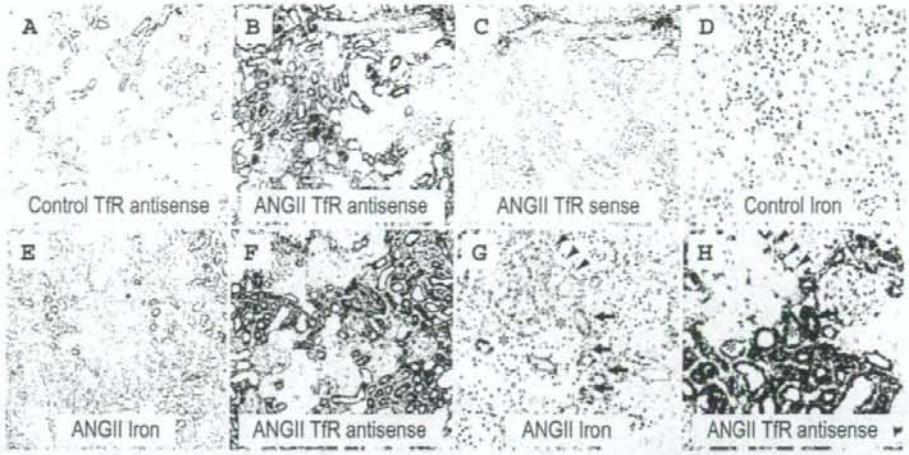

Fig. 1. *Expression and localization of TfR mRNA. Samples were from the kidney of control (A, D) and angiotensin II (ANGII)–infused (B, C, E–H) rats. B-C, E-F, and G-H are serial specimens. A, B, F, H:* In situ *hybridization using the TfR antisense probe. C:* In situ *hybridization using the TfR sense probe (background). D, E: Prussian blue staining for iron. TfR mRNA staining was weak in control rat kidney (A). After angiotensin II infusion, TfR mRNA expression became more widely distributed in the tubular and glomerular cells (B). No staining for iron was observed in the untreated control rat kidney (E). Localization of iron deposits (E) and TfR mRNA (F) showed overlap, although not completely. Higher magnification microscopy showed that some tubular cells that were positive for iron were also positive for TfR mRNA (G, H, arrows). By contrast, some tubular cells that were positive for iron deposition were negative for TfR (G, H, arrowheads), and* vice versa *(G, H, asterisks). Original magnifications, ×100 (A–C, E, F) and ×200 (D, G, H).*

AATGCAGTCTG-3′ for IRE(+)DMT1; 5′-TCTACCTCC TGAACACCGTG-3′ and 5′-CGTTAGCTTTACCCGACT CC-3′ for IRE(−)DMT1; 5′-CCAGATTATGACATTCGGT-3′ and 5′-TTGGCTCAGTATCTTTAGGT-3′ for FPN; and 5′-GGCAACAGACGAGACAGACT-3′ and 5′-ATGCAA CAGAGACCACAGGA-3′ for hepc. The primers used for TfR and GAPDH have been described previously (*19*). The mRNA expression of these genes was normalized to GAPDH mRNA expression and is presented here as the percentage of the values from the aortas of untreated animals.

Statistical Analysis

Data are expressed as the mean±SEM. We used ANOVA followed by a multiple comparison test to compare raw data, before we expressed the results as a percentage of the control value using statistical analysis software, StatView ver. 5.0 (SAS Institute, Cary, USA). A value of $p<0.05$ was considered to be statistically significant.

Results

Localization of the Expression of Iron Metabolism–Related Genes

In situ hybridization revealed that TfR mRNA was weakly expressed, primarily in the tubular and glomerular cells in the kidneys of untreated animals (Fig. 1A). After angiotensin II treatment, TfR mRNA expression was more widely distributed in these regions (Fig. 1B, C). As we reported previously (*15*), angiotensin II infusion led to iron deposition, primarily in the proximal tubular epithelial cells, as detected by Prussian blue staining (Fig. 1D, E). Staining of serial specimens showed the possible overlap of iron and TfR staining (Fig. 1E, F). Higher magnification microscopy showed that levels of TfR mRNA expression were also increased in the glomerular cells and that some TfR-positive cells were positive for iron (Fig. 1G, H, arrows), whereas some iron-positive cells were negative for TfR (Fig. 1G, H, arrowheads), and some TfR-positive cells were negative for iron (Fig. 1G, H, asterisks). DMT1 mRNA expression could be observed in the tubular and glomerular cells in the untreated rat kidney and was markedly increased after angiotensin II infusion (Fig. 2A–C). Similar to TfR mRNA expression, some tubular cells were positive for both iron and DMT1 (Fig. 2D, E, arrows), whereas others were positive for iron but negative for DMT1 (Fig. 2D, E, arrowheads) or *vice versa* (Fig. 2D, E, asterisks). Staining for FPN mRNA was very weak in the control kidney (Fig. 2F), but was substantially increased after angiotensin II infusion (Fig. 2G, H). Some tubular cells were positive for both iron and FPN (Fig. 2I, J, arrows), whereas others were positive for iron but negative for FPN (Fig. 2I, J, arrowheads)

