Flavonoids have antioxidant properties (1) and have been reported to prevent the development of free-radical-induced diseases, including forms of cancer (2), coronary heart disease (CHD) (3–5), and gastric mucosal injury (6).

Oxidized low density lipoprotein (LDL) is considered to be an important risk factor in the atherogenic progression of cardiovascular disease (7). Oxidized LDL can attract monocytes into the arterial wall where they can be transformed into macrophages—the precursors of foam cells, leading to the formation of early atherosclerotic lesions (8, 9). Protection against LDL oxidation is critical in the prevention of the initiation and progression of atherosclerosis.

We previously reported that red wine and cocoa, which contain dietary polyphenolic components, inhibited LDL oxidation (10, 11). These antioxidant foods are enriched in various types of flavonoids such as catechin and quercetin. In the Zutphen Elderly Study (5), quercetin, kaempferol, myricetin, apigenin, and luteolin were compared as dietary antioxidant flavonoids. It is still unclear, however, which flavonoid has the most beneficial effects in preventing LDL oxidation.

Previous studies in vitro have demonstrated that the flavonoids act as hydrogen-donating free radical scavengers, and their function is dependent on their structural properties (1, 12, 13), of which there are three key features: 1) the o-dihydroxyl (catechol) structure in the B ring, which confers higher stability to radical form; 2) a 2,3-double bond in conjunction with the 4-oxo function in the C ring, which is responsible for electron delocalization; and 3) the 3 and 5-hydroxyl groups responsible for maximal radical scavenging power.

The aim of the present study was to determine the antioxidant power of flavonoids against aqueous radicals and lipophilic radicals. In this study, we examined the antioxidant capacity of 10 structurally related flavonoids by measuring their free-radical-scavenging...
Fig. 1. The chemical structures of the flavonoids used in this study.

Flavonols (Catechin)

EC: \( R_1=R_2=H \)

EGC: \( R_1=OH, R_2=H \)

ECg: \( R_1=H, R_2=gallic \) acid

EGCg: \( R_1=OH, R_2=gallic \) acid

Materials and Methods

Materials. (-)-Epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg), and (-)-epigallocatechin gallate (EGCg) were donated by Itoen Ltd., Japan. (+)-Catechin, myricetin, quercetin, apigenin, kaempferol, and luteolin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The general formulas for flavonoids used in this study are shown in Fig. 1. 2,2'-Azobis(4-methoxy-2,4-dimethylvaleronitrile) [AMVN-CH₃O] and 1,1-diphenyl-2-picrylhydrazyl [DPPH] were purchased from Wako Pure Chemical Industries (Osaka, Japan). A bicinchoninic acid (BCA) assay kit for protein was from Pierce Laboratories Inc. (Rockford, IL, USA) and \( \alpha \)-toc was a gift from Eisai Co., Ltd. (Tokyo, Japan).

Free radical scavenging capacity of flavonoids. The free radical scavenging capacity of flavonoids was measured by using DPPH (14). An aliquot of the various flavonoids dissolved in ethanol was mixed with 2 mL of 0.1 mM DPPH solution (in ethanol). Following 20 min of incubation at 37°C, the absorbance at 516 nm was measured with a Beckman Model DU 650 spectrophotometer.

Isolation and preparation of LDL. LDL was isolated from human plasma with EDTA (1 mg/mL) prepared from fasting normolipidemic volunteers, who were someone of authors obtained informed consent. It was separated by a single-spin density gradient ultracentrifugation (417,000×g, 40 min, 4°C) by using the TLA-100.4 rotor fixed-angle rotor (Beckman Instruments Inc., CA, USA) (15) and protein was determined by the BCA method (16). Before the start of the oxidation experiments, LDL samples were diluted with PBS to give final concentrations of 35 or 70 \( \mu \)g/mL LDL protein as indicated in Fig. 4 and Tables.

Oxidative modification of LDL. The oxidation of LDL was performed as previously reported (17). LDL solutions in the absence (control) or presence of flavonoids and/or \( \alpha \)-toc were incubated with freshly prepared AMVN-CH₃O (200 \( \mu \)M), using acetonitrile as the solvent. The kinetics of LDL oxidation was obtained by monitoring the absorbance of conjugated dienes formation at 234 nm with a Beckman Model DU 650 spectrophotometer at 5 min intervals at 37°C.

The lag time of lipid peroxidation, measured as previously described (18), is defined as the time interval between initiation and the intercept of the two tangents
Antioxidant Ability of Flavonoids

Table 1. Effects of various flavonoids on LDL oxidation lag time and propagation rate.

