Review

Dietary Antioxidants for Prevention of Cardiovascular Disease

Takuya Katsube¹, ², Mamiko Watanabe³, Masayuki Yamasaki⁴, Keiko Kitajima⁵, Yosuke Yamane² and Kuninori Shiwaku²
¹ Shimane Institute for Industrial Technology, Shimane, Japan
² Department of Environmental and Preventive Medicine, Shimane University School of Medicine, Shimane, Japan
³ Tsuyama Central Hospital, Okayama, Japan
⁴ Shimane University, Shimane, Japan

Abstract

The traditional Japanese diet with its high intake of fruits and vegetables that are rich in antioxidants is believed to effectively ward off cardiovascular disease. Oxidative stress, related to reactive oxygen and nitrogen species produced by aerobic organisms, is responsible for the pathogenesis of most chronic diseases. Oxidative low density lipoprotein (LDL) is thought to play a key role in the pathogenesis of early atherosclerosis. Oxidized LDL attracts monocytes, induces monocyte adhesion to endothelium, induces migration and proliferation of smooth muscle cells, impedes endothelial cell migration, and promotes procoagulant properties of vascular cells. Oxidized LDL is taken up by scavenger receptors of macrophages in the subendothelial space, gradually leading to the formation of foam cells and fibrous plaques. Immunochemical studies have demonstrated that oxidized LDL is present in atherosclerotic lesions and the plasma of humans.

I. Oxidative Stress and Oxidized LDL

Oxidative stress, related to reactive oxygen and nitrogen species produced by aerobic organisms, is responsible for the pathogenesis of most chronic diseases, including cardiovascular disease. Oxidized LDL is thought to play a key role in the pathogenesis of early atherosclerosis. Oxidized LDL attracts monocytes, induces monocyte adhesion to endothelium, induces migration and proliferation of smooth muscle cells, impedes endothelial cell migration, and promotes procoagulant properties of vascular cells. These modified LDL induce gene expression in endothelial cells and smooth muscle cells that result in the acceleration of atherogenesis. Oxidized LDL is taken up by the scavenger receptors of macrophages in the subendothelial space, gradually leading to the formation of foam cells and fibrous plaques. Immunochemical studies have demonstrated that oxidized LDL is present in atherosclerotic lesions and the plasma of humans.

It was generally assumed that a small amount of oxidized LDL is present in plasma and that oxidized LDL in atherosclerotic lesions is produced locally in the arterial wall after entry of normal LDL from the plasma. This concept is supported by the findings that plasma contains a variety of antioxidants and that oxidized LDL is removed extensively and rapidly from plasma after intravenous injection. However, numerous studies have provided evidence that oxidized LDL and thiobarbituric acid-reactive substances (TBARS), a nonspecific measure of lipid peroxidation, can be detected in human plasma and atherosclerotic lesions. This suggests that oxidized LDL is an important mediator of atherosclerosis.
peroxidation, occur in the plasma, and their concentrations are higher in subjects with coronary artery disease. It is suggested that oxidized LDL can circulate in the plasma for periods long enough to enter and accumulate in the arterial wall.

II. Antioxidants and Cardiovascular Disease

The Japanese have enjoyed the longest life expectancy and lowest mortality from cardiovascular disease in the world for 30 years. The traditional Japanese diet with its high intake of fruits and vegetables is believed to effectively ward off cardiovascular disease. Plants are high in numerous antioxidant compounds such as polyphenols, carotenoids, tocopherols, tocotrienols, glutathione, and ascorbic acid, as well as enzymes with antioxidant activity, because the use of solar energy and its conversion into chemical energy would not have been possible without a mechanism that effectively prevents oxidative damage of the plant cell. There has been increasing interest in antioxidant substances derived from edible plants. A large body of observational data suggests that diets high in fruits and vegetables are associated with a lower risk of cardiovascular diseases. Animal work has suggested that antioxidants found in abundance in fruits and vegetables, prevented atherosclerotic lesion development.