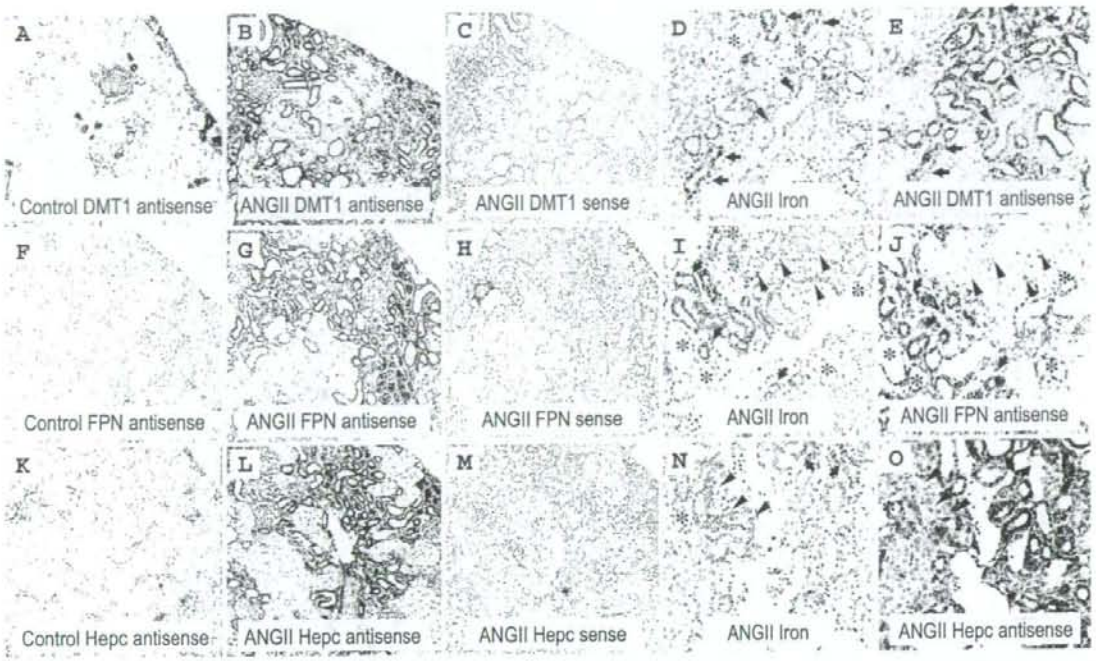

Fig. 2. *Expression and localization of DMT1, FPN, and hepc mRNA and iron deposits. Samples were from the kidney of control (A, F, K) and angiotensin II (ANGII)–infused (B–E, G–J, L–O) rats. B-C, D-E, G-H, I-J, L-M, and N-O are serial specimens. A, B, E:* In situ *hybridization using the DMT1 antisense probe. C:* In situ *hybridization using the DMT1 sense probe (background). F, G, J:* In situ *hybridization using the FPN antisense probe. H:* In situ *hybridization using the FPN sense probe (background). K, L, O:* In situ *hybridization using the hepc antisense probe. M:* In situ *hybridization using the hepc sense probe (background). D, I, N: Prussian blue staining for iron. mRNA expression of DMT1, FPN, and hepc mRNA was more widely distributed after angiotensin II infusion (A, B, F, G, K, L). As in the case of TfR mRNA, some tubular cells that were positive for iron were also positive for DMT1, FPN, or hepc mRNA (D, E, I, J, N, O, arrows). By contrast, some tubular cells that were positive for iron deposition were negative for TfR (D, E, I, J, N, O, arrowheads), and* vice versa *(D, E, I, J, N, O, asterisks). Original magnifications, ×100 (A–C, F–H, K–M) and ×200 (D, E, I, J, N, O).*

or *vice versa* (Fig. 2I, J, asterisks). Hepc mRNA was also expressed weakly in the tubular and glomerular cells in the untreated control rat kidney, and it was also upregulated by angiotensin II (Fig. 2K–O).

Quantification of Iron Metabolism–Related Gene Expression in the Kidney

Real time RT-PCR showed a ~1.9-fold increase in TfR mRNA expression after the infusion with angiotensin II; this increase was suppressed by both hydralazine and losartan (Fig. 3A). The expression of IRE(−)DMT1 mRNA also increased after angiotensin II infusion, which was suppressed by both hydralazine and losartan (Fig. 3B). IRE(+)DMT1 expression was not significantly increased by angiotensin II infusion (Fig. 3C). The expression of FPN mRNA was increased by angiotensin II; this increase was not affected by hydralazine, but was suppressed by losartan. Hepc mRNA expression showed more than a four-fold increase after angiotensin II infusion; this increase was suppressed by both hydralazine and losartan (Fig. 3E). Norepinephrine infusion increased the levels of expression of hepc mRNA, but not those of TfR, IRE(−)DMT1, IRE(+)DMT1, or FPN.

