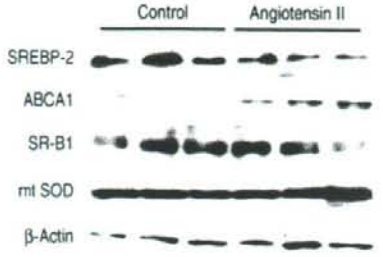

Fig. 5. Western blot analysis of proteins related to lipid metabolism. Shown are the results of the expression in the heart of control and angiotensin II-infused rats of the following proteins: Sterol regulatory element-binding protein (SREBP)-2, ATP-binding cassette transporter subfamily A-1 (ABCA1), scavenger receptor class B type 1 (SR-B1), mitochondrial superoxide dismutase (mt SOD).

infused ($n=4$) rats, the expression of these proteins was, respectively (% control): SREBP-2: 100±17 versus 78±14 (P=NS); ABCA1: 100±10 versus 172±7 ($P<0.001$); SR-B1: 100±17 versus 127±18 (P=NS); mit SOD: 100±5 versus 110±18 (P=NS) (Fig. 5).

4. Discussion

In the present study, we showed that administration of angiotensin II, but not catecholamines, caused accumulation of lipids in myocardial, vascular wall, and perivascular cells in the rat heart. Such angiotensin II-induced lipid deposition, as well as the increases in tissue triglyceride content in the heart, was suppressed completely by losartan, but only partially by hydralazine. These findings collectively indicate that the accumulation of intracardiac lipids induced by angiotensin II was, at least in part, independent of the pressor properties of angiotensin II.

Intracardiac lipid accumulation, which is sometimes designated 'cardiac steatosis' (McGavock et al., 2007), is known to occur in humans in certain diseased conditions, such as diabetes and heart failure (McGavock et al., 2007, Sharma et al., 2004). By means of genetic engineering, several animal models showing an amount of intracardiac lipids have been generated; these models include mice with cardiac-specific overexpression of acyl CoA synthase (Lee et al., 2004), fatty acid transport protein 1(Chiu et al., 2005), and PPAR-α (Finck et al., 2003), and mice with cardiac-restricted deletion of PPAR-delta (Cheng et al., 2004). The observation that accumulation of excessive fatty acids aggravates, whereas reduction of cardiac lipid content ameliorates, the structural and functional damage in these models supports the notion that accumulation of excessive lipid may indeed be cardiotoxic. In our previous studies, we found that administration of angiotensin II, but not catecholamines, caused marked accumulation of neutral lipids in the kidney (Ishizaka et al., 2006, Saito et al., 2005), leading us to investigate whether these two pressor agents affect cardiac lipid content differently in the current study.

What would be the mechanism underlying angiotensin II-induced intracardiac lipid deposition? We found that angiotensin II upregulated the expression of SREBP-1c, FAS, and HMG-CoAR, and downregulated that of UCP2, and UCP3; in addition, the pattern of regulation paralleled intracardiac lipid accumulation. It has been reported that angiotensin II upregulates the expression of SREBP-1c and FAS, resulting in increased lipogenesis in adipocytes in vitro (Jones et al., 1997, Kim et al., 2001). In addition, although the physiological functions of UCP2 and UCP3 are not well-established, downregulation of these new UCPs may augment the production of reactive oxygen species and decrease the catalysis of transported fatty acids (Affourtit et al., 2007). We also found that angiotensin II upregulated PPAR-α mRNA expression. Overexpression of PPAR-α in the heart may also cause lipotoxic cardiomyopathy (Finck et al., 2003, Vikramadithyan et al., 2005), suggesting that PPAR-α upregulation might be an underlying mechanism linking angiotensin II administration and cardiac lipid deposition.

Several previous studies have shown that PPAR-α activator may ameliorate myocardial damage induced by angiotensin II (Fujita et al., 2008, Ichihara et al., 2006). In the current study, we also found that PPAR-α expression was increased by norepinephrine infusion, which did not cause apparent cardiac lipid accumulation, indicating that upregulation of cardiac PPAR-α may not solely account for lipid accumulation in the heart. Whether or not PPAR-α activator acts to enhance or to suppress angiotensin II-induced lipid accumulation in the heart should be examined in future studies.

Activation of AMPK may result in the phosphorylation of acetyl CoA carboxylase, followed by the reduction of malonyl CoA and the subsequent activation and upregulation of CPT-1, leading to the stimulation of fatty acid oxidation (Affourtit et al., 2007). In the current study, we found that angiotensin II activated cardiac AMPK; however, it downregulated CPT-1 mRNA expression. Tian et al. (2001) have recently reported that pressure overload-induced cardiac hypertrophy causes a significant increase in AMPK activity in the heart that is, unexpectedly, accompanied by a downregulation of CPT-1 expression. They presumed that, unlike short-term activation, prolonged activation of AMPK might result in a downregulation of the enzymes that would be critical to fatty acid oxidation. With regard to this, it may be of note that, in the current study, both AMPK activation and CPT-1 downregulation by angiotensin II were suppressed not only by losartan, but also by hydralazine, and that CPT-1 mRNA downregulation was also induced by norepinephrine-induced hypertension, suggesting that these events were induced not in an angiotensin II-specific manner, but rather by hypertension itself.

It has been reported that UCP2 may reduce the generation of ROS, and conversely, downregulation of uncoupling proteins may increase the generation of ROS (Arsenijevic et al., 2000). On the other hand, enhanced oxidative stress or increased amounts of ROS may activate or upregulate SREBP-1 and FAS (Furuta et al., 2008, Gharavi et al., 2006). In addition, CuZn-SOD deficiency has been reported to increase lipid accumulation in the liver (Uchiyama et al., 2006). We found in our previous study (Saito et al., 2005) and the current one that superoxide is histologically co-localized with lipid deposition in the heart and kidney of angiotensin II-infused rats. Taken together, these findings may collectively suggest that angiotensin II-induced deposition of lipid in the heart may be evoked, at least in part, by enhanced oxidative stress (Fig. 5). This hypothesis should be examined in future studies (Fig. 6).

In conclusion, administration of angiotensin II to rats induced intracardiac lipid accumulation in regions where superoxide

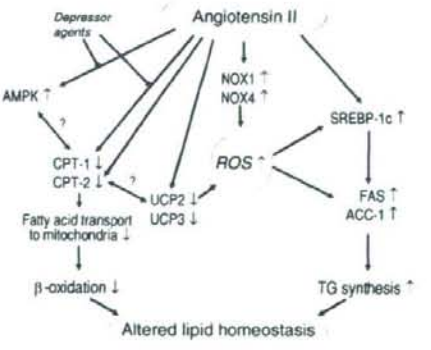

Fig. 6. Working hypothesis on angiotensin II-induced altered lipid homeostasis. Abbreviations are same as in Table 1. ROS indicates reactive oxygen species.

production was found to be increased. The angiotensin II-induced accumulation of intracardiac lipids, in addition to regulation of the expression of several lipid metabolism-related genes (SREBP-1c, FAS, HMG-CoAR, PGC-1α, UCP2, and UCP3), events that were not mimicked by catecholamine infusion, were found to be dependent on the angiotensin AT_1 receptor. The physiological significance of angiotensin II-induced cardiac lipid accumulation and the role of enhanced oxidative stress on this phenomenon await further investigation.

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Limitation of immunoaffinity column for the removal of abundant proteins from plasma in quantitative plasma proteomics

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ABSTRACT: In plasma proteomics, before a proteome analysis, it is essential to prepare protein samples without high-abundance proteins, including albumin, via specific preparation techniques, such as immunoaffinity capture. However, our preliminary experiments suggested that functional changes with use alter the ability of the immunoaffinity column. Thus, in this study, to evaluate the changes of the removal ability of abundant proteins from plasma by the immunoaffinity column, plasma proteome analysis was performed for the long-term test for the reproducibility of the affinity column using the fluorogenic derivatization–liquid chromatography–tandem mass spectrometry method combined with an IgY column. The specific adsorption for albumin decreased with an increase in the number of the column usage before its expiration date. Moreover, it was demonstrated that hydrophobic high molecular weight compounds in plasma adsorbed onto the column materials surface contributed to the functional changes from specific immunoaffinity adsorption into hydrophobic interaction. These results suggested that, in quantitative plasma proteomics studies, it is important to keep in mind the risk of not only the nonselective loss but also the changes in the adsorption ability of the immunoafinity column. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: plasma; proteomics; immunoaffinity column; abundant protein; FD-LC-MS/MS method

Introduction

Blood samples can be taken at a particular point in time with little burden on patients and the constituents of the blood samples could reflect a developing or existing illness because tissue-specific proteins may be released into the brood stream from the damaged or dead cells. Therefore, it is generally recognized in proteomics studies that blood samples represent the greatest potential source of information on the proteins related to human diseases. However, plasma proteome analysis aiming at quantitative protein profiling and biomarker discovery is not easily done. Since several high-abundance proteins, such as albumin, typically constitute greater than 90% of total protein mass, the detection of lower-abundance proteins which presumably are the biologically interesting population is interfered with by the dominant proteins. To address the complexity of these samples, it is essential to prepare samples via specific preparation techniques to remove high-abundance proteins from the samples before the proteome analysis (Linke *et al.*, 2007; Martosella *et al.*, 2005; Qian *et al.*, 2006; Steel *et al.*, 2003). There are a number of approaches for removing proteins based on their biochemical and biophysical features, such as molecular weight, mass, density, hydrophobicity, surface change and isoelectric point. Among these techniques, immunoaffinity capture using antibodies is rapidly becoming the prefractionation method of choice in proteomics analysis. Commercial kits using an avian immunoglobulin yolk (IgY) have recently become available due to its high avidity and lesser cross-reactivity with heterologous human proteins (Huang *et al.*, 2005; Linke *et al.*, 2007; Qian *et al.*, 2006). A number of researchers have already indicated its utility and the improvement of the detection of low-abundance proteins by the elimination of the high-abundance proteins using the IgY affinity column (Gong *et al.*, 2006; Huang *et al.*, 2005; Linke *et al.*, 2007; Liu *et al.*, 2006; Qian *et al.*, 2006).