Antioxidants that prevent LDL from oxidation may interrupt the progression of atherosclerosis in humans. Such activity mainly depends on absorption of potentially important antioxidants from plants. Antioxidant activity in edible plants has been analyzed through the evaluation of serum total antioxidant capacity following consumption of the target plant. Cao et al. evaluated total antioxidant capacity in the serum of elderly women and found significant increases in antioxidant capacity after consumption of red wine, strawberries, and spinach. Consumption of alcohol-free red wine caused significant increases in plasma antioxidant capacity and polyphenol concentration in 10 healthy subjects 50 min after ingestion. In a study with 20 volunteers, Wang et al. found that epicatechin levels and the ability to scavenge free radicals in plasma increased significantly six hours after ingestion of epicatechin-rich chocolate.

Evaluation of the susceptibility of LDL to oxidation after the consumption of antioxidants has been studied in humans and experimental animals. Ingestion of grape seed extract (150 mg/day) for four weeks in 24 healthy male heavy smokers significantly reduced TBARS concentration in plasma and prolonged the oxidation lag phase of LDL. Diets supplemented with quercetin and catechin for four weeks lengthened the oxidation lag time of VLDL+LDL induced by copper ions in rats. These two findings indicate that antioxidant components in diets are absorbed into the blood and bind LDL, resulting in reduced susceptibility of LDL to oxidation. In fact, consumption of either red wine or quercetin by apolipoprotein E-deficient mice reduced susceptibility of LDL to oxidation as well as the progression of atherosclerosis, in relation to the binding of quercetin to LDL particles via the formation of an ether bond. Consequently, the evaluation for susceptibility of LDL to oxidation after consumption of edible plants is physiopathologically important. Consumption of black tea, green tea, olive oil, and red wine have been shown to be associated with an increased resistance of plasma LDL to oxidation.

Epidemiological studies have indicated that consumption of fruits and vegetables, olive oil, red wine, and tea is inversely correlated with heart disease rates. Vitamins E from plants decreases the morbidity of coronary heart disease. Consumption of black tea, green tea, olive oil, and red wine have been shown to reduce the risk of coronary heart disease. Intervention studies of human subjects with Brussel sprouts, onions, and tomatoes resulted in a significant decrease in the urinary excretion of free radical DNA damage biomarkers. These three plants and their products contain large amounts of natural antioxidant nutrients, including vitamin C, vitamin E, zeaxanthin, lutein, carotenoids, and polyphenols. More than 600 naturally occurring carotenoids and more than 4,000 polyphenols have been identified.

III. Screening Methods for Antioxidant Activity in Plants In Vitro

It is important to identify the most beneficial edible plants using a feasible method for measuring total amounts of antioxidant activity of edible plants. The method of measurement of total capacity of antioxidant activity in crude extracts of edible plants derived from varieties of individual compounds is also important. Several such measurement methods have been used and can be grouped according to their respective measurement principles. The Trolox equivalent antioxidant capacity assay (TEAC assay) and the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay (DPPH assay) are based on the antioxidant's ability to scavenge free radicals generated in the assay systems. These assays are based on discoloration of ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) radicals or DPPH radicals. The activity of hydroxy radicals (·OH) or superoxide radicals (·O2−), which are short-lived and unstable and whose scavenging activity can be measured using ESR equipment for measuring stable spin adducts, are thought to be physiologically...
important as they exist and act as prooxidants in vivo. The oxygen radical absorbance capacity (ORAC) assay\textsuperscript{29} and the β-carotene bleaching assay\textsuperscript{30} prevent target compounds from oxidation by free radical attack. The ferric-reducing ability (FRAP) assay measures the reduction of Fe\textsuperscript{3+} to Fe\textsuperscript{2+} using antioxidants as reductants in a redox-linked colorimetric reaction\textsuperscript{31}. The values in the FRAP assay correspond to the propensity to donate hydrogen. Color development using a Folin-Ciocalteu reagent (Folin-Ciocalteu assay)\textsuperscript{32} is the generally preferred method for measuring phenolics, since most plant-derived antioxidants contain large amounts of polyphenols. This method is also based on the reducing ability of the subject plant extract against phosphomolybdic and phosphotungstic acids that exist in the reagent.