Comparison of the Localization of Lipid Deposits and Iron Metabolism–Related Gene mRNA

We previously found that angiotensin II infusion causes a marked accumulation of lipids in the tubular epithelial cells, and this lipid deposition co-localized with the expression of transforming growth factor-β1 (TGF-β1) mRNA (*17*). We therefore characterized the localization of lipid deposition in relation to the expression of iron metabolism–related genes (Fig. 4). Only a small fraction of TfR, DMT1, and hepc mRNA was found to co-localize with lipid deposition in the angiotensin II–treated rat kidney. By contrast, there was con-

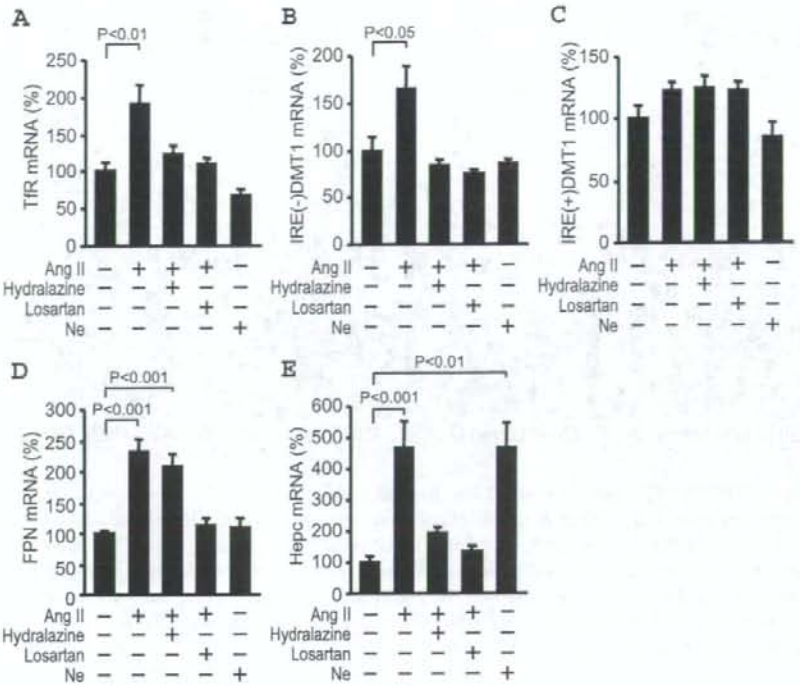

Fig. 3. *Quantification of the expression of iron metabolism–related genes. Expression of TfR (A), IRE(−)DMT1 (B), IRE(+)DMT1 (C), FPN (D), and hepc (E) mRNA was analyzed by quantitative PCR using the HibriProbe system and a LightCycler (Roche). Shown is a summary of the results from quantitative RT-PCR from 6 to 10 samples. Ne, norepinephrine.*

siderable co-localization of FPN mRNA and lipid particles (Fig. 4E, F).

Discussion

Here we have investigated the expression patterns of several iron metabolism–related genes and their regulation by angiotensin II at the mRNA level. We found that the expression of all genes tested (TfR, IRE(−)DMT1, FPN, hepc), with the exception of IRE(+)DMT1, was upregulated at the mRNA level by angiotensin II infusion. Angiotensin II infusion induced slightly different regulatory effects according to the genes tested, in terms of localization and dependency on hypertension *per se*. The causal or resultant relationship between iron deposition and regulation of the expression of these genes remains to be elucidated; however, our results suggest that expression of these iron metabolism–related genes in the kidney may play a role in the modulation of the homeostasis of iron at either the whole-body or the local level. We found that after angiotensin II infusion, some proximal tubular cells exhibiting iron deposition showed increased expression of the iron metabolism–related genes, although some discrepancies were observed (Figs. 1, 2); these results stood in contrast with the exclusive co-localization of ferritin and heme oxygenase-1 (*15*). In addition, only a fraction of the mRNA expression of the genes tested was co-localized with lipid deposition, again in contrast to the exclusive co-localization of TGF-β1 mRNA and lipid deposition in the kidney (*16, 17*).

TfR, which facilitates the efficient cellular uptake of holotransferrin (a ferric-iron bound transferrin) (*20*) is expressed in the kidney. Recent studies have suggested that, in addition to reabsorbing the iron compounds filtered from the glomerulus, TfR might act as an immunoglobulin (Ig)A1 receptor and might be involved in the pathogenesis of IgA nephropathy (*21, 22*). It remains of interest whether or not the modulation of TfR expression underlies the renoprotective effects of angiotensin II converting enzyme inhibitor and the AT_1 receptor blocker in IgA nephropathy. DMT1 is expressed at the absorptive epithelium of the duodenum. A mutation of the transmembrane domain of DMT1 in anemic *mk* mice and Belgrade (b) rats (*2, 3*) causes impaired iron uptake at the intestinal brush border, thus indicating that DMT1 plays a pivotal role as an iron transporter. DMT1 has been shown to be expressed in other organs, including the placenta, brain, and kidney. Alternative splicing of the DMT1 gene produces two different mRNAs, namely, IRE(−)DMT1 and IRE(+)DMT1 (*2, 3*). In the current study, *in situ* hybridization