We have recently developed a highly sensitive and quantitative proteomics method called fluorogenic derivatization–liquid chromatography–tandem mass spectrometry (FD-LC-MS/MS) (Masuda *et al.*, 2004; Toriumi and Imai, 2003). The method consists of separation of the fluorogenic derivatized proteins by high-performance liquid chromatography (HPLC), isolation of the target protein obtained by HPLC, hydrolysis and identification of the target protein by LC-MS/MS with the probability-based protein identification algorithm. This highly selective, sensitive and reproducible method enables the post-translational proteins and isoforms to be distinguished. The method was applied to the extracts of *Caenorhabditis elegans*, mousee liver and breast cancer cell lines, and revealed the proteins related to early-stage Parkinson's

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Abbreviations used: DAABD-Cl, 7-Chloro-*N*-[2-(dimethylamino)ethyl]-2,1,3-benzoxadiazole-4-sulfonamide; FD, fluorogenic derivatization; HCCA, α-cyano-4-hydroxycinnamic acid; TCEP, Tris (2-carboxyethyl) phosphine hydrochloride; TFA, trifluoroacetic acid; TOF, time-of-flight.

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disease (Ichibangase *et al.*, 2008), hepatocarcinogenesis (Ichibangase *et al.*, 2007) and tumor progression and metastasis (Imai *et al.*, 2008). During the course of our studies, we applied the FD-LC-MS/MS method to plasma proteomics. To detect plasma biomarkers that are probably masked by the high-abundant proteins, an IgY affinity column was utilized for the removal of the dominant proteins, such as albumin, from plasma before the fluorogenic derivatization (FD) of the plasma proteins. On the preliminary experiments, the quantitative changes of the peaks on the chromatograms obtained from the same samples were observed on every occasion of sample treatment with the affinity column. Since the detectability of the fluorogenic derivatized proteins by the HPLC-fluorescence detector is always constant, the change in the removal ability of the IgY column for the abundant proteins could be monitored during the usage of the column. Although it was reported that there was a risk of loss by inadvertent capture of low-abundance proteins (Bjorhall *et al.*, 2005; Gong *et al.*, 2006; Linke *et al.*, 2007; Plavina *et al.*, 2007; Yocum *et al.*, 2005), there are no reports of long-term tests for the reproducibility of the affinity column in quantitative proteome analysis.

In this study, to evaluate the removal ability of abundant proteins from plasma by the affinity column, we performed proteome analysis of plasma sample and protein standards by FD-LC-MS/MS combined with the IgY technique and investigated the cause of the quantitative changes of the chromatograms mentioned above.

Experimental

Materials and Methods

Reagents. 7-Chloro-*N*-[2-(dimethylamino)ethyl]-2,1,3-benzoxadiazole-4-sulfonamide (DAABD-Cl) and 6.0 M guanidine hydrochloride (pH 8.7 buffer solution) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid sodium salt (Na_2EDTA) and 3-[(3-Cholamidopropyl) dimethyammonio]-1-propanesulfonate (CHAPS) were from Dojindo Laboratories (Kumamoto, Japan). Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was obtained from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile and trifluoroacetic acid (TFA) for the HPLC-fluorescence detection were HPLC grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other reagents used were of analytical grade.

Affinity Columns

The prepacked IgY-R7 Spin Column and Seppro®-IgY12 were obtained from Beckman Coluter (Fullerton, CA, USA) and GenWay Bioteck (San Diago, CA, USA), respectively. The IgY-R7 Spin Column (1.2 mL spin column) removes seven high-abundance proteins in rodent plasma (albumin, IgG, α2-antitrypsin, IgM, transferring, haptoglobin and fibrinogen) and utilizes centrifugation as the force for affinity separation. The column is said to be reusable 100 times under proper conditions. The Seppro®-IgY12 column is optimized for human plasma and removes 12 high-abundance proteins (IgA, α1-acid glycoprotein, α2-macroglobin, apolipoproteins A-I and apolipoproteins A-II besides the above seven proteins). This column is used with the high-throughput automated proteomic sample processing instrument (Magtration System SA-1; Precision System Science, Chiba, Japan) and is said to be able to be used 30 times. Both companies are corporate partners for the exclusive marketing of the IgY microbeads technology, and both column materials are the same except for recognized animal species to the IgY.

Plasma Samples

For the IgY-R7 Spin Column, plasma sample were obtained from C57BL/6N male mice (10 and 19 months; Clea Japan, Tokyo, Japan) by centrifugation at 5510 rpm for 10 min at 4°C, and frozen at −80°C until use. On the other hand, the human control plasma sample purchased from Sigma-Aldrich was used for the Seppro®-IgY12 column. The control plasma sample was passed through a 0.45 μm filter before use.

Treatment of Mouse Plasma with the IgY-R7 Spin Column

Mouse plasma treated with the spin column was carried out according to the manufacturer-instructed column usage and loading capacity [10 μL plasma diluted with dilution/washing buffer: 10 mM Tris–HCl, 150 mM NaCl, pH 7.4 (TBS)]. Three buffers (dilution/washing buffer; stripping buffer: 100 mM glycine, pH 2.5; neutralization buffer: 100 mM Tris–HCl, pH 8.0) were used under the separation scheme that consisted of sample loading–washing–eluting–neutralization followed by a re-equilibration scheme for a total cycle time of 40 min. To increase the recovery of the non-specific proteins, the resulting flow-though fraction and the washing fractions were collected and concentrated to 10 μL with 3.0 kDa molecular weight cutoff device according to the manufacturer's instructions (Microcon YM-3; Millipore, Billerica, MA, USA).

Treatment of Protein Standards and Control Human Plasma Sample with the Seppro®-IgY12 Column

The molecular weight standards, consisting of phosphorylase B, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme for electrophoresis (Table 1; 12 mg/mL protein amount; low range; Bio-Rad, Herccules, CA, USA), were employed in sample processing without dilution to evaluate the recovery of non-specific proteins from the column. The injected amount of the protein standards was 2.4 mg protein per injection to the affinity column. Since, in the instructions, 15 μL of plasma (generally corresponding to 70–80 mg proteins/mL) was diluted to 500 μL and loaded to the affinity column, the injected amount of the standards was compatible. According to the manufacturer's instructions, the protein standards were set in the sample holding and then the flow-though fraction was obtained.

The control human plasma sample was also treated according to the manual. Briefly, 15 μL of plasma sample was diluted with the dilution/washing buffer to 500 μL, and the sample was set in the sample holding, as in the case of the protein standards. The resulting flow-though fraction of the plasma was concentrated

Table 1. Protein standards and the number of labeled region with DAABD-Cl

Protein	Source	MW (kDa)	No. of rabeled region
Phosphorylase B	Rabbit muscle	97.4	10
Serum albumin	Bovine	66.2	35
Ovalbumin	Hen egg white	45	5
Carbonic anhydrase	Bovine	31	2
Trypsin inhibitor	Soybean	21.5	5
Lysozyme	Hen egg white	14.4	6

to 15 μL with a Microcon YM-3 device. A series cycle including the sample loading–washing–eluting–neutralization finished in 65 min.

FD-LC-MS/MS Conditions

Each FD condition was optimized for the protein standards and for plasma samples in order to obtain the highest peak on the chromatograms. For the protein standards, a 2.5 μL aliquot of the flow-though fraction was mixed with 30 μL of a mixture of 0.83 mM TCEP, 3.3 mM Na_2EDTA and 16.6 mM CHAPS in the pH 8.7 buffer solution, 12.5 μL of the buffer solution and 5.0 μL of 8.0 mM DAABD-Cl in acetonitrile. The mixture was reacted at 50°C for 5.0 min, and the reaction was stopped with 1.5 μL of 20% TFA. A 20 μL aliquot of the above reaction mixture was injected to the HPLC system (Hitachi L-2000 series; Hitachi Instruments, Tokyo, Japan) using a column of Intrada WP-RP (30 nm pore size, 250 × 4.6 mm i.d., Imtakt Co, Kyoto, Japan) at 60°C with a flow rate of 0.55 mL/min. The eluent (A) and eluent (B) were water–acetonitrile–TFA (90:10:0.15, v/v/v) and water–acetonitrile–TFA (30:70:0.05, v/v/v), respectively. The gradient condition was established from 5 to 100% eluent (B) over a period of 60 min. For mouse and control plasma samples, a 6.0 μL aliquot of the flow-though fraction was mixed with 30 μL of the above mixture of TCEP, Na_2EDTA and CHAPS, 10 μL of the buffer solution and 4.0 μL of 825 mM DAABD-Cl in dioxane. After the FD reaction (50°C for 5.0 min), the reaction was stopped with 2.0 μL of 20% TFA. An aliquot (10 μL) of the reaction mixture was injected, and the longer column (Intrada WP-RP 30 nm pore size, 500 × 4.6 mm i.d., Imtakt Co) together with a precolumn (Intrada WP-RP 30 nm pore size, 5.0 × 2.0 mm i.d., Imtakt Co) at 60°C was adopted with a flow rate of 0.55 mL/min on the HPLC system. The mobile phases consisted of water–acetonitrile–TFA (A) 90:10:0.15 and (B) 30:70:0.05. Mobile phase (C) was the same as (A), except with 0.05% TFA. The gradient condition is described in Fig. 1. Fluorescence detection was carried out at 395 and 505 nm for the excitation and emission wavelengths, respectively. The peak height of each protein peak obtained from the HPLC chromatograms was calculated by HITACHI EZChrom Elite™ Chromatography Data System (Hitachi Instruments) and the identification of the standard proteins was accomplished according to the previous report (Ichibangase *et al.*, 2007).