LDL antioxidant activity of edible plants can be assessed by measurement of the protective effects of antioxidants on lipid peroxidation, using purified LDL\textsuperscript{33} or unFractionated plasma\textsuperscript{34}. In vitro LDL antioxidant activity has been studied using the measurement of various indications for oxidative substances. The kinetics of lipid oxidation induced by copper ions in LDL monitored at 234 nm allow for the measurement of the continuous changes in the LDL oxidation state. The three phases (lag, propagation, and decomposition) are identified in Figure 1. During the lag phase of LDL oxidation, polyunsaturated fatty acids (PUFA) in LDL are protected from oxidation by LDL-associated naturally-occurring antioxidants. After complete consumption of any antioxidants in LDL, the increase in absorbance at 234 nm reflects mostly the formation of conjugated dienes derived from PUFA during the propagation phase. After the propagation phase, the cholesterol in LDL becomes completely oxidized, followed by an accumulation of oxysterols made up mainly of 7-ketocholesterol. Since 7-ketocholesterol also has an absorption maxima at 234 nm, the monitoring at 234 nm shows an apparent plateau.

These three phases offer many points at which measurement of the LDL oxidation is possible using various methods. The content of TBARS mostly reflects aldehyde formation and can be measured using a spectrophotometer or a fluorescence meter\textsuperscript{35}. The amounts and composition of lipid hydroperoxides are determined using HPLC with a chemiluminescence detector\textsuperscript{36}. The negative charge of LDL apo-B protein and tryptophan fluorescence reflects the oxidation state of LDL protein\textsuperscript{37}.

In in vitro studies, water-soluble free radical generators such as 2, 2' azobis (2-aminopropane) dihydrochloride (AAPH) or transition metal ions are used as initiators of LDL oxidation. The application of these reagents is problematic, since they are unnatural in the LDL oxidation process. The concentration of copper in the intima where LDL oxidation occurs in vivo is unlikely to reach the micromolar levels required for in vitro LDL oxidation. It is believed that LDL oxidation in the intima is initiated by free radicals that are catalyzed by enzymes such as lipoxygenase and myeloperoxidase\textsuperscript{38}. Once free radicals occur in LDL-associated lipids, peroxidation is easily catalyzed by transition metal ions such as iron and copper. Iron ions can promote LDL oxidation only in the presence of free radical initiators\textsuperscript{39}, whereas copper ions at submicromolar concentrations can form free radicals on the LDL surface by interacting with LDL-associated antioxidants, mainly α-tocopherol or LDL-associated hydroperoxides\textsuperscript{40}. On the other hand, oxidation induced by AAPH is thought to be less relevant to the in vitro oxidation than that induced by copper ions. Furthermore, continuous monitoring at 234 nm is of little value when oxidation is induced by AAPH because its decomposition products have UV absorption. Therefore, the use of copper ions, the most abundant transition metal ion in vivo, as an inducer of oxidation can be regarded as a reasonable model for in vitro LDL oxidation.

IV. Screening of Edible Plants

Measurement of the exact oxidation lag time is possible by the successive monitoring of conjugated diene formation through measurement of the increase in absorbance at 234 nm. We found that oxidation lag time was representative of antioxidant activity levels of plant products\textsuperscript{41}. Fifty-two kinds of edible plants (medicinal plants, fruits, vegetables, roots and tubers, spices, and other plant forms) from Shimane prefecture were extracted using 70% aqueous ethanol solution, and the antioxidant activity of the extracts was determined. We measured antioxidant activities of epigallocatechin 3-gallate (EGCG) (0, 0.25, 0.5, 0.75 µM) as a standard compound.

Antioxidant activity ranged widely (296.9 to 0.4
μmol EGCG-equivalent/g). Of the medicinal plants, akamegashia (Mallotus japonicus) leaf, Japanese privet (Ligustrum japonicum) leaf, and green tea (Camellia sinensis (L.) O. Kuntze) showed the most antioxidant activity. Other medicinal plants showing relatively high antioxidant activity were the Japanese silverleaf (Farfugium japonicum) leaf, spicebush (Lindera umbellata) leaf, kawaraketsumei (Cassia mimosaoides) fruit and shell, shirakashi (Quercus myrsinifolia) leaf, sarutoriibara (Smilax china) leaf, udo (Aralia cordata Thunb) leaf, and hamaboufu (Glehnia littoralis Fr) leaf. Antioxidant activity in the non-medicinal plants was lower than that of the medicinal plants. Only the astringent persimmon (Diospyros kaki) fruit showed high antioxidant activity using the LDL oxidation assay, while the non-astringent persimmon (Diospyros kaki) showed little activity, suggesting that tannin is a component of antioxidant activity.40

Because different antioxidant compounds may act in vivo through different mechanisms, more than one method may be needed for evaluation of antioxidant activity. Using three different methods (TEAC, TRAP and FRAP) pellegrini et al.41 assessed the antioxidant capacity of vegetables, fruits, beverages, and oils commonly consumed in Italy, and found that among vegetables spinach had the highest antioxidant capacity using the TEAC and FRAP assays, whereas asparagus showed the greatest antioxidant capacity using the TRAP assay.