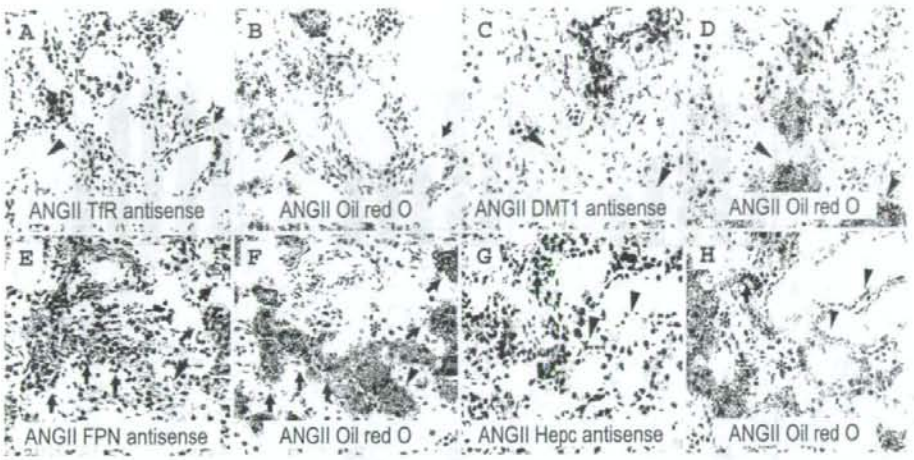

Fig. 4. *Localization of DMT1, FPN, and hepc mRNA and lipid deposition. Unfixed, frozen samples from the kidneys of angiotensin II–infused rats were used. A-B, C-D, E-F, and G-H are serial specimens. A:* In situ *hybridization using the TfR antisense probe. B, D, F, H: Oil red O staining. C:* In situ *hybridization using the DMT1 antisense probe. E:* In situ *hybridization using the FPN antisense probe. G:* In situ *hybridization using the hepc antisense probe. Some tubular cells that were positive for lipid deposition were also positive for the mRNA tested (arrows). By contrast, some tubular cells that were positive for iron deposition were negative for the mRNA tested (arrowheads), and some tubular cells that were negative for iron deposition were positive for the mRNA tested (asterisks). Original magnification, ×200.*

was used to reveal that DMT1 was expressed in the cortex in the kidney of untreated rats, and we demonstrated that both IRE(−) and IRE(+) splicing variants were expressed by RT-PCR; these findings are consistent with those of previous studies (*11*, *12*, *23*, *24*). Because of its localization in the kidney, DMT1 is thought to play a role in the reabsorption of iron in the kidney (*11*). If this is indeed the case, then angiotensin II–induced upregulation of renal DMT1 might enhance the reuptake of filtered iron into the tubular cells, resulting in tubular iron deposition, which would be in accordance with the co-localization (albeit partial co-localization) of DMT1 mRNA and iron deposition in the kidney of angiotensin II–infused rats. This possibility must be carefully validated, however, because DMT1 expression has been found to be reduced in the kidney of an animal model of diabetes (*10*), in which iron accumulation in the tubular cells has also been reported (*25*).

FPN, an iron exporter, is involved in the release of iron from enterocytes of the duodenum and tissue macrophages, and mutation of FPN results in a hemochromatosis-like phenotype (*26*, *27*). It is presumed that in the duodenum, iron is transported into enterocytes across the apical membrane by DMT1, and is then exported out of the cell and into the portal circulation across the basolateral membrane *via* FPN. As we found in the current study, FPN is expressed in the kidney (*4*, *5*); however, little is known about the regulation of renal FPN expression. It has been reported that FPN expression may not be affected by an altered dietary intake of copper (*13*). In the current study, FPN was clearly upregulated in the renal cells after angiotensin II infusion. It is possible that FPN mRNA was upregulated in response to the deposition of iron in some tubular epithelial cells in the kidney of angiotensin II–infused rats. However, a recent study has shown that mutation of the FPN gene causes iron accumulation in hepatic macrophages (*5*, *28*), but not in the enterocytes (*29*), suggesting that FPN haploinsufficiency affects iron export from Kupffer cells, but not from enterocytes. Therefore, the iron export system in parenchymal cells may differ from that in the tissue macrophages.

Hepc, which is expressed most abundantly in the liver, plays a pivotal role in the development of anemia associated with inflammation, innate immunity, and iron metabolism (*30*). Kulaksiz and co-workers have reported that hepc protein is also expressed strongly in the thick ascending limb of the cortex and in the connecting tubules in the rat kidney (*14*). Our findings demonstrated that the expression of both FPN and hepc mRNA was induced by angiotensin II. The role of the angiotensin II–induced upregulation of hepc mRNA awaits further investigation.

