Antioxidative Activity of Green Tea Treated with Radical Initiator 2,2′-Azobis(2-amidinopropane) Dihydrochloride

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This study investigated the antioxidative activity of green tea extract, and a green tea tannin mixture and its components, under conditions of radical generation using the hydrophilic azo compound, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) to generate peroxyl radicals at a constant and measurable rate in the cultured renal epithelial cell line, LLC−PK_1, which is susceptible to oxidative damage. Treatment with AAPH decreased cell viability and increased the formation of thiobarbituric acid-reactive substances. However, green tea extract, and the tannin mixture and its components, comprising (−)-epigallocatechin 3-*O*-gallate (EGCg), (−)-gallocatechin 3-*O*-gallate (GCg), (−)-epicatechin 3-*O*-gallate (ECg), (−)-epigallocatechin (EGC), (+)-gallocatechin (GC), (−)-epicatechin (EC), and (+)-catechin (C), showed protective activity against AAPH-induced cellular damage. The tannin mixture and its components exhibited higher antioxidative activity than the green tea extract. Furthermore, EGCg and GCg had higher activity than EGC and GC, respectively. In particular, EGCg exerted the most significant cellular protective activity against AAPH. These results indicate that green tea tannin may inhibit cellular loss and lipid peroxidation resulting from the peroxyl radical generated by AAPH, and that the chemical structure of tannin is also involved in the activity, suggesting that the *O*-dihydroxy structure in the B ring and the galloyl groups are important determinants for radical scavenging and antioxidative potential.

Keywords: *Antioxidative activity; 2,2′-azobis(2-amidinopropane) dihydrochloride; green tea; tannin; (−)-epigallocatechin 3-O-gallate; LLC−PK_1*

INTRODUCTION

Free-radical-mediated cell injury and lipid peroxidation may be of critical importance in various pathological phenomena (Halliwell, 1987). Therefore, antioxidants that prevent damage caused by free radicals are considered to be worthy of study.

The antioxidant activity of green tea, a widely enjoyed beverage, has been focused upon. In particular, the polyphenol antioxidants present in tea are attracting increasing attention, because of the wide variety of beneficial biological effects ascribed to them. Considerable numbers of studies have shown that polyphenol suppresses the occurrence of lipid peroxidation in biological tissue and subcellular fractions (Graham, 1992; Ho et al., 1992; Salah et al., 1995; Pannala et al., 1997). Green tea tannin, a polyphenolic compound of green tea, has exhibited not only radical scavenging activity in several assay systems, but also an inhibitory effect on oxidative stress-induced apoptosis. We have also reported previously that green tea tannin exerted radical scavenging activity, and attributed this activity to the active component flavan-3-ol and its gallate compounds (Yokozawa et al., 1993, 1996a,b, 1997a,b, and 1998; Yokozawa, 1998).

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The reactions of free radicals in biological systems are complicated. To study these reactions, a well-designed in vitro model system is required. Numerous factors induce oxidative stress, such as irradiation, redox decomposition by metal ions of hydroperoxides or hydrogen peroxide, and thermal decomposition of free-radical initiators, including peroxides, hyponitrites, and azo compounds. To generate free radicals at a known, constant and well-defined rate, thermal decomposition of free-radical initiators is preferred. It has been suggested that this can be achieved by the use of 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH), one of the hydrophilic azo compounds. In an appropriate model system, hydrophilic antioxidants can be used to scavenge radicals and suppress the oxidation initiated by AAPH within cells (Terao and Niki, 1986; Dooley et al., 1990). It is well-known that AAPH generates peroxyl radicals rapidly via interaction with carbon-centered radicals and molecular oxygen, eventually causing the oxidation of lipid and protein in biomolecules. In addition, AAPH administration in vivo damages biological tissues such as lymphocytes, kidney, and liver, leading to pathological conditions including atherosclerosis, ischemia-reoxygenation injury, and inflammatory disease.

