Macrophage as a Target of Quercetin Glucuronides in Human Atherosclerotic Arteries
IMPLICATION IN THE ANTI-ATHEROSCLEROTIC MECHANISM OF DIETARY FLAVONOIDS

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Epidemiological studies suggest that the consumption of flavonoid-rich diets decreases the risk of cardiovascular diseases. However, the target sites of flavonoids underlying the protective mechanism in vivo are not known. Quercetin represents antioxidative/anti-inflammatory flavonoids widely distributed in the human diet. In this study, we raised a novel monoclonal antibody 14A2 targeting the quercetin-3-glucuronide (Q3GA), a major antioxidative quercetin metabolite in human plasma, and found that the activated macrophage might be a potential target of dietary flavonoids in the aorta. Immunohistochemical studies with monoclonal antibody 14A2 demonstrated that the positive staining specifically accumulates in human atherosclerotic lesions, but not in the normal aorta, and that the intense staining was primarily associated with the macrophage-derived foam cells. In vitro experiments with murine macrophage cell lines showed that the Q3GA was significantly taken up and deconjugated into the much more active aglycone, a part of which was further converted to the methylated form, in the activated macrophages. In addition, the mRNA expression of the class A scavenger receptor and CD36, which play an important role for the formation of foam cells, was suppressed by the treatment of Q3GA. These results suggest that injured/inflamed arteries with activated macrophages are the potential targets of the metabolites of dietary quercetin. Our data provide a new insight into the bioavailability of dietary flavonoids and the mechanism for the prevention of cardiovascular diseases.

Flavonoids are widely distributed in plant foods and beverages and therefore are regularly ingested with the human diet. In 1936, Rusznyak and Szent-Gyögyi (1) found citrus flavonoids reduced capillary fragility and permeability in blood vessels. Thereafter, a large number of biological activities of flavonoids have been described which overall are believed to be beneficial for good health. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a prime example of such a flavonoid and is bound to sugars in foods, mainly as β-glycosides. The quercetin glycosides occur in broccoli, apples, and especially in onions, with an abundance as high as 0.25–0.5 g/kg (2). The average daily intake of the flavonoids subclasses in The Netherlands is 23 mg (calculated as aglycones) of which quercetin supplies 16 mg (3). Epidemiological evidence links diets rich in quercetin with decreased incidence of cardiovascular and neoplastic diseases (4–9). Because oxidative stress has been implicated in the pathogenesis of these diseases, the bioavailability of quercetin and other flavonoids has been investigated in relation to their antioxidant activities in vivo. The antioxidant potential of quercetin is related to the number and position of the free hydroxyl groups in the molecule (10); therefore, the regioselectivity of conjugation of the hydroxyl groups can be expected to modulate the biological activity of quercetin. Upon ingestion with the diet, quercetin glycosides are rapidly hydroyzed during passage across the small intestine or by bacterial activity in the colon to generate quercetin aglycone, which is further metabolized in the so-called phase II reactions into the glucuronidated and/or sulfated derivatives. Alternatively, 3′- or 4′-hydroxyl group in the B-ring catechol moiety can also be methylated by catechol-O-methyltransferase (COMT) activity. Previous reports have clearly shown that quercetin-3-glucuronide (Q3GA) and quercetin-3-sulfate are the major quercetin conjugates in rat and human plasma, in which aglycone could not be detected (11, 12). Although the biological activities of quercetin generally attenuate after conversion to the metabolites, the physiologically conceivable activities associated with oxidative stress for various quercetin metabolites have been reported (13). It is expected that Q3GA represents the radical scavenging actions of quercetin metabolites in vivo (12), because it retains the cat-

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2 The abbreviations used are: COMT, catechol-O-methyltransferase; Q3GA, quercetin-3-O-β-D-glucuronide; HPLC, high performance liquid chromatography; HSA, human serum albumin; LPS, lipopolysaccharide; KLH, keyhole limpet hemocyanin; BAECs, bovine aortic endothelial cells; ECD, electron chemical detection; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; GDP, glucose; glycerol; lysophosphatidylcholine; phospholipase C; RT, reverse transcription; SR-A, the class A scavenger receptor; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; LDL, low density lipoprotein; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; SR-A, scavenger receptor A; HBSS, Hanks’ balanced salt solution; IR3G, isorhamnetin-3-glucoside.

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Echol moiety (3’, 4’-o-dihydroxy group) responsible for radical scavenging of quercetin even after the enzymatic metabolism.