In the present study, the expression of all tested genes except for IRE(+)DMT1 was upregulated in the kidney of angiotensin II–infused rats, although the pressor-dependency may differ slightly. For example, the expression of hepc was upregulated in response to both angiotensin II and norepinephrine, suggesting that hypertension *per se* may play a role in the regulation of the hepc expression. To date, little is

known about the regulation of iron homeostasis in hypertensive patients. Piperno *et al*. have reported that increased serum ferritin was more frequent in subjects with essential hypertension than in normotensive subjects (*31*). In their study, increased resistance appeared to be among the possible mechanisms underlying iron overload in an animal model of hypertension (*32*). Urinary transferrin excretion has been reported to be increased when albuminuria is present, not only in diabetic hypertensive cases (*33*), but also in non-diabetic hypertensive cases (*34*); this increase might also in part account for the link between altered iron homeostasis in the kidney and hypertension. It has been reported that treatment of diabetic patients with low-dose candesartan slightly decreased blood pressure, and this in turn reversed the increase in the urinary excretion of transferrin over time (*35*).

In the current study, we targeted the regulation of the expression of several newly discovered iron metabolism–related genes in the kidney. It is well known that the whole-body iron balance is maintained by the regulation of iron absorption by the intestine, as essentially no pathway for iron excretion is present in humans (*20*). Considering that anemia is among the possible side effects of all commercially available AT_1 receptor blockers in Japan, regulation of the expression of iron metabolism–related genes by angiotensin II or by the renin angiotensin system in intestinal cells should also be closely investigated in future studies.

In conclusion, we have characterized the expression patterns of several iron metabolism–related genes, including TfR, DMT1, FPN, and hepc, and their regulation by angiotensin II in the rat kidney. Further studies are necessary for analyzing the relative contribution of these genes to renal iron homeostasis and, presumably, to tubular iron reabsorption in terms of the activity and involvement of the renin angiotensin system.

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ORIGINAL ARTICLE

Ningen Dock 2007 ; **21** : 47－49

Carotid Ultrasonography in General Health Screening : Non-Invasive Assessment of Early Atherosclerosis

Nobukazu Ishizaka

As carotid ultrasonography is an easy, immediate, and non-invasive diagnosing modality that does not involve radiation exposure, it has gained considerable recognition for use in the screening of carotid atherosclerosis even in asymptomatic subjects. Carotid plaque may be defined when the maximum of the IMT measured at the several arterial segments surpasses a certain cut-off value, and, although not always, has a focal protrusion. The size, number, surface morphology, and echogenicity of such carotid plaque may provide useful information for estimating the likelihood of future ischemic cerebrovascular events. Although significant luminal narrowing (≥70% stenosis) of carotid arteries that may increase the incidence of stroke will rarely be encountered in the setting of Ningen Dock, the presence of carotid atherosclerosis may also indicate an increased likelihood of the presence of coronary artery stenosis or future cardiac events. In addition, visualization of the presence of plaque may also increase the subjects' motivation for making lifestyle modifications, such as smoking cessation. Due to its non-invasive nature, carotid ultrasonography has become reasonable and feasible modality for the diagnosis of early atherosclerosis in the setting of general health screening or Ningen Dock. (*Ningen Dock* 2007 ; **21** : 47－49)

Key Words : ultrasonography, intima-media thickness (IMT), screening, ningen dock

Duplex carotid ultrasonography, which enables the observation of both B mode ultrasonography and Doppler mode ultrasonography, has gained considerable recognition for use in the screening of carotid atherosclerosis. Because of its non-invasive nature, this method is appropriate for use on asymptomatic subjects, such as those who undergo general health screening or Ningen Dock. Although the exact screening region of the carotid artery that is screened may differ according to the institute, it usually includes the common carotid artery and bilateral internal carotid artery. We can also get images of vertebral arteries by ultrasonography, although some of these arteries may be obscured by vertebral bones. Ultrasonography can observe three layer structure, and the first high echoic layer and the second low echoic layer are collectively considered to represent the intima-media complex (**Fig. 1**). Carotid IMT increases with age and is larger in men than in women[1].

Diagnosis of Carotid Atherosclerosis

Carotid atherosclerosis may be diagnosed when there is a plaque or a thickening of the intima-media complex within the internal carotid arteries, bifurcations, and common carotid arteries[2]. When the thickness of the intima-media complex, commonly referred to as the IMT, is greater than a certain cut-off point, the subjects is said to have intima-media thickening. The cut-off value for diagnosing intima-media thickening may vary slightly across different studies.

Plaque may be defined when the maximum of the IMT measured at the several arterial segments surpasses a certain cut-off value, and, although not always, has a focal protrusion. Here again, cut-off values may differ slightly according to the study[3-9]. In Japan, guidelines for diagnosing carotid plaque have been advocated by several committees and study groups, including the Joint Committee on Guidelines for Management Stroke, the Japan Academy of Neurosonology (http : //wwwsoc.nii.ac.jp/jan/index.html), and the Society for the Study of Early Atherosclerosis (http : //www.imt-ca.com/).