LLC−PK_1, a renal-tubular epithelial cell line, is susceptible to oxidative stress, resulting in cell death or injury. Therefore it is widely used to study renal injury. With these points in mind, we attempted to generate peroxyl radicals in cells that are vulnerable

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to oxidative injury, and then to investigate the cellular protective activity of green tea extract, and a green tea tannin mixture and its components, by measuring cell viability and lipid peroxidation.

MATERIALS AND METHODS

Green Tea Extract and Tannin Mixture and Its Components. Fifty grams of dry green tea leaves, which were produced in the Haibara district (Shizuoka, Japan), were added to 1 L of hot distilled water (70 °C) and shaken for 5 min. The resulting supernatant was freeze-dried to obtain green tea extract. The green tea tannin mixture was prepared from a hot-water extract of green tea. It was composed mainly of (−)-epigallocatechin 3-*O*-gallate (EGCg, 18.0%), (−)-gallocatechin 3-*O*-gallate (GCg, 11.6%), (−)-epicatechin3-*O*-gallate (ECg, 4.6%), (−)-epigallocatechin (EGC, 15.0%), (+)-gallocatechin (GC, 14.8%), (−)-epicatechin (EC, 7.0%), and (+)-catechin (C, 3.5%). For purification of these components, recycling high-performance liquid chromatography (HPLC) was done on a JAI-LC-908 high-performance liquid chromatograph (Japan Analytical Industry Co., Tokyo, Japan) equipped with JAI RI and JAI UV detectors, operating at 280 nm, as described previously(Sakanaka et al., 1989). A prepacked PVA HP-GPC column (JAIGEL GS-320, 50 × 2 cm i.d.) was used. Methanol was employed as the eluting solvent at a flow rate of 3 mL/min. Each component isolated was identified by analysis using fast atom bombardment mass spectroscopy (FABMS) and HPLC. FABMS was recorded on a mass spectrometer (JMS-DX 303, JEOL, Tokyo, Japan) using glycerol as the matrix. The chemical structures of these constituents are illustrated in Figure 1.

Reagents and Medium. AAPH was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle medium/nutrient mixture F-12 (D-MEM/F-12) and fetal calf serum (FCS) were purchased from Life Technologies, Inc. (Grand Island, NY) and Cell Culture Laboratories (Cleveland, OH), respectively.

Experiment with Cultured Cells. Commercially available LLC−PK_1 cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air (routine conditions) in culture plates with 5% FCS-supplemented D-MEM/F-12 medium. After confluence had been reached, the cells were seeded into 96-well culture plates at 10^4 cells per well. Two hours later, 1 mM of AAPH was added to all wells, together with standardized samples of either green tea extract or tannin in the test wells. The plates were then incubated under routine conditions for 24 h. The proper concentration of AAPH and incubation time was determined by the preliminary experiment. Fifty microliters of MTT (1 mg/mL) solution was added to each well. After incubation for 4 h at 37 °C, the MTT solution was removed from the medium. The resultant formazan crystals in the renal cells were solubilized with 100 μl of dimethyl sulfoxide. The absorbance of each well was then read at 540 nm using a Microplate Reader (Model 3550-UV, BIO-RAD, Tokyo, Japan). The level of lipid peroxidant released from the cultured cells was estimated as thiobarbituric acid-reactive substances (TBARS) according to the methods of Yagi (1976) and Yokode et al. (1988) with a slight modification. One aliquot of medium was mixed with 1.5 mL of 0.67% TBA aqueous solution and 1.5 mL of 20% trichloroacetic acid, and boiled at 95−100 °C for 45 min. The mixture was cooled with water and shaken vigorously with 3.0 mL of *n*-butanol. After the mixture was centrifuged at 4000*g* for 10 min, the *n*-butanol layer was removed, and the fluorescence was measured on a fluorescence spectrophotometer (Model RF-550, SHIMADZU, Kyoto, Japan).