Although the tissue distributions of quercetin and its metabolites have been assessed by a number of authors, only limited information on the localization of quercetin metabolites in the aorta is presently available. We have previously demonstrated that quercetin metabolites were present in the aorta of high cholesterol/quercetin glucoside-fed rabbits (14), in which cholesterol accumulation in the aorta was significantly inhibited, suggesting the anti-atherosclerotic action of the quercetin metabolites in the aorta. To further understand the mechanism of the anti-atherosclerotic action of quercetin, it is necessary to know the target sites of the quercetin metabolites in the aorta. At present, a chromatographic technique such as high performance liquid chromatography (HPLC) or gas chromatography combined with mass spectrometry or electrochemical detection is the only analytical approach for evaluating quercetin and other flavonoids in biological samples. The immunochemical technique is a powerful tool for evaluating the localization of target molecules in tissue/cellular components; therefore, we developed a monoclonal antibody directed to a quercetin metabolite, Q3GA, and identified the activated macrophage cells as the potential target of dietary flavonoids in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**—Quercetin dihydrate, acetobromo-α-D-glucuronic acid methyl ester, human serum albumin (HSA), lipopolysaccharides (LPS, from Escherichia coli), saccharic acid 1,4-lactone, and dinitrocatechol were purchased from Sigma. Quercetin-3-O-β-D-glucoside, quercetin-4’-O-β-D-glucoside, quercetin-3-O-sulfate, hyperoside, rutin, isorhamnetin (3’-methyl quercetin), and cyanidin-3-O-β-D-glucoside were obtained from Extrasynthese (Genay, France). Succinic anhydride was purchased from Wako Pure Chemicals (Osaka, Japan). Keyhole limpet hemocyanin (KLH), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, and N-hydroxysuccinimide were obtained from Pierce. Quercetin-3′-O-β-D-glucuronide (Q3GA) was chemically synthesized as reported previously (12). Monoclonal murine antibody to scavenger receptor A (SR-A) was obtained from TransGenic Inc. (Hyogo, Japan). Rabbit polyclonal antibodies to CD36 and β-actin were obtained from Santa Cruz Biotechnology and BioLegend, respectively.

**Cell Culture**—Bovine aortic endothelial cells (BAECs) (15) were cultured in Medium 199 (Sigma) containing 20% fetal bovine serum (FBS). RAW264 cell line was obtained from the Riken Cell Bank (Tsukuba, Ibaraki, Japan) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS. J774-1 was obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Miyagi, Japan). Cells were cultured in an atmosphere containing 5% CO2 at 37 °C. All media contain 100 μg/ml penicillin and 100 units/ml streptomycin.

**Analysis of Quercetin Metabolites in Human Plasma**—Healthy volunteers (provided informed consent) that fasted overnight were served 350–500 g of cooked onion paste (provided by Kagome Research Institute, Tochigi, Japan) roasted with salad oil. The onion amounts were designed so as to be almost the same per their body weights (~7 g/kg). The quercetin content in the onion paste was analyzed by HPLC (16) after the acid hydrolysis of the quercetin mono- and di-glucosides in the methanolic extract of the paste and determined to be ~32.9 mg/100 g to onion paste (as the equivalent for the quercetin aglycone). Before and 1.5 h after intake, heparinized blood was collected from each subject, and the plasma was obtained by centrifugation. The quercetin metabolites in the plasma were extracted with 5 volumes of methanol, and the methanolic fractions were evaporated under an N2 stream and dissolved in 20% aqueous acetonitrile containing 0.5% phosphoric acid. For analysis of aglycone, plasma samples were mixed with an equal volume of ethyl acetate and centrifuged. After two extractions, the ethyl acetate layers were collected, evaporated under an N2 stream, and dissolved in HPLC solvent. Ten μl of the sample was injected into an HPLC-electrochemical detection (ECD) system (ESA, Cambridge, MA) equipped with a TSK-gel ODS-80Ts column (4.6 × 150 mm). The separation of the compounds was carried out by a gradient elution. Solvent A was 20% aqueous acetonitrile containing 0.5% phosphoric acid, and solvent B was 100% acetonitrile containing 0.5% phosphoric acid. The gradient program was as follows: 0–10 min, 1% B; 10–20 min, linear gradient to 25% B; 20–25 min, linear gradient to 1% B; 25–30 min, hold; flow rate, 0.8 ml/min. Electrochemical detection was performed with a coulometric electrode at 150 mV.

**Cell-mediated LDL Oxidation**—LDL (d = 1.063–1.093 g/ml) was isolated from healthy volunteers by sequential ultracentrifugation and dialyzed in phosphate-buffered saline. The LDL concentrations were determined by measuring the protein contents using a BCA protein assay kit (Pierce). BAECs in a 60-mm dish were treated with LDL (200 μg/ml in PBS-free Medium 199) in the presence of 5 μM Cu2+ and the different concentrations of Q3GA and the related compounds at 37 °C. After incubation, the oxidation reaction was terminated by adding 0.1 volume of 1 mM EDTA, 10 μM 2,6-di-tert-butyl-p-cresol solution. The LDL oxidation was measured as the thiobarbituric acid-reactive substances with fluorescent detection (excitation 515 nm, emission 553 nm). Tetraethoxypropane was used as the standard compound that readily decomposes into the thiobarbituric acid-reactive malondialdehyde, a representative aldehyde formed in oxidized LDL, during the assay processes.