Scanning Electron Microscopy and Matrix-assisted Laser Desorption/Ionization MS Analysis

Scanning electron microscopy (SEM) and matrix-assisted laser desorption/ionization (MALDI) MS analyses were conducted in Jeol Datum (Tokyo, Japan) and Bruker Daltonics Japan (Kanagawa, Japan), respectively.

For SEM analysis, the column materials were diluted with phosphate buffer (300 mOsm) and fixed with fixative (2.5% glutaraldehyde in PBS, pH 7.0) for 10 min. The fixed sample was captured on the filter (SEM-Pore: 0.6 μm i.d.; 10 μm) and washed with the buffer. After the osmium-fix (2% OsO_4) and a brief rinse with the fixative solutions, specimens were dehydrated in a series of graded ethanol (30–100%). The immersed specimens in ethanol were replaced with isoamyl phenylacetate and subjected to critical point drying. The dried samples were coated with osmium using a plasma coater (OPC80N, Jeol). Images were acquired using a Jeol JSM-7401F in normal SEM mode. For the low power microscope images for the whole picture of a material, a lower electron image (LEI) was applied.

For MALDI MS analysis, the column materials were washed and spotted on a plate. α-Cyano-4-hydroxycinnamic acid (HCCA) was used as matrix. MALDI mass spectra were acquired with time-of-flight (TOF) MS (autoflex III, Bruker Daltonics) in positive linear mode.

Results and Discussion

Quantitative Functional Changes of the IgY-R7 Spin Column after a Number of Treatments with Mouse Plasma

For the detection of low-abundance proteins in mouse plasma, the removal of high-abundance proteins from mouse plasma with the affinity column was performed prior to the FD-LC-MS/MS proteome analysis. Typical chromatograms are shown in Fig. 1(A), obtained from the same mouse plasma sample treated with 80 and 86 cycles of the same spin column, respectively. All peak heights obtained from the 86 cycles of the spin column were clearly higher compared with those obtained from the 80 cycles. Although a difference between column lots might exist, there were also significant differences in the removability of the affinity column between the second (column lot no. 2) and the 44th cycles (column lot no. 1) of the treated spin column [Fig. 1(B)]. The relative standard deviation (RSD) of the protein peaks was calculated between-day ($n = 3$) using samples provided by the same treatment number of the column. The RSD values were less than 21.6%, obtained from the peak in Fig. 1(A), suggesting that the detectability of the fluorogenic derivatized proteins by HPLC is constant. Therefore, it was considered that the quantitative changes of the peaks on the chromatograms might result from the changes in the affinity column by the sample treatment.

Evaluation of Seppro®-IgY12 Column for the Adsorption of Protein Standards using an Automatic Instrument

To eliminate a manual usage error from the sample processing, a high-throughput automated instrument, SA-1, for the removal of high-abundance proteins from human plasma samples with a Seppro®-IgY12 column was investigated. For the evaluation of the exact adsorption ratio of specific and non-specific proteins to the affinity column, the affinity column was periodically treated with protein standards after treatment with a control human plasma sample some dozen times. Since the other investigator has reported the non-specific adsorption of the protein by concentration methods such as a centrifugal filter to be about 15% (Linke *et al.*, 2007), the flow-through fraction of the injected protein standards from the affinity column was subjected directly to the FD-LC-MS/MS analysis without a protein concentration step in this study.

At first, to eliminate the non-specific adsorption to the affinity column, the protein standards mixture was divided into two parts and one was subjected into the untreated affinity column, and the flow-though fraction was derivatized with the fluorogenic reagent, DAABD-Cl, and separated by the HPLC system (4.8 μg protein/HPLC injection). Another part of the protein standards mixture was diluted and derivatized with DAABD-Cl, and injected onto the HPLC system (4.8 μg protein/HPLC injection). The obtained chromatograms are depicted in Fig. 2. Each protein peak was collected, digested in peptide mixtures, and identified by applying the peptides to HPLC-MS/MS with a

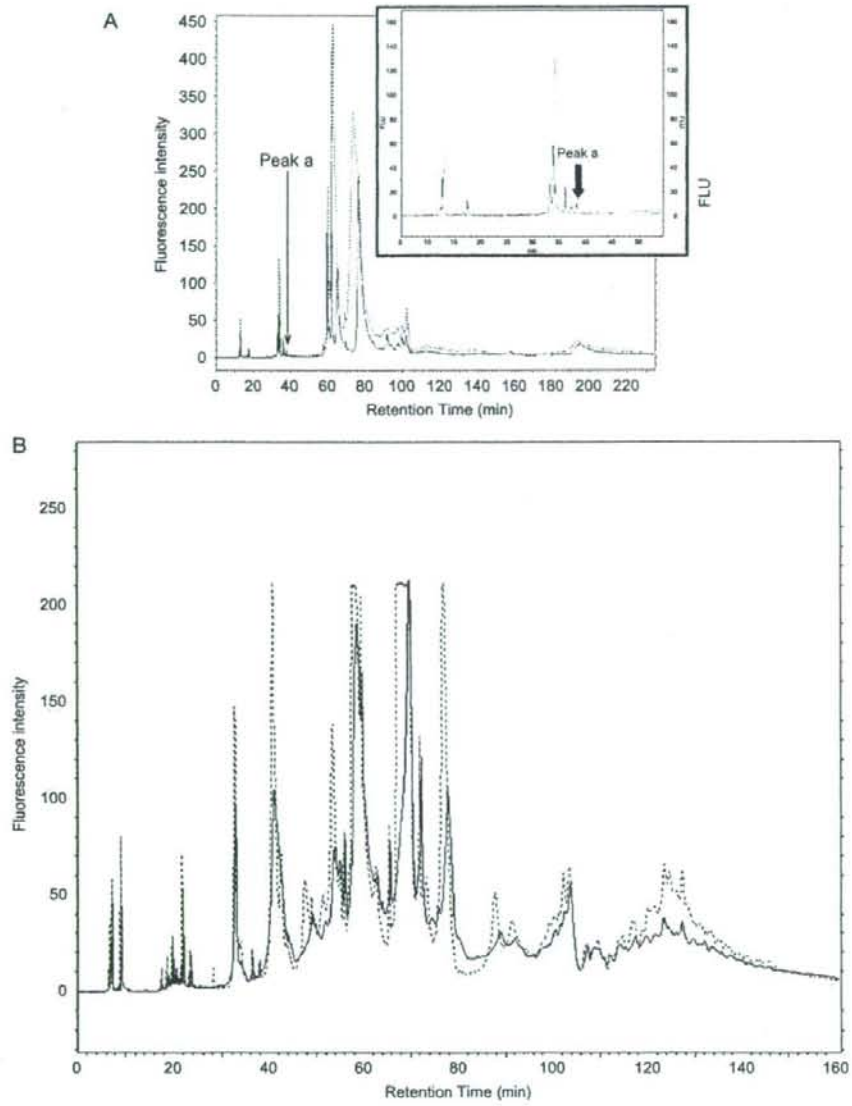

Figure 1. Chromatograms of mouse plasma samples treated with IgY-R7 Spin Columns. (A) The mouse plasma (19 months, C57BL/6N) treated 80 times (solid line) and 86 times (dotted line) in the same spin column. The gradient conditions were as follows: time (min), 0 → 10 → 20 → 44 → 48 → 68 → 80 → 90 → 108 → 120 → 130 → 160 → 180 → 230; B (%): 5 → 5 → 30 → 30 → 35 → 38 → 39.2 → 39.2 → 42 → 43 → 44 → 45 → 47 → 58; C (%): 0 → 0 → 0 → 0 → 0 → 0 → 0 → 60.8 → 58 → 57 → 56 → 55 → 53 → 42. (B) The mouse plasma samples (10 months, C57BL/6N) were treated with the second cycle (column lot no. 2; solid line) and the 44th cycle (column lot no. 1; dotted line) of each spin column. The gradient conditions were as follows: time (min), 0 → 5 → 10 → 22 → 24 → 34 → 54 → 60 → 60.1 → 80 → 130 → 140 → 150 → 160 → 170; B(%): 5 → 5 → 30 → 30 → 35 → 38 → 42 → 43 → 43 → 47 → 58 → 60 → 60 → 75 → 78; C(%): 0 → 0 → 0 → 0 → 0 → 0 → 0 → 0 → 57 → 53 → 42 → 40 → 40 → 25 → 22.