We compared the results of antioxidant activity using three assay methods (LDL oxidation assay, DPPH radical scavenging assay, Folin-Ciocalteu assay)42. The greatest antioxidant activity levels were found, in order, in the akamegashia leaf, astringent persimmon, green tea, and Japanese privet leaf. Within this antioxidant group, the first three plants showed similar activity levels using all three assays, while the Japanese privet leaf showed much higher activity using the LDL oxidation assay compared to that with the other two assays. The LDL oxidation assay/DPPH radical scavenging assay activity ratios ranged from 1.0 to 1.4 for the first three plants, while the ratio for the Japanese privet leaf was 4.4. Regression analysis was used to correlate the results obtained by the three assay methods, and each comparison indicated a significant correlation between methods (R=0.946-0.887).

It is believed that the antioxidant activity for LDL oxidation is caused by a combination of free radical scavenging activity, the binding to critical sites on LDL, and metal chelation43. The free radical scavenging activity of each edible plant was confirmed by a DPPH radical scavenging assay. The coefficient between the LDL oxidation assay and the DPPH radical scavenging assay was significant (R=0.887), suggesting that LDL antioxidant activity depends mainly on radical scavenging activity. Antioxidant activity for LDL oxidation of butylated hydroxyanisole (BHA), a synthetic hydrophobic antioxidant, is 1.2 times higher than quercetin44, whereas its DPPH radical scavenging activity is approximately one-fifth that of quercetin44. The reason for the relatively high LDL antioxidant activity of BHA compared to DPPH radical scavenging activity is the former’s high lipophilicity and affinity to lipoprotein45. The relatively lower correlation coefficient between the LDL oxidation assay the and DPPH radical scavenging assay compared to other assays is likely a consequence of the affinity of antioxidants to LDL, a mechanism characteristic of the LDL oxidation assay. Antioxidant activity for LDL by metal chelation reportedly causes a decrease in the rate of oxidation during the propagation phase46; however, none of our samples showed any significant decrease in the oxidation rate. Since the effective phenolic concentrations, known to act as chelating agents, are from one to two orders of magnitude lower than that of Cu2+ in the reaction mixture, chelation was most likely not a mechanism of action in our model.

Our results of the methods comparison study reveal the importance of conducting antioxidant activity evaluation using a variety of assays as well as the value of the LDL oxidation assay since certain samples showed high LDL antioxidant activity in spite of their remarkably lower activity levels using the other assay methods. The LDL antioxidant activity greatly depends on the affinity of antioxidants with LDL particles, a characteristic of the LDL oxidation assay. Ivanov et al.47 found that preincubation of LDL with red wine followed by gel filtration to remove unbound red wine components resulted in a significant decrease in LDL oxidation induced by copper. The synergy effect between an LDL-associated antioxidant, α-tocopherol, and a hydrophilic antioxidant such as ascorbic acid is also an important mechanism in LDL antioxidant activity48. Therefore, a comparison of the results of the assay methods of preincubation and co-incubation of LDL with antioxidants is important in helping to characterize their antioxidant mechanisms.

After screening for antioxidant activity in edible plant products, there is a need to isolate and characterize the individual compounds of such edible plants to elucidate their various antioxidant mechanisms. As mulberry (Morus alba L.) leaves showed relatively high antioxidant activity in our LDL oxidation assay, we chose them for our subject of study49. Mulberry leaves, bark, and branches have long been used in Chinese medicine to treat fever, protect the liver, improve eyesight, strengthen joints, facilitate discharge of urine, and lower blood pressure50. The leaves of mulberry species are consumed in Korea and Japan as antihyperglycemic nutraceutical food for patients with diabetes mellitus since the leaves contain...
1-deoxynojirimycin, known to be one of the most potent α-glycosidase inhibitors. In Japan, consumption of mulberry-leaf tea has been increasing. The antioxidant activity of mulberry leaves has also been reported. Doi et al. reported that 1-butanol extract of mulberry leaves scavenged the DPPH radical and inhibited the oxidative modification of rabbit and human LDL. Five flavonol glycosides [rutin, isoquercitrin, quercetin 3-(6-acetylglucoside), astragalin and kaempferol 3-(6-acetylglucoside)] have been reported in mulberry leaves. However, there are few reports on quantitative antioxidant activity or specifying the amounts of antioxidants in mulberry leaves.