**Preparation of Monoclonal Antibody to Q3GA**—To prepare the immunogen, the synthesized Q3GA was conjugated with KLH by a carbodiimide procedure. Briefly, the carboxylic derivatives of Q3GA (11.0 μmol) was activated by incubating with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (11.0 μmol) in the presence of N-hydroxysuccinimide (11.0 μmol) in dimethylformamide (200 μl) at room temperature overnight. A 100-μl aliquot of the mixture was added to 360 μl of KLH (10 mg/ml) or HSA (10 mg/ml) in phosphate-buffered saline (PBS) and incubated at room temperature for 4 h. After incubation, the proteins were dialyzed to PBS at 4 °C for 2 days. The obtained Q3GA-KLH conjugate (0.6 mg/ml in PBS) was emulsified with an equal volume of adjuvant. Six-week-old female BALB/c mice were intraperitoneally immunized with this emulsion (100 μl). The mice were repeatedly boosted with...
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the immunogens (0.2 mg/ml) emulsified with an equal volume of adjuvant every 2 weeks. In the final boost, 100 μl of the immunogens (0.5 mg/ml in PBS) without adjuvant was intravenously injected. Three days after the final boost, one of the mice was sacrificed, and the spleen was removed. The spleen cells were fused with P3U1 myeloma cells in the presence of polyethylene glycol 1500 (Roche Applied Science) and cultured in the hypoxanthine/aminopterin/thymidine medium for the selection of the hybridomas. After a week, the immunoreactivities of the culture supernatants were screened by enzyme-linked immunosorbent assay (ELISA) as follows. Fifty μl of the antigens (5 μg/ml) in PBS were coated in wells and incubated at 37 °C for 1 h. After washing three times with PBS containing 0.05% Tween 20 (TPBS), the wells were blocked with a 4% aqueous solution of Block Ace (Dainihon Seiyaku, Osaka, Japan) at 37 °C for 1 h. After washing, 100 μl of the primary antibody in TPBS was added, and the wells were incubated at 37 °C for 2 h. After washing, 100 μl of the peroxidase-labeled anti-mouse IgG goat antibody (Chemicon International, Temecula, CA) with a 1:5000 dilution was added and incubated at 37 °C for 1 h. The color-developing reaction was performed by the addition of 100 μl of the TMB substrate solution (within TMB substrate reagent set, BD Biosciences). The binding of the antibody to the antigen was evaluated by measuring the absorbance at 450 nm. The immunoreactive hybridomas were then cloned by the limited dilution method. After repeated screening and cloning, a monoclonal antibody mAb14A2 was finally obtained. The antibody was purified by ammonium sulfate precipitation from the culture supernatant and used in the following experiment.

Competitive ELISA—For competitive ELISA, the reaction of the primary antibody was carried out in the absence or presence of competitors. The competitive reactions were performed in PBS containing 1% HSA at 37 °C for 90 min. The cross-reactivity of the antibody to the competitors was expressed as $B/B_0$, in which $B$ is the amount of the antibody bound to the coating antigen in the presence of the competitor, and $B_0$ is in the absence of a competitor.

Immunohistochemistry—This investigation was carried out on aortic wall samples obtained during autopsy from patients with generalized arteriosclerosis. Each autopsy was performed at Tokyo Women’s Medical University after the patients’ family members granted informed consent according to the established guideline. Each sample was prepared for 10% formalin-fixed, paraffin-embedded materials and for frozen materials embedded in the optimum cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) at ~80 °C. Multiple 6–μm-thick sections were cut from these paraffin-embedded and frozen materials and used for the histopathological and immunohistochemical examinations. The paraffin-embedded sections were deparaffinized in xylene and ethanol, rehydrated in distilled water. Frozen sections were dried, postfixed or not in 10% formalin, and rehydrated. These prepared sections were quenched for 10 min with 3% hydrogen peroxide for inhibiting the endogenous peroxidase activity, rinsed in PBS, pretreated for 30 min at room temperature with 5% skim milk in PBS, and treated with the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). Sections were then incubated overnight at 4 °C with the primary antibodies such as the mAb14A2 at a dilution of 1:200 and mouse monoclonal IgG1 against CD68 (Clone KP-1; DakoCytomation, Kyoto, Japan) at a dilution of 1:10,000. Sections processed with omission of the primary antibodies or incubated with 5% skim milk in PBS served as negative reaction controls. Antibody binding was visualized by the avidin-biotin-immunoperoxidase complex method using the appropriate Vectastain ABC kit (Vector Laboratories). Immunohistochemical localization of Q3GA was verified by comparison of consecutive sections stained with hematoxylin-eosin and immunostained for CD68. 3,3’-Diaminobenzidine tetrahydrochloride was used as the chromogen, and hematoxylin was used as the counterstain. Immunostained sections were observed with a light microscope (Olympus, Tokyo, Japan). In addition, the location of Q3GA immunoreactivity in macrophages was strictly identified by the double immunofluorescence method on frozen sections. In brief, sections were postfixed for 10 min at 4 °C in 100% acetone, rehydrated, rinsed in PBS, pretreated for 10 min at room temperature with 5% skim milk in PBS, and incubated overnight at 4 °C with the mAb14A2 and rabbit polyclonal IgG against CD68, simultaneously (catalog number sc-9139; Santa Cruz Biotechnology, Santa Cruz, CA). Sections were then rinsed in PBS and incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) at the same time. Double-immunostained sections were observed with a fluorescence microscope (Nikon, Tokyo, Japan). The appearance of yellowish signals at merging FITC (green) and Cy3 (red) was considered as the co-localization of Q3GA and CD68.

Competitive experiments to confirm the specificity of the immunostaining with mAb14A2 were also performed with the antibody in the presence of 100 μM Q3GA. Normal mouse IgG was used as the negative control. Immunohistochemical localization of the immunoreaction product deposits was verified by light microscopy on consecutive sections with hematoxylin-eosin or immunostained for CD68. To confirm the immunostaining of the conjugate metabolites with mAb14A2, the sections were treated for 60 min with β-glucuronidase (>600 units/ml) from Helix pomatia in 0.1 M sodium acetate buffer, pH 5.0, prior to the reaction with mAb14A2.

