at increased levels in IBD, is also downregulated by HSP70, which inhibits the translation step. Collectively, inflammation-related signals in IBD might exacerbate colitis at least in part through downregulation of HSP70, thereby interfering with its vital role in maintaining intestinal homeostasis.

Green tea is a widely consumed beverage that contains characteristic polyphenolic constituents, generally known as green tea polyphenols (GTPs), which include (−)-epigallocatechin-3-gallate (EGCG), (−)-epicatechin, (−)-epigallocatechin, and (−)-epicatechin. EGCG, the most abundant polyphenol, has been reported to have various preventive effects as to several chronic diseases, including cancer and disease. In addition, GTPs are strong antioxidants as against ROS and inducers of several antioxidant proteins, including heme oxygenases-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1), which are regulated by activation of nuclear factor erythroid-2-related factor 2. On the other hand, we reported recently that a diet including 1% GTP damaged pro-inflammatory cytokines, aggravated colitis, and tended to promote colon carcinogenesis in DSS-exposed colons, while it decreases...
ed the activities of superoxide dismutase (SOD) and catalase in non-treated mice. Furthermore, a 1% GTPs diet exacerbated kidney and liver functioning, presumably through downregulation of antioxidant enzymes and HSPs, in both normal mice and ones with DSS-induced colitis. Lambert et al. found that EGCG at 1% induced hepatotoxicity, as demonstrated by increased formation of malondialdehyde and 4-hydroxynonenal. In addition, several cases of hepatotoxicity in humans following the consumption of dietary supplements containing green tea extracts have been reported. Thus, GTPs have both beneficial and harmful effects as to organ homeostasis in a dose-dependent manner.

The aim of the present study was to examine the effects of oral feeding of GTPs at various doses (0.01%, 0.1%, and 1%) on liver and kidney functioning and the expression of antioxidant enzymes and HSPs in both normal and DSS-treated ICR mice.

Materials and Methods

Chemicals. Extracted green tea polyphenols (HPLC graded GTPs containing 95% polyphenols) were purchased from LKT Laboratories (St. Paul, MN). High-performance liquid chromatography analysis of the green tea extracts revealed a percentage composition of 70% total catechins, 35% EGCG, and 3% caffeine. DSS, with a molecular weight of 36–50 kDa, was purchased from MP Biomedicals (Aurora, OH). The catechins, 35% EGCG, and 3% caffeine. DSS, with a molecular weight of 36–50 kDa, was purchased from MP Biomedicals (Aurora, OH). The other chemicals and kits were from Wako Pure Chemical Industries (Osaka, Japan), unless specified otherwise.

Animals. Four-week-old male specific pathogen-free ICR mice (17–19 g) were purchased from Japan SLC (Shizuoka, Japan) and housed five per cage. All were fed rodent MF pellets (Oriental Yeast, Kyoto, Japan) were given fresh tap water and ad libitum, and were kept at 22–26°C at a relative humidity of 55–65% under a 12-hour light (06:00–18:00)/dark cycle for 6 d prior to experiments. The mice were treated in accordance with the Guidelines for the Use of Experimental Animals of Kyoto University, and the experimental protocol was approved by the Experimentation Committee (approval #21–42).

Experimental design. As shown in Fig. 1A, mice were randomly divided into eight groups: a non-treated group (group 1), GTP supplementation groups (group 2, 0.01% GTPs; group 3, 0.1% GTPs; group 4, 1% GTPs), a DSS-exposed group (group 5), and GTPs + DSS-exposed groups (group 6, 0.01% GTPs; group 7, 0.1% GTPs; group 8, 1% GTPs). Experimental colitis was induced in the DSS groups exposed to by administrating 5% DSS (w/v) through the water intake showed tendencies similar to those for body weight (Fig. 1D, E).