database-searching algorism. Table 2 shows the adsorption ratio of the proteins to the untreated column and the identified protein names. The protein names of peak 1 and 2 could not be identified since these peaks were peptides coexisting in the protein standards. Also, carbonic anhydrase and ovalbumin could not be detected. Since carbonic anhydrase has only two cycteine residue for labeling with DAABD-Cl, its detection might be difficult. The reason for the undetected ovalbumin was not clear. Since this study was aimed at investigating the changes in the adsorption of the specific and non-specific proteins using the column, this issue was not examined further. Consequently, although the affinity column was able to efficiently remove bovine serum

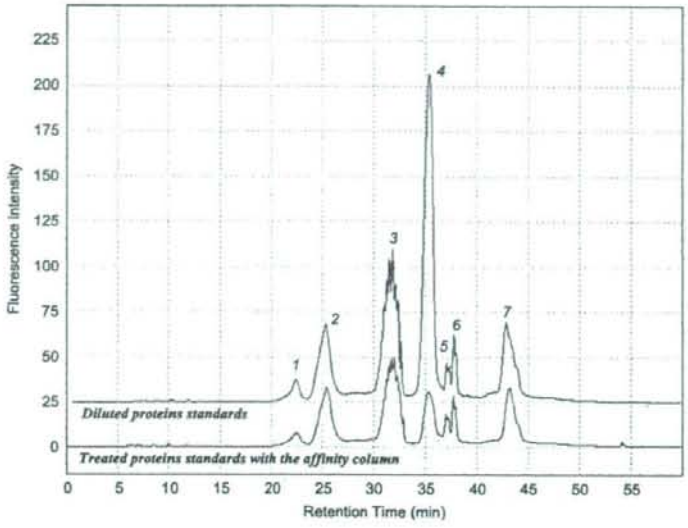

Figure 2. Chromatograms of the protein standards which were treated with Seppro®-IgY12 column and diluted to the same protein amount (4.8 μg/HPLC injection) as the amount for the column treatment. The peak numbers correspond to Table 2.

Table 2. Adsorption ratio to the untreated Seppro®-IgY12 column and protein names obtained by FD-LC-MS/MS method

Peak no.	Adsorption ratio to the untreated Seppro®-IgY12	Protein name
1	38.5%	Peptide
2	30.6%	Peptide
[a]3	45.9%	Lysozyme
4	83.9%	Bovine serum albumin (BSA)
[b]5	18.3%	Trypsin inhibitor
6	28.5%	Trypsin inhibitor
7	30.4%	Phosphorylase B

[a]3, [b]5: Most highest peak

albumin (BSA; 83.9%) as compared with the other proteins in the standards, non-specific binding to the column materials or to carrier proteins such as albumin itself was observed in the 18.3–45.9% range and could result in the loss of presumed biomarkers.

Next, the time series changes of the specific and non-specific adsorption of proteins to the column were investigated. The protein standards mixture was treated with the column periodically after 10 and 20 cycles of treatment of the control plasma sample. The relation of the changes of the protein standards adsorption to the number of uses of the affinity column is shown in Fig. 3(A). The specific adsorption of BSA decreased with an increase in the number of times the column was used. However, the non-specific adsorption for lysozyme, trypsin inhibitor and phosphorylase B reached a maximum at 11 cycles and decreased at 21 cycles. Since the affinity column was optimized for human plasma, the absorption of BSA for the column might be weaker than for plasma albumin. However, the adsorption of albumin in control plasma also decreased with an increase in the number of times the column was used (data not shown). Moreover, since the slopes of decrease differed among the protein standards, the correlation of each adsorption with molecular weight of each protein was calculated [Fig. 3(B)]. The open dots show the value of BSA in Fig. 3(B). The correlation coefficient value was the closest to 1.00 ($R^2 = 0.813$) for 21 cycles [Fig. 3(B-3)], demonstrating that the adsorption ability of the column does not depend any longer on the affinity of the antibody but on the hydrophobicity of the protein. In contrast, the correlation of the values obtained from the first cycle [Fig. 3(B-1); $R^2 = 0.077$] was not fairly observed between the adsorption ability and the hydrophobicity, and the value for BSA was apart from those for other proteins. Therefore, BSA was specifically removed as compared with the other protein standards by the immunoaffinity adsorption. Also, as shown in Fig. 3(B-2), the result obtained from 11 cycles ($R^2 = 0.012$) demonstrated that all proteins bound to the surface of the affinity column materials equally. Therefore, the present data demonstrates that the quantitative changes of the adsorption for the affinity column appear before the limited use of the column (30 times in the manufacturer's instructions).

Understanding the State of the Plasma-treated Column Materials

To understand the state of the column materials of the Seppro®-IgY12 column, the untreated and 11- and 21-times-treated column materials were subjected to electron microscopy and MALDI-TOF-MS analysis.

As shown in Fig. 4, the SEM images show an obvious difference between the untreated and treated materials. The attachment of the unknown bio-molecules to the materials surface appeared and increased with an increase in the number of treatments.

Next, in order to characterize the attached compounds, the same materials were subjected to MALDI-TOF-MS analysis. Since

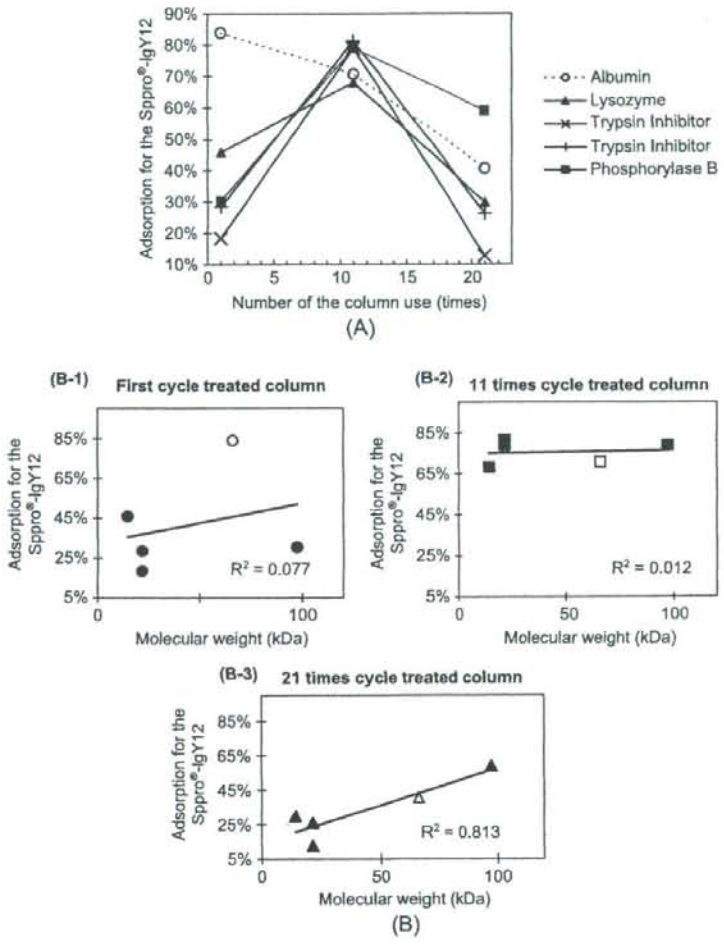

Figure 3. Changes of the adsorption ratio of protein standards for Seppro®-IgY12 column. (A) Relation of the adsorption ratio to the number of times the column was used. (B) Correlation of the adsorption ratio for the column with molecular weight of each protein standard using the column treater one (B-1), 11 (B-2) and 21 (B-3). The open dots show the value for BSA.

direct laser irradiation of the materials could affect the instrument, the positions to be irradiated were the points of the existence of many matrices on a few column materials. Although several peaks existed of less than 70,000 *m/z* in each mass spectrum, the higher molecular weight peaks (7266, 9689, 14,532 and 29,041 *m/z*) appeared in the treated but not in the untreated materials (Fig. 5). After the materials were washed with acetonitrile, the higher molecular weight peaks in the treated materials disappeared (data not shown). Therefore, the compounds attached to the material surface should be hydrophobic high-molecular-weight compounds existing in human plasma.

Two analyses of the column materials surface demonstrated that the hydrophobic high-molecular-weight compounds in plasma adsorbed onto the surface of the affinity column materials and contributed to the changes in the adsorption ability of plasma protein from immunoaffinity into hydrophobic interactions. Howevere, further studies are needed to characterize the exact details of the compounds.