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Lag time (min)</th>
<th>Propagation rate (nmol diene/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.6</td>
<td>2.37</td>
</tr>
<tr>
<td>Apigenin</td>
<td>31.6</td>
<td>2.81</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>52.4</td>
<td>1.79</td>
</tr>
<tr>
<td>Myricetin</td>
<td>106</td>
<td>2.23</td>
</tr>
<tr>
<td>Epigallocatechin</td>
<td>119</td>
<td>1.74</td>
</tr>
<tr>
<td>Epigallocatechin gallate</td>
<td>136</td>
<td>1.84</td>
</tr>
<tr>
<td>Catechin</td>
<td>182</td>
<td>1.07</td>
</tr>
<tr>
<td>Quercetin</td>
<td>190</td>
<td>0.97</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>197</td>
<td>1.07</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>250</td>
<td>1.21</td>
</tr>
<tr>
<td>Luteolin</td>
<td>434</td>
<td>0.44</td>
</tr>
</tbody>
</table>

An aliquot (2 μM) of each flavonoid solution was added in LDL solution (70 μg/mL LDL protein). Drawn to the lag and propagation phase of the absorbance curve at 234 nm and was expressed in min. The propagation rate of conjugated dienes was estimated from the linear portion of the curve, using the extinction coefficient for conjugated dienes at 234 nm (29,500 L/mol/cm) (18).

RESULTS

Radical scavenging capacity of flavonoids

The radical scavenging capacity of various flavonoids was tested with DPPH in aqueous phase (Figs. 2 and 3). As shown in Fig. 2, the absorbance of DPPH at 516 nm decreased linearly by the addition of luteolin, showing that luteolin is an effective DPPH radical scavenger. The relative effectiveness of the flavonoids in scavenging the DPPH radical is shown in Fig. 3. The data revealed that EGCg, ECg, quercetin, EC, catechin, and myricetin were most effective and sufficed at less than 3 μM for scavenging 50% of the DPPH radical in comparison with EGC (3.99 μM) and kaempferol (5.93 μM), whereas luteolin was the least effective and required 16 μM to do the same. The value of apigenin could not be estimated because no DPPH radical was trapped.

Effect of flavonoids on AMVN-CH3O-induced LDL oxidation

The oxidation of LDL was used as an indication of investigating the lipid chain-breaking antioxidant activity of flavonoids. AMVN-CH3O rather than copper ions was chosen for propagating lipid peroxy radicals to avoid the effects of flavonoids as metal chelators (13). As shown in Fig. 4, when LDL (70 μg protein/mL) was incubated with 200 μM AMVN-CH3O in the absence (control) or presence of 1 or 2 μM quercetin, quercetin prolonged the lag time in a dose-dependent manner. Compared with the control group (23.9 min), the lag time was prolonged 2.5-fold at 1 μM and 3.4-fold at 2 μM. Flavonoids examined in this study are classified into flavonols (catechin group), flavones (quercetin, myricetin, and kaempferol), or flavones (luteolin and apigenin). The inhibitory effect of each flavonoid (2 μM) for LDL oxidation was determined as shown in Table 1. Their order of prolongation of LDL oxidation lag time was ECG (250 min) > EC (197 min) > catechin (182 min) > EGCg (136 min) > EGC (119 min) in the flavonol group. Quercetin (190 min) > myricetin (106 min) > kaempferol (52.4 min) in flavonoids, and luteolin (434 min) > apigenin (31.6 min) in flavones. The propagation rates of luteolin, quercetin, EC, C, and ECg were roughly similar to one another in the range of 0.44 to 1.21 nmol diene/min/mg protein. Among them, luteolin was the most effective in breaking the lipid peroxy radical chain.

To analyze the correlation between the trapping abilities of flavonoids for aqueous phase and lipid peroxy radicals, we plotted the values of IC50 scavenging DPPH radical against LDL oxidation lag time obtained in Fig. 3 and Table 1, respectively. Luteolin scavenges lipid per-
Fig. 5. Correlation of the trapping ability of flavonoids between aqueous phase and lipid peroxyl radicals. The IC50 values for scavenging DPPH radical were plotted against LDL oxidation lag time obtained in Fig. 3 and Table 1, respectively.

Interactive effects between a-tocopherol and flavonoids

Our previous report showed that the shortened lag time observed at higher doses of a-toc was restored when water-soluble antioxidants such as ascorbic and uric acids were added simultaneously (17). Tables 2 and 3 show the interactive effects of a-toc and flavonoids on LDL oxidation. The addition of flavonoids reversed the lag time, which was shortened at a high dose of a-toc. The interaction of a-toc with catechin prolonged the lag time as compared with the control (20.5 min). The lag time (73.6 min) at 6 mg/100 mL a-toc, prolonged to 146.3 min with 10 μg/100 mL catechin and to 172.2 min with 20 μg/100 mL catechin.

In the same experiment, the lag time with 3 mg/100 mL a-toc was 100.4 min and 87.2 min with 6 mg/100 mL a-toc. This result differs from that shown in Table 2 because LDL used in each experiment was taken from individual subjects, and the conditions of the experiments also differed. When a-toc and EGCg were added together, the lag time with 10 μg/100 mL EGCg was 104.6 min (3 mg/100 mL a-toc) or 119.9 min (6 mg/100 mL a-toc). This effect is due to the regeneration of a-toc from a-toc radicals by flavonoids and ascorbate, among others.