Statistics. Results were presented as the mean ± SE of 5 determinations. The data were analyzed for statistical significance using Dunnett's test. Significance was accepted at $p < 0.05$.

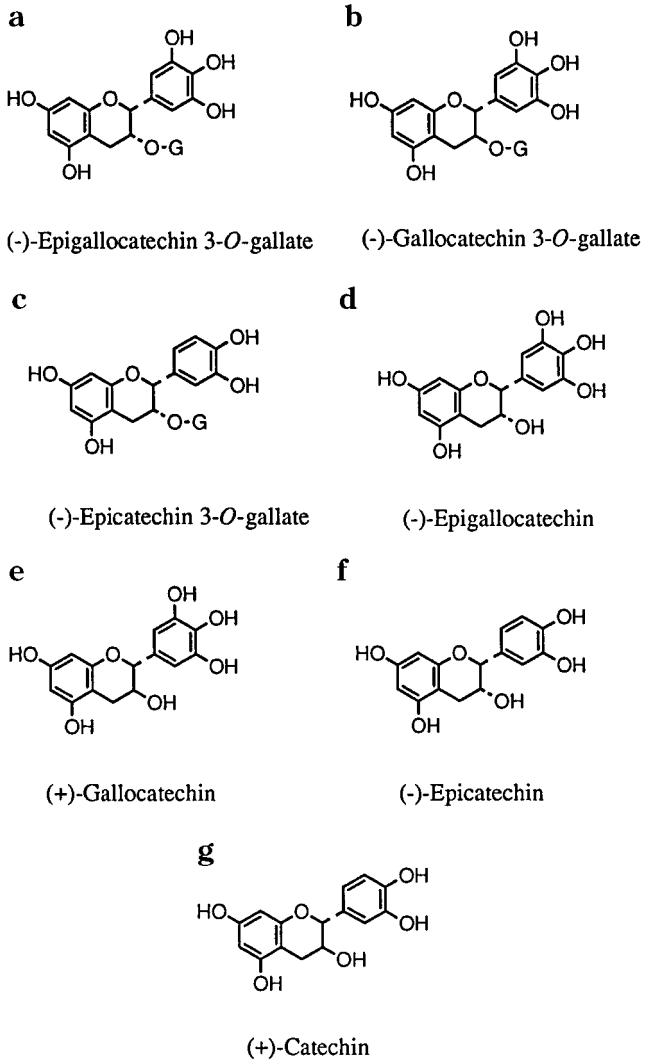

Figure 1. Chemical structures of green tea tannin.

RESULTS

Table 1 shows the effect of green tea extract and tannin mixture on the viability of LLC−PK_1 renal epithelial cells treated with AAPH which is a free-radical generator. The viability of LLC−PK_1 cells declined to 65.6% following AAPH treatment. However, the green tea extract and the tannin mixture exerted a significant degree of protective activity against AAPH-induced cellular damage. In particular, the tannin mixture showed higher activity than the green tea extract. When green tea extract (50 μg/mL) or tannin mixture (50 μg/mL) was present in samples treated with AAPH, the cell viability increased from 65.6% to 80.7% and 87.9%, respectively.

Table 2 shows the protective activity of green tea tannin components against cellular damage mediated by AAPH. Wells incorporating green tea tannin components showed higher cell viability than the control cells treated with AAPH alone. The presence of EGCg with AAPH recovered the viability of LLC−PK_1 cells to nearly 100%. Furthermore, EGC, GCg, and GC retained higher cell viability than the control. On the other hand, EC and C showed relatively low cell viability compared with the other tannin components, although they did maintain higher viability than the control.