V. Isolation and Identification of Antioxidants in Mulberry Leaves

The extraction of mulberry leaves with ethanol solution was concentrated by evaporation, and the mulberry-leaf antioxidants were isolated by liquid-liquid (water-ethyl acetate) extraction, Diaion HP20 column chromatography, and preparative ODS column chromatography. Three compounds showed the strongest antioxidant activity after preparative ODS column chromatography (Figure 2), two of which were identified as rutin (quercetin 3-rutinoside) (peak 1) and isoquercitrin (quercetin 3-glucoside) (peak 2) by LC-MS analysis and comparison with the authentic reagents. The third compound, showing the greatest LDL antioxidant activity (peak 3), was reduced by evaporation to a yellow powder, and acid hydrolysis of this powder detected quercetin and glucose, while treatment with β-glucosidase produced no hydrolyzed products, indicating that this was not a quercetin β-glucoside. H NMR and 13C NMR data of the yellow powder compound were identical to that previously reported for quercetin 3-(6-malonyl)-glucoside (Q3MG). We confirmed this by APCI-MS in which a molecular ion at m/z 549 [M-H] was observed corresponding to quercetin monoglucoside acylated with malonic acid. In addition, fragments consistent with the sequential loss of the malonyl residue (m/z 463) and glucosyl residue (m/z 301) were also observed, confirming that this was quercetin 3-(6-malonyl)-glucoside. Onogi et al. noted four flavonol glycosides in mulberry leaves: isoquercitrin and astragalin as major flavonoids and their acetylated forms as minor flavonoids. In our study, the most abundant flavonol glycoside was Q3MG (900 mg/100 g of fresh leaf), the greatest contributor to antioxidant activity of the mulberry leaf (Figure 3).

Quercetin (3, 3', 4', 5, 7-pentahydroxyflavone) is one of the most abundant flavonoids in human diets and the most powerful of the antioxidants. Data on quercetin content suggest a range of 2-250 mg quercetin/kg wet weight in fruits; 0-100 mg/kg in vegetables, with onions being especially high (200-600 mg/kg); 4-16 mg/L in red wine; 10-25 mg/L in tea; and 2-23 mg/L in fruit juices. The mulberry leaf is a promising dietary source of quercetin due to its relatively high content of that compound (260 mg as aglycone/100 g of fresh weight in our results) compared to the white onion (48-56 mg/100 g of fresh weight) and red onion (40-100 mg/100 g of fresh weight), other known sources of quercetin. Quercetin has been shown to have strong inhibitory effects on oxidative modification of human LDL in vitro. It has also been recognized in rat plasma as sulfate, glucuronide, and sulfoglucuronide conjugates after intragastric administration of quercetin aglycone, and these quercetin conjugates engage in antioxidant activity and inhibit LDL from oxidation induced by copper ions. Hayek et al. reported that dietary consumption of quercetin aglycone by apolipoprotein E-deficient mice attenuates the development of atherosclerotic lesions and prolongs the lag phase for conjugated diene formation of LDL isolated from...
plasma induced by copper ions. In plant foods, quercetin occurs mainly bound to various sugars via α-glycosidic link. Quercetin glucosides are absorbed more easily than aglycone\(^{62}\), suggesting the participation of the mechanism of lactase phloridzin hydrolase (LPH) or sodium dependent glucose transporter (SGLT1)\(^{63}\). As the bioavailability of quercetin malonylglucoside is unknown, further investigation into its metabolism is needed to clarify the effect of dietary consumption of mulberry leaves.