Analysis for Cellular Uptake of Quercetin-3-glucuronide—RAW264 macrophages were grown to confluence in DMEM containing 10% FBS on 60-mm dish in an atmosphere containing 5% CO2 at 37 °C. The cells were treated with or without LPS (1 μg/ml) in 2 ml of DMEM with 10% FBS. After a 24-h incubation, the cells were washed twice with 1 ml of FBS-free media, after which the media were exchanged with FBS-free DMEM containing 20 μM Q3GA. Following a 4-h incubation, the cell were washed three times with 1 ml of Hanks’ balanced salt solution (HBSS), scraped from the dish, and resuspended in 200 μl of methanol/acetic acid (100:1). Q3GA and its cellular metabolites were then extracted by sonication for 1 min using an Astron XL2020 ultrasonic processor (Heat Systems-Ultrasonics, Farmingdale, NY) at level 6. After centrifugation, the supernatants were collected, evaporated under an N2 stream, and dissolved in 20% aqueous acetonitrile containing 0.5% phosphoric acid. The samples were injected into the HPLC-ECD system as already described. Quantitation of quercetin...
compounds was performed using standard curves developed by the peak areas of authentic compounds (Q3GA, quercetin, and isorhamnetin).

Immunocytochemical detection of Q3GA with mAb14A2 was also performed. Cells were cultured on coverslips in a 24-well plate. After treatment of cells with or without LPS/Q3GA as described above, cells were washed with HBSS three times and then fixed for 10 min in 4% paraformaldehyde in PBS on ice. To prevent nonspecific antibody binding, the cells were washed twice in PBS and blocked for 1 h at room temperature with 1% skim milk in PBS. Membranes were permeabilized by exposing the fixed cells to PBS containing 0.2% Triton X-100 for 2 min on ice. The cells were then incubated in the primary antibody (mAb14A2) in PBS containing 3% bovine serum albumin overnight at 4 °C. The cells were then incubated for 1 h in the presence of FITC-labeled anti-mouse IgG (Dako Japan Co., Ltd., Kyoto, Japan), rinsed with PBS, and mounted on glass slides using Dako Cytomation fluorescent mounting medium. Images of cellular immunofluorescence were acquired using a Leica TCS-NT confocal laser scanning microscope. The DNA was also stained with propidium iodide.

β-Glucuronidase Activity—Intracellular β-glucuronidase activity was measured by a colorimetric analysis using phenolphthalein mono-β-glucuronide (Sigma) as the substrate. Briefly, 30 μg of cell-free extracts, prepared by repeated freezing and thawing of cells, were mixed with 0.6 mM phenolphthalein mono-β-glucuronide in 100 μL of 0.1 M sodium phosphate buffer, pH 5.0. After incubation at 37 °C for 30 min followed by adding 200 μL of 0.1 M sodium phosphate buffer, pH 11.0, the absorbance at 550 nm indicating the formation of phenolphthalein aglycone was measured.

Extracellular activity was evaluated using Q3GA as the substrate. The culture medium was removed from the dishes and then incubated with 50 μM Q3GA at 37 °C for 1 h. After incubation, the formed aglycone was extracted twice with ethyl acetate, evaporated, and dissolved in mobile phase for HPLC-ECD analysis. Ten ml of the samples was injected into HPLC-ECD system as described above. The activity was expressed as the conversion rate (%) of Q3GA into the aglycone.

Expression of Scavenger Receptors in Macrophage Cells—The RAW264 cells cultured in a 35-mm dish were washed twice with FBS-free DMEM, after which the media were exchanged with FBS-free DMEM containing the indicated concentrations of Q3GA dissolved in 0.1 M sodium phosphate buffer, pH 11.0, the absorbance at 210 nm.

Immunoblot Analysis—The cells were washed twice with HBSS and lysed with RIP lysistrain buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride. The protein samples were boiled with reducing sample buffer for 5 min. The samples (10 μg) were run on 10% SDS-polyacrylamide gels, transferred to a poly(vinylidene) fluoride membrane (Hybond-P, GE Healthcare), incubated at room temperature for 1 h with a blocking reagent (EzBlock, ATTO Corp., Tokyo, Japan) in TTBS (Tris-buffered saline containing 0.05% Tween 20) for blocking, washed in TTBS, and treated with primary antibody at 4 °C overnight. After washing, blots were further incubated for 1 h at room temperature with secondary antibody coupled to horseradish peroxidase in TTBS. After washing, the membrane was visualized by using ECL-Plus detection reagent.

Cholesterol Accumulation in RAW264 Cells—RAW264 cells in 35-mm dish were treated with or without Q3GA in FBS-free DMEM for 24 h. After washing twice with HBSS, cells were treated with oxidized LDL (0.2 mg/ml) for 4 h. After washing, total cholesterol was extracted three times with 1 ml of n-hexane/isopropyl alcohol (3:2, v/v). The extracts were dried up and then saponified in 10% KOH/ethanol (1:9, v/v) at 90 °C for 1 h. Free cholesterol was extracted with 1 ml of ether, evaporated, and then dissolved in ice-cold acetone. Then 20 μl of the supernatant was injected onto TSK-gel Octyl-80T’s column (4.6 × 150 mm) equilibrated with acetonitrile/methanol/water (46:45:9) at a flow rate of 1 ml/min with UV detection at 210 nm.