As shown in Table 3, AAPH enhanced lipid peroxidation in LLC−PK_1 renal tubular epithelial cells,

Table 1. Effect of Green Tea Extract and Tannin Mixture on Viability of Cells Treated with AAPH

material	concentration (μg/mL)	cell viability (%)
extract	0	65.6 ± 1.1^{a}
	5	$72.3 \pm 1.1^{a,b}$
	25	$79.2 \pm 4.8^{a,c}$
	50	$80.7 \pm 3.7^{a,c}$
tannin mixture	0	65.6 ± 1.1^{a}
	5	$71.8 \pm 2.0^{a,b}$
	25	$87.1 \pm 5.1^{a,c}$
	50	$87.9 \pm 2.6^{a,c}$
	−	100.0 ± 1.0

Statistical significance: ^{a} $p < 0.001$ vs AAPH nontreatment value, ^{b} $p < 0.05$, ^{c} $p < 0.001$ vs AAPH treatment control value.

Table 2. Effect of Green Tea Tannin Components on Viability of Cells Treated with AAPH

material	concentration (μg/mL)	cell viability (%)
(−)-epigallocatechin 3-*O*-gallate	0	65.6 ± 1.1^{c}
	0.5	$93.6 \pm 2.0^{b,d}$
	1	99.0 ± 2.6^{d}
	2	99.6 ± 2.2^{d}
	5	99.7 ± 2.5^{d}
	10	99.4 ± 2.6^{d}
(−)-gallocatechin 3-*O*-gallate	0	65.6 ± 1.1^{c}
	0.5	67.7 ± 1.4^{c}
	1	$78.5 \pm 0.5^{c,d}$
	2	$83.6 \pm 1.7^{c,d}$
	5	$83.9 \pm 0.8^{c,d}$
	10	$83.9 \pm 1.7^{c,d}$
(−)-epicatechin 3-*O*-gallate	0	65.6 ± 1.1^{c}
	0.5	71.5 ± 2.7^{c}
	1	$77.8 \pm 2.6^{c,d}$
	2	$79.1 \pm 5.0^{c,d}$
	5	$79.7 \pm 3.4^{c,d}$
	10	$82.5 \pm 2.0^{c,d}$
(−)-epigallocatechin	0	65.6 ± 1.1^{c}
	0.5	$85.6 \pm 2.9^{c,d}$
	1	$87.1 \pm 3.2^{c,d}$
	2	$93.3 \pm 3.6^{a,d}$
	5	94.4 ± 2.2^{d}
	10	95.9 ± 3.1^{d}
(+)-gallocatechin	0	65.6 ± 1.1^{c}
	0.5	64.0 ± 1.4^{c}
	1	$76.0 \pm 2.1^{c,d}$
	2	$80.4 \pm 2.2^{c,d}$
	5	$81.2 \pm 2.9^{c,d}$
	10	$85.0 \pm 2.2^{c,d}$
(−)-epicatechin	0	65.6 ± 1.1^{c}
	0.5	$76.3 \pm 1.2^{c,d}$
	1	$76.8 \pm 4.1^{c,d}$
	2	$76.1 \pm 1.8^{c,d}$
	5	$78.3 \pm 3.1^{c,d}$
	10	$78.9 \pm 1.2^{c,d}$
(+)-catechin	0	65.6 ± 1.1^{c}
	0.5	$76.8 \pm 2.0^{c,d}$
	1	$77.6 \pm 2.4^{c,d}$
	2	$76.9 \pm 3.3^{c,d}$
	5	$76.8 \pm 1.4^{c,d}$
	10	$78.8 \pm 2.1^{c,d}$
	-	100.0 ± 4.1

Statistical significance: ^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$ vs AAPH nontreatment value, ^{d} $p < 0.001$ vs AAPH treatment control value.