VI. Effects of Mulberry Leaves and Quercetin 3-(6-malonyl)-glucoside on Mice

In our in vitro screen for LDL antioxidant activity, the mulberry leaf showed relatively high antioxidant activity\(^{42}\). Moreover, we identified quercetin 3-(6-malonyl)-glucoside (Q3MG) as the major contributor to antioxidant activity in mulberry leaves\(^{50}\). We also investigated the effects of dietary consumption of mulberry leaves and purified Q3MG on the development of atherosclerotic lesions, in relation to the susceptibility of plasma LDL to oxidative modification (Figure 4)\(^{64}\). For these purposes, we fed an atherogenic diet to LDL receptor-deficient (LDLR-/ -) mice, as these animals were known to develop marked hypercholesterolemia and early-to-intermediate atherosclerotic lesions in response to six to eight weeks of an atherogenic diet. Male mice aged eight weeks were randomly divided into four groups (control, quercetin, Q3MG, and mulberry). The control group was fed an atherogenic diet containing 3% (wt/wt) cholesterol and 15% (wt/wt) cocoa butter. The other three experimental groups were fed the same atherogenic diet supplemented with 0.05% (wt/wt) quercetin for the quercetin group, 0.05% (wt/wt) Q3MG for the Q3MG group, and 3% (wt/wt) dried mulberry-leaf powder for the mulberry group (Fig. 4). All mice were fed their respective diets for eight weeks. The extent of atherosclerosis in the aorta was evaluated by computer-assisted image analysis, and atherosclerotic lesions were found in the aortas, mainly in the aortic arches, of all mice groups. Treatment with either Q3MG or mulberry significantly reduced the atherosclerotic lesion area (5.2%) compared to the control group (Figure 5). However, the atherosclerotic lesion areas in the quercetin-treated mice did not differ from those of the control group.

The susceptibility of LDL to oxidative modification was assessed in the lag phase of conjugated diene formation and by measuring LDL-MDA concentration (Figure 6). The incubation of LDL isolated from the control mice with copper ions resulted in a lag phase of 24 min, whereas LDL isolated from the LDLR-/ - mice treated with Q3MG or mulberry showed a significant prolongation of the lag phase (44.3% and 42.2%, respectively) compared with that of the control mice. However, the lag phase in the quercetin-treated mice did not differ from that of the controls. The LDL-MDA concentration in the Q3MG group noticeably decreased compared with the control group; however, the difference did not reach statistical significance by post hoc analyses. MDA concentration adjusted to concentrations of LDL protein did not differ among the four groups.

Given this knowledge, the following may be surmised (Figure 7). Oxidation of LDL is a free radical-driven lipid peroxidation process, that can be chronologically divided into a lag phase, a propagation phase, and a decomposition phase\(^{65}\). During the lag phase, the polyunsaturated fatty acids (PUFAs) in LDL...
are protected from oxidation by the lipophilic antioxidants, particularly \( \alpha \)-tocopherol, which is the most abundant antioxidant in LDL\(^{49}\). Similarly, flavonoids have been shown to protect LDL from oxidation at the initial stage of lipid peroxidation by acting as free radical scavengers\(^{66}\). In a previous study\(^{42}\), the mulberry leaf showed potent free radical scavenging activity as determined by the DPPH radical scavenging assay. Quercetin and other flavonoids have been shown to bind to the surface of LDL particles via the formation of an ether bond\(^{16}\), limiting access of the oxidants and their initial attack on the surface. These mechanisms are likely responsible for the delayed onset in our study of LDL oxidation in the mice groups consuming quercetin glucoside-rich mulberry leaves or pure Q3MG. In our previous study\(^{60}\), the mulberry leaf showed potent free radical scavenging activity as determined by the DPPH radical scavenging assay. Quercetin and other flavonoids have been shown to bind to the surface of LDL particles via the formation of an ether bond\(^{60}\), limiting access of the oxidants and their initial attack on the surface. These mechanisms are likely responsible for the delayed onset in our study of LDL oxidation in the mice groups consuming quercetin glucoside-rich mulberry leaves or pure Q3MG. Furthermore, in these two groups the prolongation of the lag phase of conjugated diene formation was associated with a reduction in atherosclerotic lesion formation. Studies in humans have also demonstrated the lag phase to be independently associated with severity of coronary atherosclerosis\(^{60}\).