Target Sites of Quercetin Glucuronides in Aorta
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A

for metabolites

for aglycone

B

C

Q3GA

IR3G

Statistical Analysis—Data from real time PCR and cholesterol accumulation were expressed as the mean ± S.D. Comparisons were analyzed with the Student’s t test. A p value < 0.05 was considered statistically significant.

RESULTS

Q3GA as a Major Quercetin Metabolite in Human Plasma—It is known that most of the quercetin is metabolized to the glucuronides, sulfates, and/or methylated form during absorption and circulation (17), and therefore the quercetin aglycone could not be detected in human and rat plasma (11). To examine the quercetin metabolites in vivo, we analyzed human plasma obtained before and after the intake of onion (quercetin equivalent, 2.3 mg/kg body weight), one of the major quercetin sources in foods, using HPLC with electrochemical detection. It was confirmed that Q3GA could be one of the major electrophoretically active metabolites (Fig. 1A, left). Quercetin aglycone and the methylated form (3′- or 4′-methylated quercetin) could not be detected in the plasma (Fig. 1A, right). The concentration of Q3GA in human plasma after intake was estimated to be 264 ± 46 nM (n = 4). An electrochemical response is associated with the oxidizability of compounds that may reflect the free radical scavenging activity, and therefore Q3GA may represent the major antioxidative metabolites of quercetin in human plasma. We have shown that Q3GA significantly inhibited the LDL oxidation mediated by BAECs in a dose-dependent manner (Fig. 1B, left). Endothelium-mediated LDL oxidation is accepted to be an experimental model that reflects the LDL oxidation in vivo, because it is known that vascular cells metabolically modify the LDL (reviewed in Ref. 18), generating a form that is taken up by the macrophages. The uncontrolled uptake of the modified LDL by macrophage scavenger receptors leads to foam cells and plaques. It is generally known that the catechol moiety (3′,4′-dihydroxyl group) is responsible for radical scavenging of quercetin and other polyphenols. Indeed, a 3′-methylated analogue isorhamnetin-3-glucoside (IR3G, see Fig. 1C) could not inhibit the LDL oxidation (Fig. 1B, right). These results suggest that Q3GA is a major quercetin metabolite that plays a protective role against the oxidative modification of plasma LDL constantly exposed to endothelial cells. Several electrochemically active peaks were also detected in Fig. 1A. Although we have not yet identified these peaks, Day et al. (11) have reported the identification of quercetin metabolites in human plasma. In addition to Q3GA, the report identified the B-ring glucuronides (3′- or 4′-), 3′-methylated glucuronides, di-glucuronides, and 3′-sulfate as the major metabolites. Based on the report previously published (11), the peak eluted at 6 min is presumed to be a quercetin-diglucuronide. The two peaks at 16–17 min are presumed to be glucuronide isomers (3′- or 4′-glucuronide), methylated quercetin glucuronides, or 3′-sulfate. Further characterization is required in the future.

Development of mAb to a Quercetin Glucuronide—To further examine the localization and actions of quercetin metabolites in the aorta, we developed a novel monoclonal antibody targeting Q3GA. The immunogen was chemically synthesized by the conjugation of Q3GA with KLH (Fig. 2A) and injected into BALB/c mice. We finally obtained a monoclonal antibody (mAb14A2) and examined the specificity by competitive ELISA. The mAb14A2 significantly recognized Q3GA, but not the quercetin aglycone, quercetin-3-sulfate, and 3′-methylated metabolite isorhamnetin (Fig. 2B). It was found that the mAb14A2 also reacted with several quercetin-3-glycosides, including glucoside, galactoside (hyperoside), and rutinoside (rutin) (data not shown). The antibody could distinguish quercetin-3-glucoside from quercetin-4′-glucoside and from cyanidin-3-glucoside, an anthocyanin glucoside (Fig. 2C). Sugar moiety itself was not recognized by the antibody (data not shown). It was also confirmed that the antibody did not react with the antioxidative molecules present in vivo (catechins, vitamins E and C, reduced/oxidized glutathiones, and β-carotene) (Fig. 2, D and E). These results suggest the requirement of both a flavon backbone and its 3-glycosidation for the recognition of the mAb14A2. The 3′-methylated quercetin-3-glucoside (isorhamnetin-3-glucoside, IR3G) is also recognized by the antibody (supplemental Fig. S1A), indicating that the presence or absence of methylation might not be important for the recognition of the antibody. It has been reported that the quercetin glycosides in foods are absorbed as the aglycone after hydrolysis in the small intestine and metabolized via glucuronidation, sulfation, and/or methylation after absorption (19); therefore, no detectable glycosides (plant form) and aglycone could be observed in human plasma (11). These observations indicate
that Q3GA and the methylated analogue (including IR3GA) are the potential epitope of the mAb14A2 in vivo.