RNA extraction and reverse transcription PCR (RT-PCR) analysis. After isolation of the kidneys and livers, total RNA was prepared using Trizol (Invitrogen, Tokyo) as described in the manufacturer’s protocol. For RT-PCR analysis, 1 μg of RNA was reverse transcribed with an RNA PCR kit (Takara, Shiga, Japan) with oligo dT-adaptor primer, as recommended by the supplier. PCR was done using a thermal cycler (PTC-0100; MJ Research, Cambridge, MA) with mouse hypoxanthine phosphoribosyl transferase (HPRT), HO-1, HSP70, and HSP90 primers. The PCR products were subjected to electrophoresis in 3% agarose gels and were stained with 0.01% SYBR Gold stain (Molecular Probes, Eugene, OR). Band intensities were quantified by NIH image, and an absence of PCR saturation was confirmed.

AST, ALT, and creatinine measurements. Blood was collected from the inferior vena cava, and serum was obtained by centrifugation at 3,000 × g for 10 min at 4°C for analysis of biomarkers. AST and ALT (Wako Pure Chemical Industries) were used as biochemical markers, of hepatotoxicity and were measured using by commercially available spectrophotometric assay (λmax = 555 nm) following the manufacturer’s instructions. Creatinine was quantified by means of commercial kits (Wako Pure Chemical Industries, Osaka).

Lipid peroxidation, as determined by thiobarbituric acid-reactive substances (TBARS). The kidney and liver samples (about 25 mg each) were homogenized in 250 μL of RIPA buffer (25 mm Tris–HCl pH 7.6, 150 mm NaCl, 1% NP-40, 1% deoxycholic acid, and 0.1% sodium dodecyl sulfate) on ice. The homogenate was centrifuged at 1,600 × g for 10 min at 4°C and the supernatant was used in the assay. Lipid peroxidation in the kidney and liver was assessed by measuring TBARS using a TBARS Assay kit (Cayman Chemical, Ann Arbor, MI).

Statistical analysis. Differences in survival were analyzed by χ2 analysis. Results were expressed as mean ± SD for each group of 3–7 mice. After examining equality by Levene’s test, if there was a significant difference (p < 0.05), each value was converted to the logarithmic value for ANOVA. Data were analyzed by two-way ANOVA to determine the effects of DSS treatment (D), GTP administration (G), and their interaction (D × G). Tukey’s test was used to determine the significance of differences resulting from multiple comparisons among the groups, and differences considered to be significant at p < 0.05.

Results

General observations

Although no mice in groups 1–4 died during the 6 d of observation, the survival rates in groups 5–8 decreased on day 6 (Fig. 1B). In particular, group 8 exhibited a drastic decrease in survival rate, by 80%, on day 6 as compared to group 1. Mean body weights in groups 5–8 were lower than that group 1, whereas GTP supplementation in normal mice (groups 2–4) resulted in no marked differences (Fig. 1C). Data for water and food intake showed tendencies similar to those for body weight (Fig. 1D, E).

Effects of GTPs on colon, spleen, kidney, and liver weights

Colorectal shortening reflects the extent of colonic damage in DSS-exposed mice. As shown in Fig. 2A, colorectal lengths in groups 5–8 were significantly shortened as compared to group 1, while GTPs supplementation in normal mice had no effect (group 1 vs. 2, 3, 4). In addition, we observed that colorectal length in group 8 was significantly shortened as compared group 5 (Fig. 2A). Spleen weight in group 5 was significantly higher (2-fold) that in group 1 (Fig. 2B). On the other hand, GTP supplementation (groups 2–4, and 6–8) did not affect weight as compared groups 1 and 5 (Fig. 2B). Kidney weight in group 8 was notably increased as compared to group 5 (Fig. 2C), while those in groups 1–7 did not vary significantly. In contrast, liver weight was nearly constant in all groups (Fig. 2D).