In addition to the plaque size, the number of plaques, surface morphology, and echogenicity may provide useful information for estimating the likelihood of future ischemic cerebrovascular events[10]. Surface morphology may be classified as smooth, irregular or ulcerated. Heterogeneous plaque is considered to be at higher risk of rupture than homogenous plaque. Plaque may be categorized according to its density (hypodense, isodense, hyperdense, or calcified)[5,11]. Calcification within the plaque will make an acoustic shadow.

Screening for High-Grade Carotid Stenosis

Although significant luminal narrowing (≥70% stenosis) of carotid arteries increases the incidence of stroke, such high-grade carotid stenosis, which may require surgical or catheter intervention, will rarely be encountered in the setting of general health screening. Even when moderate-grade carotid stenosis is found in

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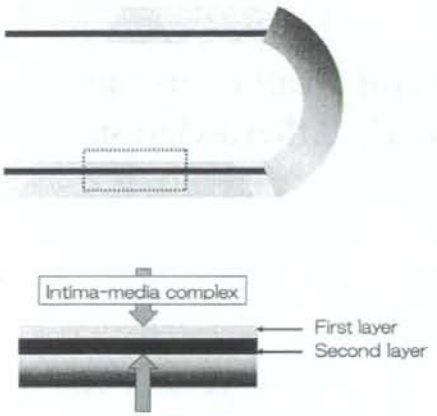

Fig. 1. *Schematic of the intima-media complex*

asymptomatic subjects, the indication for carotid artery endarterectomy should be carefully assessed because peri-operative mortality and/or morbidity following this procedure may not be negligible. Some investigators argue that carotid ultrasonography may not be cost-effective as a method of screening for significant carotid stenosis in the *asymptomatic* population[12], whereas other investigators have proposed that ultrasonographic screening for carotid artery screening may be more cost effective especially in the senior population, if a new and more rapid protocol of carotid artery screening protocol is adopted[13].

Although we may need to be aware of the cost-effectiveness of the ultrasonographic screening of extracranial carotid artery in *asymptomatic* individuals, cost-effectiveness may not be the chief concern of those undergoing general health screening in our country. As the presence of carotid plaque is a risk factor for ischemic stroke and coronary heart disease[7,14,24], anti-platelet and/or vasodilating agents might be recommended for a subset of subjects with such low-grade carotid stenosis. We may be able to identify the subjects at higher risk for ischemic stroke and/or coronary artery disease by plaque morphology combined with other diagnostic tests, such as a treadmill exercise test. At present, however, there is no standard therapeutic protocol is present for the treatment of subjects with such low-grade carotid stenosis.

Risk Factor Properties of Carotid Atherosclerosis

Atherosclerosis can develop simultaneously in different vascular beds. In this sense, it is reasonable to assume that the presence of carotid atherosclerosis may indicate an increased likelihood of the presence of coronary artery stenosis or future cardiac events. Indeed, much evidence shows that the presence of carotid intima-media thickening[15-17] and carotid plaque[18,19] are predictors for future cardio- and cerebrovascular events, and thus represent a subclinical atherosclerosis. A recent study showed that in elderly community-dwelling Japanese people, a 0.3 mm increase in the left and the right, respectively, carotid IMT was associated with a relative risk of 1.7 and 3.3 for all cause mortality, and that of 2.4 and 2.9 for cardiovascular mortality[20].

Craven *et al*. have reported that in individuals older than 50 years old, the B-mode score was associated with coronary stenosis, which was independent of other traditional coronary risk factors, and that considering the results of the B mode score, in addition to conventional risk factors, may increase the sensitivity and specificity for determining coronary artery disease status in such a population[21]. Simon *et al*. reported that the presence of carotid atherosclerosis in *asymptomatic* subjects was associated with a coronary heart disease with an incidence of 1.2% to 3.3% per year, which was, surprisingly, greater than the incidence associated with major risk factors, such as hypertension, diabetes, and smoking[22]. Importantly, they found that an absence of intima-media thickening was associated with a yearly incidence of coronary heart disease of 0.1% to 0.8%. Collectively, these studies indicate that the findings of carotid ultrasonography can provide useful information as an aid to the diagnosis of coronary heart disease.

Comparison with Other Non-Invasive Diagnostic Modalities

In addition to carotid ultrasonography, several non-invasive or least-invasive diagnostic modalities for atherosclerotic diseases have become available with recent advances in technology, including computed tomography (CT) and magnetic resonance (MR) angiography. In a recent meta-analysis, Wardlaw *et al.* compared the power and accuracy of several diagnostic tools for the diagnosis of carotid stenosis. They found that both the sensitivity and specificity of carotid ultrasonography are comparable to those of MR and CT angiography when 70–99% stenosis is present. On the other hand, the sensitivity of carotid ultrasonography and MR angiography for carotid stenosis may not be satisfactory for detecting carotid stenosis when there is an angiographically-proven level of 50–69% stenosis[23].