Localization of Quercetin Glucuronides in Human Aorta—To examine the localization of quercetin metabolites associated with the anti-atherosclerotic effects in the aorta, human aortic tissues were immunohistochemically examined using mAb14A2. The immunoreaction products with the antibody were detectable in atherosclerotic lesions (Fig. 3, A and B). The immunoreactivity was mainly localized in the cytoplasm of the macrophage-derived foam cells identified by the specific marker CD68 (Fig. 3C). The localization in macrophages was also shown at higher magnifications (Fig. 3, G and H). The endothelial cells, smooth muscle cells, and extracellular matrix in the subendothelial layer, the so-called intima, were also positively stained with mAb14A2. In contrast, no significant immunoreactivity with the antibody was obtained in normal-appearing regions (Fig. 3, E and F). The preabsorption of mAb14A2 with an excess of Q3GA abolished the positive staining (Fig. 3D), indicating the specific reactivity of this antibody with the epitope. Although we here demonstrated the immunoreactivity with the antibody was obtained in normal aorta are shown: immunostaining with mAb14A2 (A), anti-CD68 (C), hematoxylin-eosin staining (B), and competitive immunostaining with mAb14A2 in the presence of Q3GA (D). Consecutive sections of human normal aorta are shown: immunostaining by mAb14A2 (E) and hematoxylin-eosin staining (F). Original magnification is ×20. Representative photomicrographs showing the co-localization (indicated with arrowheads) of the immunoreactive materials for anti-Q3GA (G) and anti-CD68 (H) extracted by magnification from A and C, respectively. The intimal area with intense staining is indicated in A.

Atherosclerotic lesion contains many inflammatory cells that express many peroxidase-like enzymes. In addition, excessive fixation with formalin may lead to nonspecific background staining. To strictly confirm the specific staining and localization of Q3GA in the lesions, we performed additional immunostaining of nonfixed frozen sections using fluorescence-labeled secondary antibody. Immunofluorescence microscopy revealed the co-localization of the Q3GA and CD68 determinants on the macrophages in the lesions (Fig. 4A). No immunoreaction product was detectable on negative reaction control sections.

We further examined whether the mAb14A2 indeed recognized the glucuronide metabolites of quercetin in the human aorta, because the antibody weakly cross-reacted with higher concentrations (>100 µM) of quercetin aglycone (data not shown). The pretreatment of aortic sections with β-glucuronidase prior to incubation with mAb14A2 significantly attenuated the staining in the atherosclerotic lesions (Fig. 4B), showing the presence of the glucuronide metabolites in the lesions.

FIGURE 2. Development of anti-Q3GA monoclonal antibody. A, scheme for the preparation of Q3GA-protein conjugate as the immunogen. Q3GA was chemically synthesized by Koenigs-Knorr reaction and then coupled with KLH by a carbodiimide procedure. B, cross-reactivity of mAb14A2 with quercetin metabolites determined by competitive ELISA. Q3GA, solid circles; quercetin-3-O-sulfate, solid squares; quercetin, open circles; isorhamnetin (3′-O-methylated quercetin), open triangles. C, cross-reactivity of mAb14A2 with flavonoid glycosides. Quercetin-3-glucoside, solid circles; quercetin-4′-glucoside, open circles; cyanidin-3-glucoside, open triangles; daizein-7-glucoside, solid triangles. D, cross-reactivity of mAb14A2 with catechins (four major green tea catechins). E, cross-reactivity of mAb14A2 with various antioxidants found in vivo.

FIGURE 3. Immunohistochemical detection of Q3GA in human aorta. Photomicrographs of human aortic sections processed by hematoxylin-eosin staining and immunohistochemical staining. Consecutive sections of human atherosclerotic aorta are shown: immunostaining with mAb14A2 (A), anti-CD68 (C), hematoxylin-eosin staining (B), and competitive immunostaining with mAb14A2 in the presence of Q3GA (D). Consecutive sections of human normal aorta are shown: immunostaining by mAb14A2 (E) and hematoxylin-eosin staining (F). Original magnification is ×20. Representative photomicrographs showing the co-localization (indicated with arrowheads) of the immunoreactive materials for anti-Q3GA (G) and anti-CD68 (H) extracted by magnification from A and C, respectively. The intimal area with intense staining is indicated in A.
Our data clearly showed that the quercetin glucuronides specifically accumulated in atherosclerotic lesions in human aorta, especially in the macrophage-derived foam cells. The reproducibility of the immunostaining with mAb14A2 was also confirmed in several separate examinations.