Conclusions

To investigate the ability to remove abundant proteins from plasma by immnoaffinity using the IgY column, FD-LC-MS/MS method was applied to the long-term test of the reproducibility of the column. It was demonstrated that the immunoaffinity column was effective in removing BSA from the protein standards mixture, but, in addition, removing other proteins in the 18.3–45.0% range. The results suggested that the proteins of possible biomarkers could be lost and their quantification made difficult. Moreover, the specific adsorption of BSA in the protein standards mixture and of albumin in the control human plasma samples decreased with an increase in the number of times the column was used with both samples before its use expired. To examine the cause of the functional changes of the immunoaffinity, the correlations between the adsorption ratio for the affinity column and molecular weight of the adsorbed proteins were calculated, and the column materials surface was also investigated by SEM and

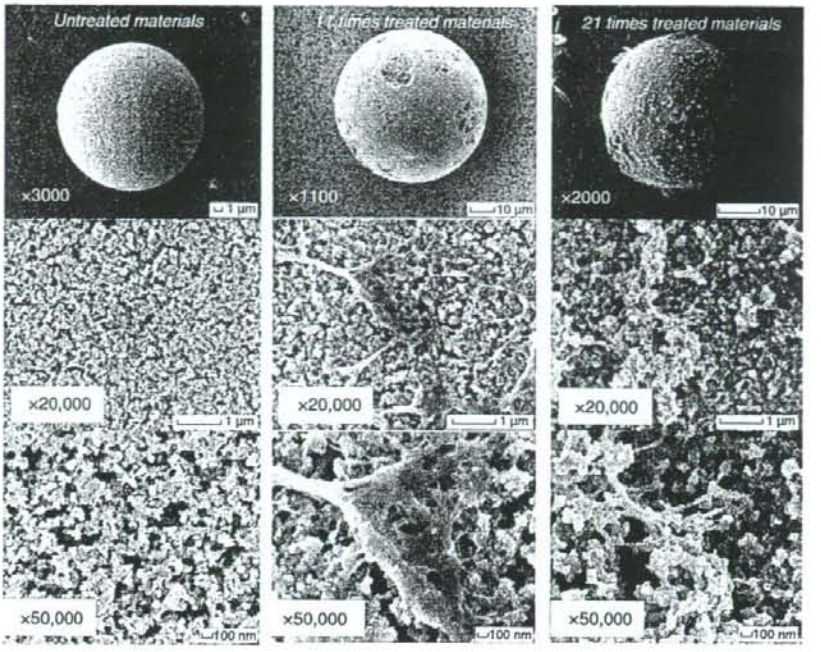

Figure 4. SEM images of the untreated and 11- and 21-times-treated column material surfaces. Magnification in SEM was controlled in a range of × 1100–3000 to show the whole picture of the material.

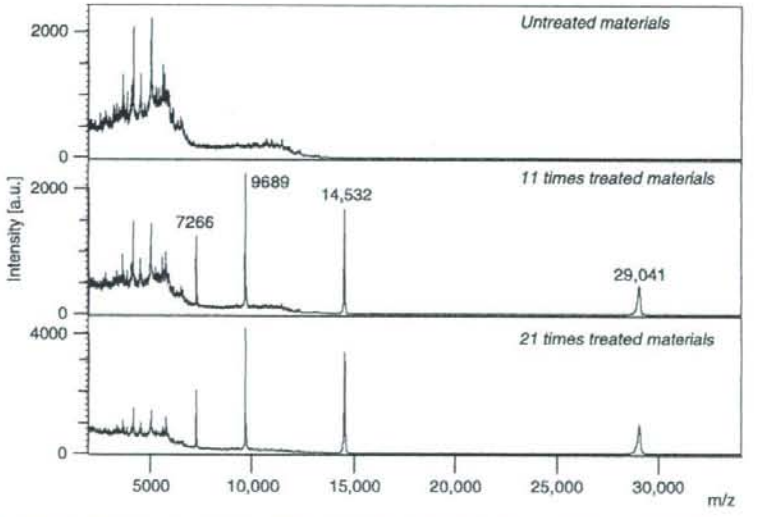

Figure 5. MALDI-TOF-mass spectrum of the untreated and 11- and 21-times-treated column material surface.

MALDI MS analysis. These data demonstrated the attachment of the hydrophobic high-molecular-weight compounds in plasma to the surface, suggesting that on every sample treatment with the affinity column, the adsorption ability of plasma protein changed into hydrophobic interactions. Further studies to characterize the attached compounds are required, and the elucidation of the compounds might lead to the improvement of the affinity column technique and contribute to progress in quantitative plasma proteomics.

Reproducibility is prerequisite for accurate quantitative proteome analysis of clinical samples for biomarker identification and quantification. For this purpose, it is generally essential to

prepare protein samples without high-abundance proteins via specific pre-fractionation techniques to enhance the detection of low-abundance proteins in plasma, and thus, immunoaffinity separation is now chosen as a reliable pre-fractionation method. However, this study indicated that, in quantitative plasma proteomics studies, it is important to keep in mind the risk of not only nonselective loss but also functional changes of the adsorption ability for the immunoaffinity column.

Acknowledgements

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Original Article

Association between hepatitis B/C viral infection, chronic kidney disease and insulin resistance in individuals undergoing general health screening

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Aim: Previous studies have shown that hepatitis B virus (HBV) and hepatitis C virus (HCV) infection may be associated with glomerulonephritis.

Methods: In the current study, we investigated the possible association between HBV/HCV infection, estimated GFR (eGFR) and albuminuria by analyzing cross-sectional data from individuals undergoing general health screening.

Results: Of 12 535 individuals enrolled, 130 (1.0%) and 72 (0.6%) tested positive for HBV surface antigen and HCV core antigen, respectively. In comparison with hepatitis-negative individuals, the prevalence of low eGFR and albuminuria was significantly greater in individuals with HCV infection, but not in those with HBV infection. Logistic regression analysis adjusted for age, sex, systolic blood pressure and fasting plasma glucose showed that HCV infection was positively associated with low eGFR (odds ratio 1.63 [95% CI 0.95–2.80, $P = 0.077$]) and with albuminuria (odds ratio 2.00 [95% CI 1.06–3.76, $P = 0.003$]). By contrast, prevalence of neither low eGFR nor albuminuria was greater in individuals with HBV infection than in hepatitis-negative subjects. Further adjustment for either HOMA-IR or serum alanine aminotransferase levels abolished the statistical significance in the association between HCV infection and albuminuria.

Conclusion: Our data suggest that although both HCV and HBV infection are associated with increased insulin resistance, the different viruses may have different impacts on chronic kidney disease among Japanese individuals undergoing general health screening.

Key words: aminotransferase, chronic kidney disease, health screening, insulin resistance, viral hepatitis

INTRODUCTION

IN JAPAN, MORE than 1 million people are estimated to be infected with hepatitis B virus (HBV) and over 2 million with hepatitis C virus (HCV);[1] HBV infection has been reported to be found in 0.8% and HCV infection in 0.5% of Japanese workers.[2] Although a major target organ of HBV and HCV infection is the liver, extrahepatic manifestations are also frequently observed in patients with acute and chronic viral hepatitis. In HCV infected patients, even without clinical evidence of liver involvement, renal complications can occur, most commonly membranoproliferative glomerulonephritis (MPGN) and membranous glomerulonephritis (MGN), which are clinically characterized by hematuria, proteinuria and variable grade renal dysfunction. One study has reported that HCV antibody was found to be positive in a large proportion (60%) of Japanese patients with MPGN.[3] El-Serag *et al.* reported that HCV-infected subjects had a sevenfold increase in the odds of MPGN compared with control subjects without HCV infection.[4] HBV infection may also be associated with MGN and MPGN,[5,6] and about 3% of HBV-infected patients were reported to have glomerulonephritis.[7]

Until recently, few data have been available on the prevalence of chronic kidney disease (CKD) and its components in individuals with HBV or HCV infection

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in a population-based study. Tsui *et al.* reported that HCV infection was associated with albuminuria, but not with decreased GFR, in a US population.[8] Huang *et al.* reported a significant association between proteinuria and HCV, but not HBV, infection in an HBV/HCV endemic area.

In the present study, we investigated whether HBV infection, diagnosed by HBV surface antigen (HBsAg) positivity, and HCV infection, diagnosed by HCV core antigen (HCcAg) positivity, were associated with CKD components in Japanese individuals who underwent general health screening.

METHODS

Study population

THE STUDY WAS approved by the Ethical Committee of the Mitsui Memorial Hospital. Between April 2004 and August 2006, 12 535 people (4481 women and 8054 men) underwent a general health screen at Mitsui Memorial Hospital, including an estimation of urinary excretion of albumin, and were enrolled in the present study. In Japan, regular health check ups for employees are a legal requirement; all or most of the costs of the screening are paid for either by the employee's company or by the subject himself.

Laboratory analysis

Blood samples were taken from the subjects after an overnight fast. Serum levels of total cholesterol (TC), HDL-cholesterol (HDL-C) and triglycerides (TG), alanine aminotransferase (ALT) and creatinine were determined by the enzymatic method. Serum uric acid was measured by the uricase–peroxidase method and hemoglobin A1C was determined by latex agglutination immunoassay. The levels of HBsAg and HCcAg in the sera were determined using commercially available enzyme immunoassay kits, AxSYM HBsAg Dynapack (Abbott Japan, Osaka, Japan) and Lumispot "Eiken" HCV antigen (Eiken Chemical, Tokyo, Japan), respectively, according to the manufacturer's instructions. HCcAg of >8.0 pg/mL was considered to be positive. Plasma glucose was measured by the hexokinase method and serum insulin was measured by enzyme immunoassay. Homeostasis model assessment insulin resistance (HOMA-IR) was calculated in these individuals according to the following formula: HOMA-IR = (fasting immunoreactive insulin [μU/mL] × fasting plasma glucose [FPG; mg/dL])/405. The median (range) ALT values in each ALT quartile (IU/mL) were 12 (4–14), 17 (15–19), 23 (20–27) and 37 (28–677).