Carotid Ultrasonography in General Health Screening

In the setting of general health screening or Ningen Dock, many other hemodynamic and metabolic data can be obtained from the health screening participants at the time of carotid artery screening ; therefore, such data may enable us to analyze the possible association between early atherosclerosis and various non-traditional putative risk factors such as metabolic syndrome, microalbuminuria, CRP, and circulating WBC count (**Fig. 2**).

Carotid ultrasonography is an easy, immediate, and non-invasive diagnosing modality that does not involve radiation exposure. Visualization of the presence of plaque may help to assess the extent of atherosclerosis in the subjects, and may also increase the subjects' motivation for making lifestyle modifications, such as smoking cessation[25]. Considering that the presence of carotid plaque and intima-media thickening not only increases the risk of stroke, but also increases the like-

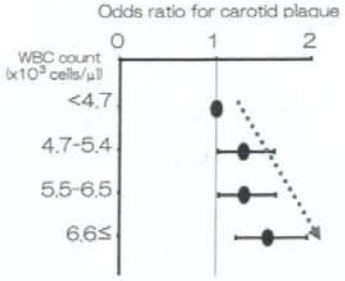

Fig. 2. *Increased WBC count is a risk factor for carotid plaque in men. Odds ratios were calculated after adjusting for age, BMI, systolic BP, total and HDL cholesterol, TG, and fasting glucose.*

lihood of the coronary artery disease, carotid ultrasonography is a feasible tool for detecting early atherosclerosis in the carotid arterial wall in the setting of general health screening or Ningen Dock.

Acknowledgement

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ORIGINAL ARTICLE

Ningen Dock 2007 ; **21** : 51−55

Albuminuria in General Health Screening in Japan : Relationship with Insulin Resistance and Atherosclerosis

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Background and Purpose The presence of microalbuminuria is a risk factor for advanced renal failure and atherosclerotic diseases. In the current study, we investigated the association between albuminuria, insulin resistance, and carotid atherosclerosis.

Methods and Results We analyzed data from 3674 individuals (female 1228, male 2446) who underwent general health screening. Microalbuminuria was defined as a urine albumin to urine creatinine ratio, termed the albumin excretion index (AEI), of 30 and 299 mg/g ; macroalbuminuria was defined as an AEI ≥300 mg/g. The prevalence of micro- and macroalbuminuria was 11.7% and 1.5%, respectively. When compared to the lowest AEI quartile (AEI <4.5 mg/g), the highest AEI quartile (AEI ≥150 mg/g) was found to be associated with metabolic syndrome (MetS) with an odds ratio of 5.7 (95% CI 1.7−19.3) in women and 3.9 (95% CI 2.9−5.3) in men after adjusting for total cholesterol (TC) and smoking status. In addition, after adjusting for sex, age, TC, smoking status, systolic BP, and fasting glucose, the highest AEI quartile was associated with carotid plaque with an odds ratio of 1.33 (95% CI 1.06−1.67).

Conclusion Our data show that the presence of albuminuria in individuals undergoing general health screening, even when it is below the cut-off value for "micro-" albuminuria, is a risk factor for MetS and carotid plaque. (*Ningen Dock* 2007 ; **21** : 51−55)

Key Words : albuminuria, ningen dock, metabolic syndrome (MetS), carotid atherosclerosis

The presence of microalbuminuria and macroalbuminuria is an established risk factor for cardiovascular morbidity and mortality as well as for end-stage renal disease in individuals with hypertension or diabetes mellitus[1−3]. Albuminuria is thought to reflect endothelial dysfunction, and this may explain the observed association between albuminuria and cardiovascular disease, although there might be other interlinking factors. Importantly, a link between albuminuria and cardiovascular disease may be present also in low-risk subjects, such as non-diabetic individuals[3] and those that are both non-hypertensive *and* non-diabetic[4]. In the current study, we analyzed the prevalence of microalbuminuria and macroalbuminuria in individuals undergoing general health screening, and assessed whether such albuminuria was a risk factor for insulin resistance, metabolic syndrome (MetS), and carotid plaque in this population.

Methods

Study Subjects

We have analyzed the data from 3674 individuals (female 1228, male 2446) who underwent general health screening at our institute between 2004 and 2006. Blood samples were taken from subject who had been fasting overnight. Serum levels of total cholesterol (TC), HDL cholesterol (HDL-C), and TG were determined enzymatically. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to the following formula : HOMA-IR=[fasting serum insulin (μU/ml) × fasting plasma glucose (mg/dl)]/405.

Diagnosis of Micro- and Macroalbuminuria

For the diagnosis of albuminuria, spot urine samples were collected and analyzed ; albuminuria was expressed the albumin excretion index (AEI). Normoalbuminuria, microalbuminuria, and macroalbuminuria were defined as an AEI of <30 mg/g, 30−299 mg/g, and 300 mg/g respectively[5]. Interquartile cut-off values of AEI were 4.5 mg/g, 7.5 mg/g, and 15.0 mg/g, respectively.