**In Vitro Accumulation of Q3GA in Macrophage Cells**—Immunohistochemical staining suggested that the quercetin glucuronides specifically accumulate in the atherosclerotic lesions especially in macrophage-derived foam cells (Fig. 3). We then examined the accumulation of Q3GA in macrophage cells using the RAW264 murine macrophage-like cell line, and we confirmed the dose- and time-dependent accumulation of Q3GA (Fig. 5A). In addition to Q3GA, we also detected the quercetin aglycone and the methylated quercetins (the mixture of 3'- and 4'-methylated quercetin) (Fig. 5B), showing the presence of β-glucuronidase and COMT activity. The β-glucuronidase-catalyzed deconjugation of flavonoid glucuronides has been suggested at the site of inflammation (20, 21). We found that LPS stimulation of RAW cells for 24 h resulted in the significant accumulation of Q3GA and the aglycone (Fig. 5, B and C). In contrast, the accumulation of the methylated quercetins was significantly attenuated in the stimulated cells, showing that the 24-h stimulation down-regulated the COMT activity. However, co-treatment of Q3GA and LPS resulted in the time-dependent increase in both deconjugation and methylation (data not shown). The increased uptake of Q3GA in LPS-stimulated RAW cells was also confirmed by immunocytochemical staining with mAb14A2 (Fig. 5D). The localization of Q3GA in the cytoplasm was confirmed by comparison with the nuclei staining with propidium iodide (data not shown). The increased β-glucuronidase (both extracellular and intracellular) activity of RAW cells was also confirmed during the stimulation with LPS (Fig. 5E). These results showed that the quercetin glucuronides preferentially accumulate and are converted into the more active aglycone or the methylated form in the activated macrophages. Similar results
Q3GA Inhibits the mRNA Expression of Macrophage Scavenger Receptors—Early atherosclerotic lesions are characterized by the massive accumulation of lipid-laden foam cells in the subendothelial space of the arteries. To examine the biological consequences of the co-localization of Q3GA with the macrophage-derived foam cells, the effects of Q3GA on the expression of two major scavenger receptors, SR-A and CD36, were determined in RAW264 cells. We have shown that Q3GA dose-dependently suppressed the mRNA basal expression of SR-A and CD36 (Fig. 6A, left) by RT-PCR analysis. In addition, Q3GA inhibited the overexpression of these scavenger receptors induced by oxidized LDL (Fig. 6A, right). The inhibitory effect was more significant for SR-A in both experiments. The similar results were also obtained using quantitative real time RT-PCR (Fig. 6B). Furthermore, we confirmed the effect of Q3GA on the protein expression by immunoblot analysis (Fig. 6C). To determine whether Q3GA indeed inhibits the formation of foam cells via the suppression of scavenger receptor expression, we analyzed the cellular accumulation of cholesterol derived from oxidized LDL. As shown in Fig. 6D, the significant accumulation of cholesterol in cells was observed upon treatment of oxidized LDL. Pretreatment of cells with Q3GA dose-dependently inhibited the LDL cholesterol accumulation in RAW cells. These observations suggest that quercetin metabolites, if accumulated in macrophage cells, may exert their anti-atherosclerotic activities, including the inhibition of scavenger receptor expression and the subsequent foam cell formation in aorta.

As shown above, glucuronide metabolites could be deconjugated into the aglycone with or without methylation (Fig. 5). A time course RT-PCR experiment showed that the SR-A expression in RAW cells treated with Q3GA was suppressed after a 12-h incubation (supplemental Fig. S1B), indicating the requirement of deconjugation reaction as observed in Fig. 5E. The contribution of the metabolic pathway to the inhibition of the expression of scavenger receptors was examined using specific inhibitors for β-glucuronidase (saccharic acid 1,4-lactone) and COMT (dinitrocateloch). It is of interest that the COMT inhibitor attenuated the inhibitory effect of Q3GA on the inhibition of SR-A expression (Fig. 7A, left). A similar observation was also observed in the treatment of cells with quercetin aglycone (Fig. 7A, right). Upon the same experimental conditions, we also analyzed the accumulation of quercetin compounds in the cells and found that methylated aglycone (3’- or 4’-methylated quercetin) is the major form accumulated in cells (Fig. 7B, left). Because the maximal concentration of Q3GA was quite lower than that of methylated quercetin, the extracellular deconjugation rather than intracellular deconjugation might be the major pathway for the accumulation of aglycone and the methylated form. Unfortunately, we found that the β-glucu-
ronidase inhibitor could not fully act during the incubation periods because it was relatively unstable (data not shown). We confirmed the complete inhibition of COMT activity upon treatment of the inhibitor by the observation that methylated products were scarcely detected in the cell extracts (Fig. 7B, right). These observations suggest the requirement of methylated metabolites for the inhibition of the expression of scavenger receptors.

**DISCUSSION**

Although numerous experimental studies indicating the beneficial effects of dietary flavonoids for health have been reported, the cellular distributions of the flavonoids in vivo underlying their biological activities have not yet been clarified. Flavonoids, if the aglycone shows biological activities in vitro, are generally metabolized via the phase II detoxification pathway during absorption by lowering their activities because of conjugation of the phenolic hydroxyl groups (22). Indeed, the quercetin aglycone could not be detected in human plasma (11). The question now arises: How do flavonoids prevent diseases in vivo? To resolve the question, we developed a novel monoclonal antibody (mAb14A2) targeting the quercetin metabolite, Q3GA, and successfully demonstrated the target sites of the metabolite that specifically accumulates in atherosclerotic lesions in human arteries (Figs. 3 and 4). We have also shown that the intense staining with mAb14A2 was primarily co-localized with macrophage-derived foam cells (Fig. 3, G and H). These results demonstrated for the first time that immunochemical detection is a powerful tool for evaluating the localization of flavonoid metabolites in vivo and that the macrophage cells could be the potential cellular target of the metabolites in the aorta.