Table 3. Effect of Green Tea Extract and Tannin Mixture on TBARS Treated with AAPH

material	concentration (μg/mL)	TBARS (nmol/well)
extract	0	0.131 ± 0.004^{b}
	5	0.125 ± 0.013^{b}
	25	$0.108 \pm 0.005^{a,c}$
	50	0.101 ± 0.008^{d}
tannin mixture	0	0.131 ± 0.004^{b}
	5	$0.110 \pm 0.008^{b,d}$
	25	0.091 ± 0.003^{d}
	50	$0.084 \pm 0.001^{a,d}$
	-	0.093 ± 0.003

Statistical significance: ^{a} $p < 0.05$, ^{b} $p < 0.001$ vs AAPH nontreatment value, ^{c} $p < 0.01$, ^{d} $p < 0.001$ vs AAPH treatment control value.

Table 4. Effect of Green Tea Tannin Components on TBARS Treated with AAPH

material	concentration (μg/mL)	TBARS (nmol/well)
(−)-epigallocatechin 3-*O*-gallate	0	0.177 ± 0.005^{c}
	0.5	$0.132 \pm 0.004^{c,f}$
	1	$0.103 \pm 0.003^{b,f}$
	2	0.093 ± 0.008^{f}
	5	0.092 ± 0.005^{f}
	10	0.090 ± 0.004^{f}
(−)-gallocatechin 3-*O*-gallate	0	0.177 ± 0.005^{c}
	0.5	$0.155 \pm 0.014^{c,f}$
	1	$0.136 \pm 0.005^{c,f}$
	2	$0.121 \pm 0.001^{c,f}$
	5	$0.114 \pm 0.001^{c,f}$
	10	$0.110 \pm 0.005^{c,f}$
(−)-epicatechin 3-*O*-gallate	0	0.177 ± 0.005^{c}
	0.5	0.172 ± 0.011^{c}
	1	$0.163 \pm 0.003^{c,d}$
	2	$0.146 \pm 0.008^{c,f}$
	5	$0.143 \pm 0.011^{c,f}$
	10	$0.134 \pm 0.003^{c,f}$
(−)-epigallocatechin	0	0.177 ± 0.005^{c}
	0.5	$0.147 \pm 0.010^{c,f}$
	1	$0.110 \pm 0.002^{c,f}$
	2	$0.101 \pm 0.007^{a,f}$
	5	0.099 ± 0.008^{f}
	10	0.093 ± 0.004^{f}
(+)-gallocatechin	0	0.177 ± 0.005^{c}
	0.5	$0.156 \pm 0.008^{c,e}$
	1	$0.124 \pm 0.016^{c,f}$
	2	$0.123 \pm 0.003^{c,f}$
	5	$0.122 \pm 0.007^{c,f}$
	10	$0.117 \pm 0.007^{c,f}$
(−)-epicatechin	0	0.177 ± 0.005^{c}
	0.5	$0.156 \pm 0.008^{c,f}$
	1	$0.150 \pm 0.004^{c,f}$
	2	$0.154 \pm 0.003^{c,f}$
	5	$0.149 \pm 0.006^{c,f}$
	10	$0.148 \pm 0.001^{c,f}$
(+)-catechin	0	0.177 ± 0.005^{c}
	0.5	0.166 ± 0.021^{c}
	1	0.165 ± 0.020^{c}
	2	0.157 ± 0.008^{c}
	5	0.157 ± 0.008^{c}
	10	$0.149 \pm 0.006^{c,f}$
	-	0.089 ± 0.003

Statistical significance: ^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$ vs AAPH nontreatment value, ^{d} $p < 0.05$, ^{e} $p < 0.01$, ^{f} $p < 0.001$ vs AAPH treatment control value.

whereas both treatments (green tea extract and tannin mixture) significantly decreased the formation of TBARS by AAPH. As the treatment concentration was increased, the formation of TBARS by AAPH appeared to asymptotically reach a minimum of about 0.09 nmol/well. In particular, the tannin mixture suppressed lipid peroxidation mediated by AAPH more effectively than green tea extract did. At concentrations of 50 μg/mL green tea extract or tannin mixture treated with AAPH for the same length of time, 0.101 nmol/well and 0.084 nmol/well of TBARS was formed respectively, whereas 0.131 nmol/well of TBARS was produced in wells treated only with AAPH.