Kidney and liver function parameters

Semen AST and ALT levels, which reflect hepatic function, were also measured. Group 5 (AST, 324.03 ± 25.04 U/L; ALT, 71.76 ± 15.31 U/L) showed a marked increase in both parameters as compared to group 1 (AST, 98.93 ± 15.6 U/L; ALT, 16.25 ± 3.52 U/L). Low and medium doses of GTPs (group 6, 281.75 ± 14.57 U/L; group 7, 279.92 ± 15.22 U/L) significantly decreased AST levels, by 13% and 14% respectively, while a high dose (group 8, 326.05 ± 32.52 U/L) was ineffective. (Fig. 3A, B). In addition, group 6 (52.57 ± 2

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7.24 U/L) and group 7 (44.4 ± 8.67 U/L) significantly decreased in ALT levels by 27% and 38% respectively, as compared to group 5 (71.76 ± 15.31 U/L). Subsequently, we measured serum creatinine levels as a biomarker of renal function. DSS exposure (group 5) did not affect the level of serum creatinine, whereas that in group 8 doubled as compared with group 5 (Fig. 3C).

Next we compared TBARS levels, a reliable indicator of lipid peroxidation that might be related to oxidative tissue damage. As shown in Fig. 3D and E, both hepatic and renal TBARS levels in group 8 were significantly higher than those in group 5. Likewise, those levels in both the liver and the kidney in group 4 were also markedly increased as compared group 1 (Fig. 3D, E). Low-dose GTPs did not affect TBARS levels in the normal or the colitis mice.

Effects of GTPs on the expression levels of antioxidant enzymes and HSPs

We also determined whether DSS and/or GTPs supplementation would affect the expression levels of HO-1, HSP70, and HSP90 in the kidney and the liver by semi-quantitative RT-PCR (Fig. 4A). Both renal and hepatic HO-1 mRNA expressions in groups 6 and 7 were significantly increased as compared with group 5. In contrast, high-dose GTPs (group 8) abolished them (Fig. 4B). Similarly, renal HO-1 mRNA levels were significantly increased by supplementation with GTPs at 0.01% and 0.1%, but not at 1% (Fig. 4B, left). On the other hand, the hepatic HO-1 mRNA level was not different among groups 1, 2, and 3, while that in group 4 was significantly changed (Fig. 4B, right).

Renal HSP70 expression in groups 2–4 did not change as compared with group 1, whereas it decreased drastically in group 5 (Fig. 4C, left panel). The renal HSP70 level in group 7 was significantly higher than in group 5 (Fig. 4C, left panel). Also, the hepatic HSP70 expression levels in groups 6 and 7, but not in group 8, were significantly increased as compared with group 5 (Fig. 4C, right panel). Likewise, those in groups 2 and 3 were remarkably higher than that in group 1, whereas the level in group 4 was decreased drastically (Fig. 4C). Both renal and hepatic HSP90 expression decreased markedly, in a dose-dependent manner, in groups 2–4,
and similar tendencies were seen for the DSS-exposed mice (Fig. 4D).

Discussion

GTPs and EGCG have a variety of beneficial health functions, including preventive effects on inflammation and carcinogenesis, and have been reported to have no serious adverse effects. However, several studies have revealed that excess intake of green tea supplements induced hepatotoxicity in both rodents and humans. 

Similarly, our group has also reported that a 1% GTP diet enhanced pro-inflammatory cytokines, aggravated colitis, and tended to promote colon carcinogenesis in DSS-exposed colons. On the other hand, a low-dose GTP (0.1–0.25%) diet had a tendency to improve both ulcers and inflammation in a colitis model. Furthermore, we reported recently that a 1% GTP diet damaged the kidney and the liver functioning via downregulation of antioxidant enzymes and HSPs in DSS-induced colitis, but the molecular mechanisms are poorly understood, and the effects of low- and medium-dose GTP diets on these functions have yet to be fully elucidated. Therefore, our aim was to determine the effects of oral feeding of GTPs at various doses on the liver and the kidney functions in DSS-induced colitis.

Several groups have reported that intragastric administration of high-dose EGCG caused hepatotoxicity in

Fig. 2. Effects of DSS and/or GTP Supplementation on Symptomatic Changes in Several Organs. Colon length (A), spleen weight (B), kidney weight (C), and liver weight (D) were determined. Data are means ± SD. Differences were considered to be significant at \( p < 0.05 \).