The endothelial injury, activation, or dysfunction is an early event during the development of atherosclerosis (23). During the endothelial injury, the increased permeability of the endothelial cells has been observed with the reduced barrier function (24). Our current data showed that a quercetin metabolite specifically accumulates in the injured aorta with atherosclerotic plaques. It is of interest that immunoreactive materials with mAb14A2 could not be observed in the normal aorta. These results suggest that the quercetin glucuronides specifically interact with damaged sites in arteries. Mochizuki et al. (25) has reported similar in vitro observations that Q3GA can pass through the human aortic endothelial cells stimulated with interleukin-1α. These observations suggest that quercetin metabolites in circulating blood can permeate through the injured/activated endothelial cells and interact with the subintimal cells, such as the macrophages and smooth muscle cells. The accumulation of Q3GA in the macrophages in the atherosclerotic lesions was also reproduced in the cultured macrophage-like cell lines in vitro. We have found that the LPS-stimulated RAW264 cells, as compared with nonstimulated cells, significantly accumulated Q3GA (Fig. 5). LPS induces the inflammatory responses associated with the process of the atherosclerosis development. These results suggest the presence of a specific transport pathway for the flavonoid glucuronides in the activated macrophages. Recently, a receptor for (−)-epigallocatechin-3-gallate (a major tea polyphenol), 67-kDa laminin receptor, has been identified as a trigger molecule that mediates the biological activity of (−)-epigallocatechin-3-gallate (26). The report raises the possibility for the presence of receptors and/or target molecules of flavonoids and the metabolites in vivo. The precise molecular mechanism for the accumulation of flavonoid metabolites in the activated macrophages should be clarified in the future.

We have shown that Q3GA down-regulated the expression of several scavenger receptors (SR-A and CD36), established as the receptors for oxidized/modified LDL (27–29), in RAW264 cells (Fig. 6), suggesting that the quercetin metabolites may decrease the formation of foam cells. We have already reported that the cholesterol accumulation in the aorta of hypercholesterolemic rabbits was significantly inhibited by the intake of a quercetin glucoside and that the quercetin metabolites were indeed present in the atherosclerotic aorta based on an HPLC-ECD analysis (14). Epidemiological studies also suggest the protective effect of quercetin on the incidence of cardiovascular diseases in human (4–9). These observations raise the possibility that the accumulation of quercetin metabolites in macrophages in the aorta may play an important role in the anti-atherosclerotic effects of dietary quercetin.

Whether the actions of the flavonoids in vivo are because of their metabolites or aglycones is still controversial. Several reports have shown that the conjugation of flavonoids results in the lowering their biological activity (12, 22). On the other hand, a scenario that the deconjugation of the glucuronide metabolites of the flavonoids by increased β-glucuronidase activity at the site of inflammation has been suggested as a plausible mechanism for the protective effects of flavonoids in vivo (20, 21). We found that Q3GA was readily converted to the aglycone in the LPS-stimulated macrophages by the increased β-glucuronidase activity (Fig. 5). A great number of studies have been made on the biological activity of quercetin aglycone associated with oxidative stress (22, 31, 32). It is of interest that a part of the deconjugated aglycone could be further converted to the methylated form in macrophages by the COMT activity. Furthermore, we found that the methylated metabolites play an important role on the inhibitory effect of Q3GA on the mRNA expression of SR-A (Fig. 7). Thus, our results also suggest that the activated macrophages utilize Q3GA more efficiently by the conversion of a part of them into the more active form. The fact that the methylated quercetin glucuronides have been detected as the major metabolites in human plasma (11) raises the possibility that they might inhibit the expression of SR-A more efficiently than Q3GA. We also confirmed that Q3GA inhibits the expression of LPS-induced cyclooxygenase-2 in RAW cells and found that the inhibitory effect requires β-glucuronidase-mediated deconjugation.3 The inhibitory effects of quercetin and/or analogous flavonoid aglycones have been reported on the activity/expression of cyclooxygenase-2 (31), inducible nitrogen-oxide synthase (31), and myeloperoxidase (33). The expression of these scavenger receptors and oxidizing enzymes closely correlated with the development of ath-

3 Y. Kawai, Y. Shiba, M. Kanayama, K. Uchida, and J. Terao, unpublished results.
eruption (28, 30, 34–37). These observations suggest that the deconjugation/methylation metabolism of flavonoid glucuronides in macrophages and the subsequent regulation of atherogenic gene expression may explain, at least in part, the molecular mechanism for anti-atherosclerotic actions of dietary flavonoids.

Most of in vitro studies previously reported using cultured cells, including our data (Figs. 1B and 6), require relatively higher concentrations (≥ 10 μM) of the flavonoid metabolites to exert their anti-atherosclerotic effects as compared with the plasma concentrations (∼ 1 μM) reported in the human studies (16). The current immunohistochemical data resolved, at least in part, this paradoxical problem by showing the presence of target sites of dietary flavonoid metabolites in vivo. Our results provide a purposeful scenario for the bioavailability of dietary flavonoids as follows: (i) the injured artery effectively recruits the quercetin metabolites from the circulating blood; (ii) the biological activities of dietary quercetin could once be inactivated by a conjugation metabolism during absorption and then safely be delivered to the target sites, such as injured/activated vascular walls; and (iii) the recruited, perhaps concentrated, metabolites are incorporated into the target cells (such as macrophages), converted to aglycone (and further to the methylated form), and exert the anti-atherosclerotic activities (Scheme 1). Further research on the immunonochemical approaches directed to dietary flavonoids would clarify the molecular mechanisms for their beneficial effects against various diseases associated with oxidative stress.

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Target Sites of Quercetin Glucuronides in Aorta

Macrophage as a Target of Quercetin Glucuronides in Human Atherosclerotic Arteries: IMPLICATION IN THE ANTI-ATHEROSCLEROTIC MECHANISM OF DIETARY FLAVONOIDS

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