In our study, quercetin, a well-known in vitro antioxidant, showed no protective effects against LDL oxidation and atherosclerotic lesion formation even though daily quercetin intake in the quercetin mice group was higher (2 mg/d) than that of the Q3MG and mulberry (1.2 and 0.4 mg/d) groups, an indication that the concentration in the diet was not material. Differences in the absorption processes of quercetin glucosides and quercetin aglycone may be responsible for the differing effects on LDL oxidation and atherosclerotic lesion formation. Absorption of onion quercetin glucosides, 45% of which is quercetin-4'-glucoside\(^{60}\), was far superior to that of quercetin aglycone\(^{62}\). Moreover, the bioavailability of quercetin-3'-glucoside (Q3G) has been shown to be as high as that of quercetin-4'-glucoside\(^{69}\). Hollman et al. reported that glucosides are transported into the enterocyte by the intestinal glucose carrier sodium-dependent glucose transporter in the small intestine\(^{62}\) and are subsequently hydrolyzed by intracellular cytosolic \( \beta \)-glucosidase\(^{70}\). Another pathway involves lactase phlorizin hydrolase, a glucosidase of the small intestine brush border membrane that catalyzes extracellular hydrolysis of certain glucosides, followed by diffusion of the aglycone across the brush border\(^{70}\). Q3G and Q3MG are not substrates for cytosolic \( \beta \)-glucosidase, but they are absorbed after hydrolysis by lactase phlorizin hydrolase\(^{72}\). Once absorbed, quercetin glucosides produce a rapid, higher plasma peak level and have a very slow elimination half-life\(^{73}\). Thus, we believe the repeated daily supplementation of pure Q3MG and quercetin-glucoside rich mulberry leaves likely leads to a buildup of a sufficient concentration to protect LDL from oxidation. The retardation of the onset of the LDL oxidation and the prevention of atherosclerosis progression in the mulberry-treated mice, despite a lower daily intake of quercetin vis a vis the Q3MG mice group, indicates the possible role of some other flavonoids and biologically active constituents present in mulberry leaves.

The antioxidant activity of quercetin aglycon is much higher than that of quercetin glycoside\(^{61}\), whereas its absorption is much lower than that of quercetin glucoside\(^{62}\). Furthermore, quercetin exists in
the blood stream as a conjugate with glucuronic acid or sulfate after digestion of its glycoside moiety in the intestine\textsuperscript{70}. As the forms of antioxidants existing in crude extracts from edible plants and in the blood after absorption \textit{in vivo} are quite different and since the absorption of quercetin glycosides depends on their glycoside moiety\textsuperscript{70}, the applications of the LDL oxidation assay \textit{in vitro} is limited. Systematic methods of measurement of the absorption of crude extract antioxidants from edible plants using an intestinal model such as cultured Caco-2 cell monolayers\textsuperscript{70} and of the antioxidant activity of absorbed compounds are needed.

VII. Conclusions

The traditional Japanese diet with its high intake of fruits and vegetables that are rich in antioxidants is believed to effectively ward off cardiovascular disease. Since a large body of observational data suggests that dietary antioxidants are associated with a lower risk of cardiovascular diseases, we found more effective antioxidants from Japanese edible plants for the prevention of cardiovascular disease. Dietary consumption of mulberry leaves and/or Q3MG enhanced the resistance to oxidative modification of LDL and attenuated atherosclerotic lesion development.

However, the outcome of intervention trials has also suggested that single antioxidants, such as vitamin E, vitamin C, or \(\beta\)-carotene had little effect on the risk of developing cardiovascular disease\textsuperscript{70}. The biological basis for decreased disease risk does not guarantee positive outcomes of targeted interventions. First, unanticipated confounding factors operating in uncontrolled environments intervene and are difficult to anticipate. Second, the atherogenic process starts early in development, so unsuccessful interventions in adults may have been successful if initiated earlier in life. Third, food is a mixture of compounds that we can classify, identify the putative compounds, and test their efficacy. The beneficial effects of the high intake of fruits and vegetables on cardiovascular disease may depend on the action of lesser-known antioxidant compounds rather than on any one well-known single compound or perhaps on the synergic effects of the different antioxidants or non-antioxidant compounds present in foods. The synergic effect of certain combinations may determine outcome. It is necessary to widen our understanding of the synergic effects of diets and nutrient-gene interactions related to nutrient /disease risk.

References


16) Hayek T, Fuhrman B, Vaya J, \textit{et al.} Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine,
or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. Arterioscler Throm Vasc Biol 1997 ; 17 : 2744–2752.