DISCUSSION

The present study demonstrated that all the flavonoids except luteolin and apigenin (flavones) can effectively donate an electron to DPPH radical and that flavonoids inhibit azo radical-initiated LDL oxidation and regenerate a-toc radical to a-toc. This effectiveness depends on the structural feature of flavonoids.

In DPPH assay, the electron-donating capacity was strongest in the following order: EGCg>ECg>quercetin>EC= catechin>myricetin>EGC>kaempferol>luteolin>apigenin (Fig. 3). This result is in agreement with the previous study (19) comparing quercetin, catechin, and myricetin.

EGC is structurally similar to catechin and EC with an additional hydroxyl group adjacent to the catechol structure in the B ring (Fig. 1). EGCg and ECg, which are esters of EC and EGC with gallic acid, enhanced the DPPH scavenging ability. An orthogonal A ring—carbonyl group conformation combined with an ability to form an intramolecular hydrogen bond is needed for DPPH scavenging activity (20). All the flavonoids tested here have this structure. Considering the chemical structures of electron-donating flavonoids, the DPPH radical scavenging ability could depend on the number of hydroxyl substitutions in its backbone structure, as indicated by Cao et al. (21). Notably, apigenin has only three hydroxyl groups, resulting in a lower effectiveness for radical scavenging.
In the case of radicals generated in the lipophilic phase, quercetin prolonged the oxidation lag time in a dose-dependent manner (Fig. 4). Flavonoids sometimes behaved as prooxidants according to the conditions (21), but certainly at a 1 or 2 μM dose they served as antioxidants. Previous studies have compared the antioxidant effect of flavonoids on LDL oxidation. Miura et al. (22, 23) reported that in a Cu²⁺-mediated oxidation system, the ability to prolong the lag time was in the order of EGCg > EGc > EC > catechin > EGC, and sesaminol > quercetin > EGCG > theaflavin > myricetin > α-toc. The inhibition of Cu²⁺-mediated oxidation by flavonoids was attributed to chelating the Cu²⁺ ions, and the lag time extension was in the order of quercetin > luteolin > rutin > kaempferol > (13). The LDL oxidation with metmyoglobin was inhibited in the order of ECG = EGC = EC = catechin > EGC > gallic acid (24). Our findings on flavanols, in part, differed from the above-mentioned results, but were in agreement with data using an azo initiator (AAPH) of oxidation (25). Kaempferol and apigenin having only one hydroxyl group on the B ring responded poorly to the LDL oxidation system (Table 1). In this study, the catechol structure of the B ring proved to be a prime determinant of the antioxidant effect of flavonoids on LDL oxidation.

Belinky et al. (26) have reported that the DPPH assay and oxidation potential (Ep1/2) correlated with Cu²⁺-induced and macrophage-mediated LDL oxidation in the presence of flavonoids. In agreement with these data, a good correlation was found between the DPPH assay data measured as an electron-donating test and the oxidation lag time, with the exception of luteolin (Fig. 5). Luteolin failed to donate an electron to DPPH, but showed the highest antioxidant activity in the LDL oxidation system for reasons still unknown. Further studies will be needed to investigate why luteolin behaves quite differently in the two assay systems.

We have previously reported that the shortened lag time induced by a high dose of α-toc is recovered in the presence of ascorbate (17). Several reports have shown that a high dose of α-toc is prooxidantive during the autoxidation of polyunsaturated fatty acids (27). Vitamin E leads to an increase in conjugated dienes concurrently with the formation of a propagation phase and results in a shortening of the oxidation lag time in LDL. In the experiment on the interaction between α-toc and flavonoids, we used catechin and EGCg as flavonoids because we are easy to ingest them with α-toc in our daily diet. As a result, flavonoids and ascorbate were both found to restore α-toc radical or to delay the consumption of α-toc (Tables 2 and 3). Our results on the interaction between α-toc and flavonoids are supported by a report from Viana et al. (28), using flavonoid-rich extracts. The cooperative effects of flavonoids and ascorbate have been recognized by Mathiesen et al. (29) and Skaper et al. (30), who described that ascorbate-protected flavonoids from oxidation. It thus seems very likely that the interaction between lipophilic and aqueous antioxidants within the body increases LDL resistance to oxidation.

In conclusion, the present study showed that the antioxidant ability of flavonoids depended on key structural properties and cooperative interactions with α-toc, effectively inhibiting lipid oxidation. Taken altogether, the higher antioxidant effect enough to prevent free-radical-related diseases can be expected by a moderate intake of such dietary antioxidants as flavonoids, α-toc, and ascorbate.

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REFERENCES