Table 4 represents the effect of tannin components on TBARS formation by LLC−PK$_1$ cells treated with

AAPH. EGCg, EGC, GCg, and GC reduced TBARS formation induced by AAPH much more than the other components. EC and C showed only relatively low inhibitory effects on TBARS formation. Of all the components, EGCg showed the strongest suppressive activity against lipid peroxidation. Treatment with EGCg reduced TBARS formation in LLC−PK_1 cells to almost the normal level.

DISCUSSION

Azo compounds have several advantages in the study of the damage to biological systems that is induced by free radicals (Terao and Niki, 1986; Dooley et al., 1990). For instance, AAPH generates free radicals at a constant and measurable rate by its thermal decomposition without biotransformation. The free radicals generated from AAPH react with oxygen molecules rapidly to yield peroxyl radicals. The lipid peroxyl radicals attack other lipid molecules to form lipid hydroperoxide and new lipid radicals. This reaction takes place repeatedly with resultant attacks upon various biological molecules, and induces physiochemical alterations and cellular damage (Miki et al., 1987). Finally, AAPH causes a diverse array of pathological changes. Therefore, an AAPH-intoxication experiment may be a promising assay system for the biological activities of antioxidants.

To investigate the antioxidative activity of green tea, we employed such an AAPH model system. Several reports documented that AAPH decreased the viability of hepatic cells, neuron, and aortic endothelial cells (Matsura et al., 1992; Rapin et al., 1998; Martin et al., 1996). In addition, treatment with AAPH induced apoptosis in the cells, causing loss of viability. In this study, we have clearly demonstrated that AAPH also leads to the decreased viability of LLC−PK_1 renal epithelial cells.

Oxidative stress was associated with the peroxidation of cellular lipids, which was determined by the measurement of TBARS. Lipid peroxidation in biological systems has long been thought to be a toxicological phenomenon, resulting in pathological consequences (Hochstein and Jain, 1981). Hence, measurement of lipid peroxidation end products such as TBARS provides a good index of cell destruction, because cells and tissues damaged by any mechanism tend to peroxidize more rapidly than normal. It was proposed that AAPH enhanced lipid peroxidation in cellular systems. Based on these views, we measured the lipid peroxidant, TBARS, to determine cellular damage induced by AAPH. The result revealed that AAPH treatment increased the formation of TBARS by LLC−PK_1 renal epithelial cells. One of the remarkable features of cellular damage is leakage of lactate dehydrogenase (LDH), a soluble cytosolic enzyme, from inside the cells. Therefore, we carried out experiments on LDH leakage from LLC−PK_1 cells. A similar effect was also found: the LDH leakage increased as AAPH was added (data not shown).

These results indicated that LLC−PK_1 cells sustained free-radical damage from AAPH, resulting in cellular loss and lipid peroxidation. Terao and Niki (1986) reported that there are three types of organ or tissue damage induced by AAPH. The most striking structural changes following the administration of AAPH include degeneration, swelling, and disruption of the endothelial cells of capillaries in various organs. The second type is death of lymphocytes in the lymphoid tissues. The third type of AAPH-intoxication is characterized by marked fatty degeneration of the kidney and liver. Although it is not completely understood which types of damage are involved in LLC−PK_1 cellular injury from AAPH, we hypothesized that AAPH treatment leads to the degeneration, disruption, and death of LLC−PK_1 kidney epithelial cells.

In AAPH-induced cell injury and peroxidation, scavenging of lipid peroxyl radicals seems to play a considerable part in antioxidative activity. From this investigation, we concluded that green tea extract and green tea tannin and its components scavenge peroxyl radicals generated from AAPH, and thereby increase viability and reduce TBARS formation in LLC−PK_1 renal epithelial cells.We considered that they might protect the cell from free radicals by either one, or a combination of, the following mechanisms. First, they may act as a chelator to inactivate catalytic cations involved in the initiation of free radicals. Second, they may function as a free-radical chain reaction interrupter by trapping the free radicals mediated by AAPH.