Estimated glomerular filtration rate, albuminuria and CKD

Serum creatinine was calibrated using the following formula: serum creatinine (Jaffe method) = 0.2 + serum creatinine (measured by enzymatic method). Serum creatinine was measured in mg/dL, and age in years; GFR was estimated using the equation from a simplified version of the Modification of Diet in Renal Disease (MDRD),[9] as follows: estimated GFR (eGFR; mL/min/1.73 m^2) = 186.3 × (serum creatinine)$^{-1.154}$ × (age)$^{-0.203}$ × 0.881 × 0.742 (if female). In this MDRD formula, 0.881 is a coefficient for eGFR specific to the Japanese population.[10] For the diagnosis of albuminuria, spot urine samples were collected and expressed as urine albumin excretion ratio (UAER), which was expressed per g-creatinine. CKD was diagnosed when individuals had an eGFR of <60 mL/min/1.73 m^2, designated as low eGFR, and/or UAER of ≥30 mg/g, designated as albuminuria.[11]

Diagnosis of metabolic syndrome

Diagnosis of metabolic syndrome was made according to the criteria of the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP-III),[12] with body mass index (BMI) used as a surrogate for waist circumference.[13] Metabolic syndrome was said to be present when three or more of the following conditions were met: TG levels ≥150 mg/d; HDL-C levels <40 mg/dL (men), <50 mg/dL (women); FPG levels ≥110 mg/dL or taking antidiabetic medication; systolic blood pressure (SBP) ≥130 mmHg or diastolic blood pressure (DBP) ≥85 mmHg or taking an antihypertensive medication; BMI ≥25 kg/m^2.

Statistical analysis

The data in this study were analyzed by one-way ANOVA with Bonferroni post hoc test, χ^2 test and by univariate and multivariate logistic regression analysis using the computer software StatView ver. 5.0 (SAS Institute, Cary, NC, USA). A value of $P < 0.05$ was taken to be statistically significant. Results are expressed as the mean ± standard deviation unless stated otherwise.

RESULTS

Baseline characteristics

THE BASELINE CHARACTERISTICS of the study subjects according to viral hepatitis infection are

Table 1 Clinical characteristics and laboratory data of study subjects

	Hepatitis negative (n = 12 333)	HBsAg positive (n = 130)	HCcAg postive (n = 72)	P-value
Male sex, n (%)	7916 (64)	93 (63)	45 (72)	0.21
Age, years	53.1 ± 10.6	55.3 ± 10.6	59.2 ± 10.5	<0.001
Body mass index, kg/m^2	22.8 ± 3.1	23.9 ± 3.2	22.3 ± 2.8	<0.001
Systolic blood pressure, mmHg	122 ± 19	126 ± 20	123 ± 22	0.024
Diastolic blood pressure, mmHg	77 ± 12	79 ± 11	77 ± 13	0.077
WBC count, ×103 cells/μL	5.3 ± 1.4	5.0 ± 1.2	5.0 ± 1.7	0.025
RBC count, ×104/μL	467 ± 43	473 ± 40	455 ± 48	0.020
Hemoglobin, g/dL	14.6 ± 1.5	14.8 ± 1.4	14.4 ± 1.5	0.17
Platelet count, ×104/μL	23.0 ± 5.1	20.1 ± 4.9	16.9 ± 5.8	<0.001
Serum data				
Total protein, g/dL	7.3 ± 0.4	7.3 ± 0.4	7.6 ± 0.5	<0.001
Albumin, g/dL	4.5 ± 0.2	4.5 ± 0.2	4.4 ± 0.3	<0.001
Total bilirubin, mg/dL	0.90 ± 0.36	0.92 ± 0.35	1.00 ± 0.47	0.040
ALT, IU/L	24 ± 19	27 ± 29	56 ± 46	<0.001
AST, IU/L	22 ± 12	25 ± 13	48 ± 27	<0.001
γ-GTP, IU/L	46 ± 67	38 ± 30	61 ± 57	0.061
Total cholesterol, mg/dL	211 ± 33	205 ± 31	175 ± 32	<0.001
HDL-cholesterol, mg/dL	59 ± 15	58 ± 14	53 ± 11	0.001
Triglycerides, mg/dL	117 ± 84	107 ± 83	89 ± 36	0.006
Fasting glucose, mg/dL	97 ± 19	98 ± 17	96 ± 15	0.82
Hemoglobin A1C, %	5.3 ± 0.7	5.3 ± 0.7	5.2 ± 0.7	0.30
HOMA-IR	1.5 ± 1.5	1.7 ± 1.1	2.4 ± 1.8	<0.001
Renal data				
Serum urea nitrogen, mg/dL	14.3 ± 3.6	14.6 ± 3.1	15.4 ± 6.4	0.031
Serum creatine, mg/dL	0.78 ± 0.26	0.78 ± 0.14	0.81 ± 0.28	0.65
eGFR, mL/min/1.73m2	70 ± 10	70 ± 9	67 ± 13	0.087
Low eGFR, n (%)	1887 (15)	13 (10)	22 (31)	<0.001
UAER, mg/g	21 ± 129	12 ± 20	94 ± 428	<0.001
Albuminuria, n (%)	1157 (9)	8 (6)	14 (19)	0.006
Smoking status				
Never/former/current, %	52/25/23	43/29/28	60/24/17	0.18
Drinking status				
Never/former/current, %	20/5/75	19/5/75	32/17/51	<0.001

ALT, alanine aminotransferase; AST, aspartate aminotransferase; eGFR, estimated glomerular filtration rate; γGTP, gamma-glutamyltransferase; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment-insulin resistance; UAER, urine albumin excretion ratio; WBC, white blood cells; RBC, red blood cells.

described in Table 1. Of the 12 535 subjects enrolled, 130 (1.0%; 37 women, 93 men) and 72 (0.6%; 27 women, 45 men) were positive for HBsAg and HCcAg, respectively; no subjects were positive for both HBsAg and HCcAg. HCcAg-positive individuals were significantly older than hepatitis-negative individuals ($P < 0.001$), whereas the age between HBsAg-positive and hepatitis-negative individuals did not differ significantly. All hepatitis-positive individuals enrolled in the current study, except one HBsAg-positive subject, underwent abdominal ultrasonography, and none was diagnosed as having advanced cirrhosis. The hematological data and aminotransferase levels of the individual who did not undergo abdominal ultrasonography were as follows: white blood cell count, 4000 (cells/microL); red blood cell count, 524 × 104 (cells/microL); Plt 25.4 × 104 (cells/microL); ALT 19 (IU/L); and AST 19 (IU/L). In the HCcAg-positive group, the mean serum TC level was lower than in the other two groups. Logistic regression analysis adjusted for sex, age, ALT, albumin and total bilirubin levels showed that an odds ratio of HBsAg-positivity and HCcAg-positivity for the lowest TC

Table 2 Logistic regression analysis for HBV/HCV infection as independent variables, and low eGFR and albuminuria as dependent variables

	Dependent variables					
	CKD Odds ratio (95% CI)	*P*-value	Components of CKD			
			Low eGFR Odds ratio (95% CI)	*P*-value	Albuminuria Odds ratio (95% CI)	*P*-value
Unadjusted						
HBV/HCV negative	1.00	–	1.00	–	1.00	–
HBsAg positive	0.63 (0.39–1.01)	0.056	0.62 (0.35–1.09)	0.098	0.63 (0.31–1.30)	0.21
HCcAg positive	2.46 (1.54–3.94)	0.0002	2.44 (1.47–4.03)	0.0005	2.33 (1.30–4.19)	0.0047
Adjusted for age and sex						
HBV/HCV negative	1.00	–	1.00	–	1.00	–
HBsAg positive	0.53 (0.32–0.86)	0.011	0.51 (0.28–0.93)	0.027	0.57 (0.28–1.18)	0.13
HCcAg positive	1.77 (1.08–2.92)	0.025	1.64 (0.96–2.82)	0.071	1.86 (1.02–3.37)	0.042
Adjusted for age, sex, SBP and FPG						
HBV/HCV negative	1.00	–	1.00	–	1.00	–
HBsAg positive	0.49 (0.30–0.81)	0.0057	0.51 (0.28–0.92)	0.026	0.50 (0.23–1.05)	0.066
HCcAg positive	1.83 (1.10–3.05)	0.020	1.63 (0.95–2.80)	0.077	2.00 (1.06–3.76)	0.034

CKD, chronic kidney disease; FPG, fasting plasma glucose; HBV, hepatitis B virus; HCV, hepatitis C virus; SBP, systolic blood pressure.

quartile (TC < 187 mg/dL) was 1.42 (95% CI 0.95–2.12, $P = 0.89$) and 7.30 (95% CI 4.39–12.13, $P < 0.001$), respectively, compared with hepatitis-negative individuals. The finding that HCcAg-positive individuals had lower TC levels than non-hepatitis or HBsAg-positive individuals was in agreement with previous observations of ours and others.[14,15] Neither FPG nor HbA1c differed significantly between individuals positive for HBsAg or HCcAg and hepatitis-negative individuals; however, HOMA-IR was significantly greater in HCcAg-positive individuals than in hepatitis-negative ($P < 0.001$) or HBsAg-positive ($P = 0.003$) individuals. Serum albumin level was statistically significantly lower in HCcAg-positive subjects than in hepatitis-negative subjects, although the difference was very small (Table 1 and 4.4 g/dL vs. 4.5 g/dL). By Bonferroni post hoc analysis, serum bilirubin levels were not statistically significantly different between HCcAg-positive and hepatitis-negative individuals or between HBsAg-positive and hepatitis-negative individuals.

eGFR and urinary albumin excretion

Of the 12 535 subjects enrolled, 1179 (9.4%, 389 women, 790 men) had albuminuria, and 1922 (15.3%, 729 women, 1193 men) had low eGFR. Both of these conditions were present in 278 individuals (2.2%); therefore, 2823 (22.5%) subjects (1023 women, 1800 men) were diagnosed to have CKD. Among the 1179 (9.4%) individuals who had albuminuria, 1062 had an UAER value between 30 and 299 mg/g (microalbuminuria), and the remaining 117 had an UAER value of ≥300 mg/g (macroalbuminuria). The median (interquartile range) of eGFR (mL/min/1.73 m^2) was 69.6 (63.2–75.8) in HBV/HCV-negative individuals, 69.5 (64.3–77.2) in HBsAg-positive individuals, and 65.9 (58.4–76.9) in HCcAg-positive individuals. The median (interquartile range) of UAER (mg/g) was 6.4 (4.2–11.8) in HBV/HCV-negative individuals, 6.4 (4.2–11.6) in HBsAg-positive individuals and 8.0 (4.1–18.6) in HCcAg-positive individuals.