Fig. 3. Effects of DSS and/or GTP Supplementation on Hepatotoxicity and Renal Toxicity. Blood was collected and serum separated for determination of serum AST (A), ALT (B), and creatinine (C) levels. The kidneys and livers were homogenized using RIPA buffer, and, then the supernatants were separated for measurement of TBARS levels in the liver (D) and kidney (E). Data are means ± SD. Differences were considered to be significant at \( p < 0.05 \).
mice, while various doses of GTPs in the diet did not damage liver function in the present study, as determined by serum AST and ALT levels (Fig. 3A, B). These contrasting results may have been due to differences in the experimental conditions imposed. We found that 1% GTPs dramatically increased kidney weight and serum creatinine, the most reliable biomarkers of nephropathy, in mice exposed to DSS, but not in normal mice (Figs. 2C, 3C). These findings confirm previous reports showing that both green tea extracts and DSS become widely distributed throughout a variety of organs in mice, including the liver and kidney, but EGCG is metabolized through methylation, glucuronidation, and sulfation under normal physiological conditions, and then excreted in the urine. Thus low- and medium-dose GTPs are metabolized and excreted in the urine, while a high dose can accumulate in various organs of normal and DSS-exposed mice, including the liver and kidney, inducing oxidative damage. Along similar lines, 1% GTPs drastically increased the level of TBARS in the kidney and liver, while 0.01–0.1% GTPs did not change those in normal or DSS-exposed mice (Fig. 3D, E). Therefore high-dose, but not low- or medium-dose, GTPs more strongly induced oxidative stress via auto-oxidation by producing ROS in the normal and DSS-exposed mice, which escapes from the inactivation processes by metabolism.

Oxidative stress is accelerated by a combination of ROS generation and impaired antioxidant capacity. Our group has reported that a high dose of EGCG reduced the expression of anti-oxidant enzymes, including HO-1, SOD, and catalase. In accordance with this observation, 1% GTPs abolished HO-1 mRNA expression in the kidney and liver, whereas 0.01–0.1% GTPs in the diet significantly increased mRNA expression (Fig. 4A, B). These findings suggest a mechanistic association of this anti-oxidative property with low and medium doses in normal and DSS-induced mice. In addition, EGCG has been found to enhance Nrf2-Keap1 signaling in vivo and to increase the expressions of other anti-oxidant enzymes, including NQO-1. Hence we speculate that low and medium doses of GTPs protect against oxidative stress by increasing anti-oxidant enzymes, whereas a high dose causes oxidative stress via downregulation of antioxidant enzymes, leading to hepatic and renal dysfunction.

The HSP family functions as a redox sensor and represents an endogenous protective mechanism against oxidative stress. Accordingly, in the present study, we found that renal and hepatic HSP70 mRNA expression was dramatically suppressed by a high dose of GTPs in the diet, while low and medium doses increased expression in normal and DSS-exposed mice (Fig. 4C), although protein levels remain to be examined. Hu et al. have reported that downregulation of HSP70 was associated with IBD development. Thus a significant increase in HSP70 expression due to low- and medium-dose GTPs, but not a high dose, might protect...
against oxidative stress. Importantly, HSP90 in the kidney and liver was identified as a chaperone protein that is downregulated by GTPs given at medium and high doses in non-treated mice (Fig. 4D). HSP90 is the most abundant molecular chaperone and it plays pivotal roles in inducing the expression of other HSP genes, resulting in organ homeostasis under various stresses.37–39 Thus high-dose GTPs may affect hepatic function via HSP90 downregulation in healthy individuals.

Taken together, our findings suggest that low and medium doses of GTPs have beneficial effects in DSS-induced hepatotoxicity and nephrotoxicity via upregulation of anti-oxidant enzymes and HSPs. In addition, attenuation of these levels can increase disease activity in DSS-induced colitis. Finally, our results provide insight into the molecular mechanisms underlying the potential toxicity of GTPs.

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