Polyphenols, depending on their precise structure, have been reported to function as hydrogen-donating antioxidants as well as chelators of metal ions, preventing metal-catalyzing formation of initiating radical species (Bors et al., 1990; Jovanovic et al., 1994). It has also been suggested that structural specificity is involved in the manifestation of antioxidative activity in green tea polyphenols (Terao et al., 1994; Yokozawa et al., 1997a,b; Zhang et al., 1997). The differences in antioxidative activity of individual tannin components on AAPH-induced cell damage and lipid peroxidation were probably related to the number and position of their hydroxyl groups. Introduction of an additional hydroxyl group to the B ring may enhance the radical scavenging activity by increasing the hydrogen available for radical scavenging. The *O*-dihydroxy (catechol) structure in the B ring is regarded as the important determinant for radical scavenging and/or antioxidative potential. It is the obvious radical target site for all polyphenols with a saturated 2, 3-bond. We found that EGCg, EGC, GCg, and GC were the effective antioxidants among the various green tea tannin components, indicating that the *O*-dihydroxy group in the B ring plays a considerable antioxidative role by conferring stability to the radical form and participating in electron delocalization.

In addition, the galloyl group associated with green tea tannin might play an important antioxidant role. In this study, gallate-free tannin proved to have relatively low activity. Based on these concepts, we assume that hydroxyl and galloyl groups in green tea tannin contribute to antioxidative activity. These functional groups make the components not only more capable of donating hydrogen but also more hydrophilic, thus increasing their power to chelate catalytic cations. With regard to the effect of these structural determinations on antioxidative activity, EGCg could be expected to be the best antioxidant, and this was the case in our investigation.

Moreover, EGCg accounts for the largest fraction of the components of green tea tannin. Taking this fact into consideration, the antioxidative activity of green tea tannin would be mainly ascribable to EGCg. Similar data have been obtained in other studies which showed that EGCg is stronger than any other catechin in providing protection from oxidation (Salah et al., 1995;

Yokozawa et al., 1997b; Chung et al., 1998). Fiala et al. (1996) also reported that the tea-derived polyphenolic antioxidant, EGCg, strongly inhibits free-radical-mediated oxidation. Furthermore, in some reports EGCg has been found to be an even better antioxidant than α-tocopherol, butylated hydroxyanisole, or butylated hydroxytoluene, which are well-known antioxidants (Yang and Wang, 1993). The antioxidative potential of green tea tannin, especially EGCg, is worthy of recognition, even though the mechanisms responsible for the activity have not been fully determined.

It is noteworthy that tannin has in vitro and in vivo antioxidative activity which may be closely related to its preventive effects on various diseases including renal disease, arteriosclerosis, and inflammation caused by lipid peroxidation and excessive free radical production (Halliwell and Gutteridge, 1990). However, limited information is available on the metabolism of the tannin components, even on their absorption in the body. Yang et al. (1998) have demonstrated that the bioavailability of tea catechins is very low after ingestion of different amounts of green tea by human. Benzie et al. (1999) have reported that although the amount of antioxidants absorbed was relatively small, some potentially polyphenolic antioxidants enter the systemic circulation and cause a significant increase in plasma antioxidant status. As the present study demonstrated that green tea tannin exerts an antioxidative activity on LLC−PK_1 renal tubular epithelial cells, further investigation on the physiological functions is needed. However, in view of the antioxidative activity as observed in the oral administration experiment using rats with renal disease and in a cell-free system (Yokozawa et al., 1996a, 1997b, 1998), green tea tannin itself seems to be effective for preventing the radical-scavenging activity.

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