Association between HBsAg/HCcAg positivity and CKD

The prevalence of both low eGFR ($P < 0.001$) and albuminuria ($P = 0.007$) was significantly greater in HCcAg-positive than in HBV/HCV-negative individuals by χ^2 test (Table 1). In contrast, compared with HBV/HCV-negative individuals, the prevalence of either low eGFR ($P = 0.12$) or albuminuria ($P = 0.27$) was not different in HBsAg-positive individuals. After adjusting for age and sex, logistic regression analysis showed that HCcAg was statistically significantly positively associated with albuminuria (Table 2) and that it tended to be positively associated with low eGFR. In contrast, HBsAg positivity was inversely associated with low eGFR, whereas it was not significantly associated with albuminuria. Essentially the same results were obtained after further adjustment for SBP and FPG.

Table 3 Logistic regression analysis for HBV/HCV infection as independent variables, and metabolic syndrome, increased insulin resistance and elevated ALT levels as dependent variables

	Dependent variables					
	Metabolic syndrome Odds ratio (95% CI)	*P*-value	Highest HOMA-IR quartile Odds ratio (95% CI)	*P*-value	Highest ALT quartile Odds ratio (95% CI)	*P*-value
Unadjusted						
HBV/HCV negative	1.00	–	1.00	–	1.00	–
HBsAg positive	1.21 (0.71–2.04)	0.49	1.60 (1.12–2.31)	0.011	1.42 (0.98–2.06)	0.068
HCcAg positive	0.25 (0.60–1.00)	0.050	3.39 (2.13–5.39)	<0.0001	10.3 (0.60–17.8)	<0.0001
Adjusted for age and sex						
HBV/HCV negative	1.00	–	1.00	–	1.00	–
HBsAg positive	1.09 (0.34–1.86)	0.75	1.57 (1.09–2.26)	0.016	1.36 (0.92–2.01)	0.12
HCcAg positive	0.23 (0.06–0.95)	0.042	3.18 (1.99–5.05)	<0.0001	16.53 (9.20–29.7)	<0.0001

ALT, alanine aminotransferase; HBV, hepatitis B virus; HCV, hepatitis C virus; HOMA-IR, homeostasis model assessment-insulin resistance.

HBsAg/HCcAg positivity, metabolic syndrome and insulin resistance

Metabolic syndrome was diagnosed in 1304 individuals (10.4%, 160 women and 1144 men). The mean values of HOMA-IR in individuals with and without metabolic syndrome were 3.1 ± 3.1 and 1.4 ± 1.0, respectively ($P < 0.001$). Age and sex-adjusted logistic regression analysis showed that HCcAg positivity was inversely associated with metabolic syndrome, whereas HBsAg positivity was not (Table 3). On the other hand, after adjusting for the same variables, both HBsAg and HCcAg positivity was positively associated with the highest sex-specific HOMA-IR quartile, which was HOMA-IR of >1.39 in women and >2.06 in men.

Relationship between metabolic syndrome, insulin resistance and CKD components

After adjusting for age and sex, logistic regression analysis showed that metabolic syndrome was positively associated with both low eGFR (odds ratio 1.43 [95% CI 1.23–1.67, $P < 0.001$]) and albuminuria (odds ratio 3.84 [95% CI 3.31–4.47, $P < 0.001$]). After adjusting for the same variables, the highest HOMA-IR quartile was also positively associated with both low eGFR (odds ratio 1.21 [95% CI 1.08–1.35, $P = 0.0012$]) and albuminuria (odds ratio 2.86 [95% CI 2.52–3.23, $P < 0.001$]).

The relationship between HBV/HCV infection and CKD components was analyzed after further adjustment for either metabolic syndrome or HOMA-IR (Table 4). The negative association between HBsAg positivity and low eGFR and the positive association between HCcAg positivity and albuminuria remained statistically significant after further adjustment for metabolic syndrome. However, in the logistic regression analysis further adjusted for HOMA-IR, the association between HCcAg positivity and albuminuria did not remain statistically significant.

Serum alanine aminotransferase activity and CKD components

Logistic regression analysis adjusted for age, sex, SBP and FPG showed that ALT was dose-dependently associated with albuminuria, but not with low eGFR (Table 5). When adjusted for age, sex, SBP, FPG and ALT, the positive association between HCcAg positivity and albuminuria did not remain statistically significant, whereas the negative association between HBsAg positivity and low eGFR remained statistically significant (Table 4).

DISCUSSION

IN THE CURRENT study, by analyzing the data from individuals who underwent general health screening, it was found that HCcAg positivity was associated with a greater prevalence of low eGFR and albuminuria, both of which are components of CKD, than hepatitis-negative individuals. By contrast, the prevalence of neither low eGFR nor albuminuria was not different between HBsAg-positive and hepatitis-negative individuals. After adjusting for age, sex, SBP and FPG, the association of HCcAg with low eGFR (tendency) or with albuminuria (statistically significant) was still present.

Table 4 Logistic regression analysis for HBV/HCV infection as independent variables, and low eGFR and albuminuria as dependent variables after further adjusting for HOMA-IR and ALT

	Dependent variables					
	CKD Odds ratio (95% CI)	*P*-value	Components of CKD			
			low eGFR Odds ratio (95% CI)	*P*-value	Albuminuria Odds ratio (95% CI)	*P*-value
Adjusted for age, sex and metabolic syndrome						
HBV/HCV negative	1.00	–	1.00	–	1.00	–
HBsAg positive	0.51 (0.31–0.84)	0.0082	0.50 (0.28–0.91)	0.024	0.54 (0.26–1.13)	0.10
HCcAg positive	1.92 (1.17–3.17)	0.010	1.70 (0.99–2.91)	0.055	2.19 (1.21–3.99)	0.010
Adjusted for age, sex, SBP, FPG and HOMA-IR						
HBV/HCV negative	1.00	–	1.00	–	1.00	–
HBsAg positive	0.49 (0.29–0.80)	0.0046	0.51 (0.28–0.92)	0.025	0.48 (0.23–1.02)	0.056
HCcAg positive	1.63 (0.97–2.74)	0.064	1.58 (0.92–2.72)	0.099	1.67 (0.88–3.19)	0.12
Adjusted for age, sex, SBP, FPG and ALT						
HBV/HCV negative	1.00	–	1.00	–	1.00	–
HBsAg positive	0.49 (0.30–0.81)	0.0050	0.51 (0.28–0.92)	0.025	0.49 (0.23–1.03)	0.060
HCcAg positive	1.55 (0.92–2.59)	0.098	1.49 (0.86–2.57)	0.16	1.59 (0.83–3.02)	0.16

ALT, alanine aminotransferase; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HBV, hepatitis B virus; HCV, hepatitis C virus; HOMA-IR, homeostasis model assessment-insulin resistance; SBP, systolic blood pressure.

Table 5 Logistic regression analysis for ALT quartiles as an independent variable and low eGFR, and albuminuria as dependent variables

	Dependent variables					
	CKD Odds ratio (95% CI)	*P*-value	Components of CKD			
			Low eGFR Odds ratio (95% CI)	*P*-value	Albuminuria Odds ratio (95% CI)	*P*-value
Unadjusted						
ALT-Q1	1.00	–	1.00	–	1.00	–
ALT-Q2	1.21 (1.07–1.36)	0.0020	1.21 (0.16–1.38)	0.0058	1.16 (0.96–1.40)	0.012
ALT-Q3	1.35 (0.20–1.53)	<0.0001	1.21 (1.06–1.39)	0.0057	1.55 (1.29–1.85)	<0.0001
ALT-Q4	1.33 (1.18–1.50)	<0.0001	0.95 (0.82–1.09)	0.45	2.05 (1.73–2.43)	<0.0001
Adjusted for age and sex						
ALT-Q1	1.00	–	1.00	–	1.00	–
ALT-Q2	1.03 (0.91–1.17)	0.63	1.02 (0.88–1.17)	0.80	1.04 (0.86–1.26)	0.66
ALT-Q3	1.27 (1.12–1.45)	0.0003	1.13 (0.97–1.31)	0.11	1.47 (1.22–1.77)	<0.0001
ALT-Q4	1.47 (1.28–1.67)	<0.0001	1.03 (0.88–1.20)	0.70	2.16 (1.80–2.59)	<0.0001
Adjusted for age, sex, SBP and FPG						
ALT-Q1	1.00	–	1.00	–	1.00	–
ALT-Q2	1.00 (0.88–1.14)	0.96	1.03 (0.89–1.19)	0.68	0.97 (0.80–1.18)	0.75
ALT-Q3	1.18 (1.03–1.34)	0.015	1.15 (0.99–1.34)	0.062	1.23 (1.02–1.49)	0.035
ALT-Q4	1.24 (1.08–1.41)	0.0023	1.08 (0.93–1.27)	0.32	1.45 (1.20–1.76)	0.0001

ALT-Q1, ALT-Q2, ALT-Q3 and ALT-Q4 indicate the first, second, third and fourth, respectively, serum alanine aminotransferase activity quartiles.
ALT, alanine aminotransferase; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; SBP, systolic blood pressure.

Both HCcAg positivity and HBsAg positivity were positively associated with increased insulin resistance. On the other hand, HCcAg positivity was inversely associated with metabolic syndrome.

Although renal involvement of hepatitis virus infection was first reported more than three decades ago,[16] knowledge of the association between HBV/HCV, proteinuria and low eGFR in the general population remains limited. Huang *et al.* analyzed data from individuals in southern Taiwan, an HBV/HCV-endemic area. They found that HBsAg and anti-HCV were positive in 13% and 7%, respectively, of the study population, and HCV infection, but not HBV infection, was associated with proteinuria.[17] Tsui *et al.* analyzed the data from a general population in the US and reported that HCV infection was associated with albuminuria, but not with low eGFR.[8] Our findings that albuminuria was positively associated with HCcAg positivity, but not with HBsAg, were therefore in agreement with these previous findings.

We showed that HCcAg positivity was associated with increased insulin resistance, defined as the highest HOMA-IR quartile. Several previous studies have shown that HCV infection was associated with diabetes as well as insulin resistance.[18,19] We have shown previously that HCV infection induces insulin resistance by the virus itself, which may influence the progression of chronic liver disease.[20,21] Compared to HCV infection, the relationship between HBsAg and insulin resistance has been less extensively studied. Custro *et al.* reported that both HBV and HCV infections increased the incidence of impaired glucose metabolism, and that the impact on glycemic homeostasis evoked by these two infections seemed to be similar.[22] In contrast, by analyzing subjects in Taiwan, where the prevalence of HBV infection is very high, Wang *et al.* showed that HBV carriers were not associated with insulin resistance.[23] We showed here that HBsAg positivity was also associated with increased insulin resistance, although to a lesser extent than HCcAg positivity (Table 3). Serum ALT levels, a marker for the extent of liver injury, is known to affect the degree of insulin resistance.[23] In the current study, the mean ALT levels were greater in HCcAg-positive than in HBsAg-positive individuals. The relative impacts of virus infection per se and liver injury for the development of hepatitis-related insulin resistance in our study population should be investigated further in future studies.

It was of note that the positive association between HCcAg positivity and albuminuria lost its statistical significance after adjusting for HOMA-IR, which suggested that the observed association between HCcAg positivity and albuminuria was confounded by insulin resistance. Insulin resistance is one of the background features of albuminuria,[24] and albuminuria is one of the diagnostic components of metabolic syndrome in WHO criteria.[12] In contrast to the positive association between HCV infection and increased insulin resistance, however, we found an apparent *negative* association between HCcAg positivity and metabolic syndrome (Table 3). Several previous studies also reported that the prevalence of metabolic syndrome was lower in HBV or HCV-infected individuals.[25,26] Together with these reports, our data suggest that increased insulin resistance, which may play a role in the development of albuminuria in HCV infection, may not be recognized as a phenotype of metabolic syndrome in HCcAg-positive individuals. In addition, our data suggest the possibility that increased insulin resistance, but not metabolic syndrome phenotype, enhances the risk for albuminuria and CKD in these individuals.

In the current study, the association between HBsAg and low eGFR or albuminuria was not statistically significant by univariate analysis (Table 1). However, after multivariate adjustment, there was an inverse mode association between HBsAg positivity and low eGFR (statistically significant) or albuminuria (tendency). Whether or not there is truly an inverse relationship between HBsAg positivity and CKD components should be investigated further after increasing the number of HBsAg-positive individuals. Nevertheless, we may be able to conclude from the current study that there is a difference in the mode of association with CKD components between HCcAg positivity and HBsAg positivity in individuals who underwent general health screening, and had, if present, only minor liver damage.

The current study had several limitations. First, GFR was not determined by a direct measurement, but instead by the MDRD formula with the Japanese coefficient of 0.881. A recent study has suggested that estimation of GFR by this method may result in an underestimation of GFR when insulin clearance is over 60 mL/min/1.73 m^2 in Japanese.[10] Second, we could not assess data of anti-HBe positivity, which might affect the prevalence of extrahepatic manifestations in HBV infection.[7] Third, due to the cross-sectional nature of the study, we could not derive the causal and resultant relationship between HBV/HCV infection and CKD components. Fourth, as the liver is the primary organ of insulin clearance, C-peptide concentration may be a better marker of secreted insulin levels and insulin resistance than parameters derived from insulin,[27] such as HOMA-IR; however, serum C-peptide data were not available in

the current study. Finally, interferon therapy may affect albuminuria and renal function, which may be either reversible or irreversible.[28–30] Although information on the history of interferon therapy was not available in the current study, this point should be taken into account in future studies.

CONCLUSION

IN CONCLUSION, BY analyzing the cross-sectional data of 12 535 individuals who underwent general health screening, we have investigated a possible association between viral hepatitis infection and CKD components. There was a positive association between HCcAg positivity, but not HBsAg positivity, and CKD components (low eGFR and albuminuria). The observed associations were confounded by the degree of insulin resistance and serum ALT levels. Although HCcAg positivity was associated with increased insulin resistance, HCcAg positivity was negatively associated with metabolic syndrome. These data collectively indicate that some differences may exist between HCV infection and HBV infection in terms of association with CKD components in Japanese individuals who undergo general health screening.

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Association between metabolic syndrome and carotid atherosclerosis in individuals without diabetes based on the oral glucose tolerance test

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ABSTRACT

Introduction: Whether or not metabolic syndrome is predictive of atherosclerotic disorders may depend on the population studied. We investigated whether metabolic syndrome is associated with carotid atherosclerosis in individuals who were shown not to have diabetes mellitus based on results of the 75-g oral glucose tolerance test (OGTT).
Methods and results: Between 1994 and 2003, 3904 individuals underwent general health screening that included the OGTT. Among these 3904 individuals, 3679 had a fasting plasma glucose of <126 mg/dL (subgroup 1), and 3488 had a 2-h post-OGTT glucose value of <200 mg/dL (subgroup 2). In both subgroups, metabolic syndrome was found to be a risk factor for carotid plaque and for carotid intima-media thickening in men, and tended to be a risk factor for carotid plaque in women after adjustment for age. Among 3473 individuals who had both a fasting plasma glucose value of <126 mg/dL and a 2-h post-OGTT glucose of <200 mg/dL, 2440 did not have hypertension, which was defined as systolic and diastolic blood pressure of <140/90 mmHg and absence of use of anti-hypertensive medication. In these non-diabetic non-hypertensive individuals, the association between metabolic syndrome and carotid plaque or carotid intima-media thickening was not statistically significant even with adjustment only for age.
Conclusions: In men who did not have impaired fasting glycemia and/or in those without impaired glucose tolerance, metabolic syndrome was a predictor of carotid atherosclerosis after age adjustment, although metabolic syndrome was not found to be a predictor of carotid atherosclerosis when hypertensive individuals were excluded from the study population.

1. Introduction

Metabolic syndrome (MetS) is a cluster of metabolic and hemodynamic abnormalities linked with insulin resistance. Since components of MetS also represent risk factors for atherosclerotic disorders, it is natural that individuals with this syndrome have an increased risk for ischemic heart disease [1] and stroke [2,3]. On the other hand, the clinical utility of MetS may depend on whether the risk conveyed by this syndrome is higher than the sum of each component utilized as diagnostic criteria for MetS [4,5].

Carotid artery intima-media thickness has been reported to be a discriminator as a surrogate of cardiovascular mortality in community-dwelling Japanese people [6] and, conversely, aggregation of established major coronary risk factors has been reported to strongly influence the presence of carotid atherogenesis in the general Japanese population [7]. Previously, we reported that the presence of MetS may not increase the risk for carotid atherosclerosis in individuals without hypertension, with hypertension defined as systolic blood pressure (SBP) of ≥140 mmHg, diastolic blood pressure (DBP) of ≥90 mmHg, or the use of anti-hypertensive medication [8]. This observation suggested that the properties of MetS that present a risk for atherosclerotic diseases may differ according to the populations selected. Consistent with this idea, it was reported that MetS was not found to be associated with cardiovascular mortality in non-diabetic non-hypertensive Chinese individuals [9], and that MetS did not significantly increase the risk of mortality from cardiovascular disease in non-diabetic Mexican Americans and non-Hispanic whites [10]. In the current study, we investigated whether MetS was associated with carotid atherosclerosis in Japanese individuals who did not have diabetes mellitus based on results of the 75-g oral glucose tolerance test (OGTT).

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