Criteria for Metabolic Syndrome

The diagnosis of MetS was made by the criteria of the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP-III)[6] with BMI as a surrogate for waist circumference. MetS was said to be present when three or more of the following conditions were met : (1) TG levels ≥150 mg/dl, (2) HDL-C levels <40 mg/dl in men or <50 mg/dl in women, (3) fasting plasma glucose ≥110 mg/dl or taking an antidiabetic medication, (4) systolic BP ≥130 mmHg or diastolic BP ≥85 mmHg or taking an antihypertensive medication, and (5) BMI >25 kg/m^2.

Carotid Ultrasonography

Carotid artery status was assessed by using a high-resolution B-mode ultrasonography instrument (Sono-

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layer SSA270A, Toshiba Medical Systems Corp., Tochigi, Japan) equipped with a 7.5MHz transducer (PLF-703ST, Toshiba Medical Systems Corp.). Here, we defined carotid plaque by the presence of portion(s) of the artery with an intima-media complex thickness of ≥1.1 mm[7] with a focal protrusion or point(s) of inflexion. This diagnostic criteria was different from the one we had used in several previous studies[8].

Statistical Analysis

The data in this study were analyzed by the χ^2 test, analysis of variance (ANOVA), and a multivariate logistic regression analysis using computer software, StatView ver. 5.0. Results are expressed as the mean±SD.

Results

Prevalence of Micro- and Macroalbuminuria

Mean ages±SD of the enrolled female and male subjects were 56.9±11.2 and 57.3±10.6 years, respectively. Mean±SD (median) value of the AEI was 27±127 (9) in women and 32±211 (6) in men. The overall prevalence of microalbuminuria was 9.8% in women and 10.4% in men, and that of macroalbuminuria was 1.4% and 1.6%, respectively (**Table 1**). Individuals with micro- or macroalbuminuria had significantly higher HOMA-IR values compared with those with normoalbuminuria. The prevalence of microalbuminuria increased with age in both genders (**Fig. 1 A**). The prevalence of micro- or macroalbuminuria according to diabetes and hypertension status is presented in **Fig. 1**

Table 1. Baseline characteristics of the subjects

Variables	Normo-albuminuria	Micro-albuminuria	Macro-albuminuria	*p* value
Women				
Subjects, *n* (%)	1090 (89)	121 (10)	17 (1)	
Age (years)	56 ± 11	62 ± 11	62 ± 11	<0.0001
BMI (kg/m^2)	21.5 ± 3.0	22.9 ± 4.2	23.8 ± 3.5	<0.0001
Systolic BP (mmHg)	120 ± 19	136 ± 23	145 ± 25	<0.0001
Diastolic BP (mmHg)	74 ± 12	82 ± 14	87 ± 12	<0.0001
Laboratory data				
CRP (mg/dl)	0.11 ± 0.39	0.19 ± 0.75	0.22 ± 0.37	0.069
Total cholesterol (mg/dl)	219 ± 35	226 ± 35	221 ± 48	0.13
HDL cholesterol (mg/dl)	68 ± 16	66 ± 17	59 ± 16	0.017
TG (mg/dl)	88 ± 49	96 ± 46	124 ± 55	0.0028
Fasting glucose (mg/dl)	92 ± 12	102 ± 27	111 ± 34	<0.0001
HOMA-IR	1.3 ± 0.9	1.8 ± 1.2	2.9 ± 2.5	<0.0001
Smoking status				
Non-smokers (%)	944 (87)	104 (86)	13 (76)	0.53
Former smokers (%)	61 (6)	9 (7)	1 (6)	
Current smokers (%)	85 (8)	8 (7)	3 (18)	
Men				
Subjects, *n* (%)	2153 (88)	255 (10)	38 (2)	
Age (years)	57 ± 11	61 ± 10	64 ± 12	<0.0001
BMI (kg/m^2)	24.0 ± 2.9	24.9 ± 2.7	24.7 ± 4.3	<0.0001
Systolic BP (mmHg)	127 ± 18	139 ± 20	142 ± 24	<0.0001
Diastolic BP (mmHg)	80 ± 11	87 ± 12	86 ± 14	<0.0001
Laboratory data				
CRP (mg/dl)	0.14 ± 0.43	0.26 ± 1.13	0.15 ± 0.20	0.0041
Total cholesterol (mg/dl)	208 ± 31	212 ± 35	213 ± 38	0.12
HDL cholesterol (mg/dl)	55 ± 13	54 ± 13	57 ± 20	0.45
TG (mg/dl)	136 ± 91	148 ± 112	153 ± 115	0.087
Fasting glucose (mg/dl)	101 ± 19	112 ± 30	123 ± 45	<0.0001
HOMA-IR	1.8 ± 1.3	2.7 ± 5.8	2.5 ± 1.8	<0.0001
Smoking status				
Non-smokers (%)	712 (33)	70 (27)	8 (21)	0.020
Former smokers (%)	878 (41)	128 (50)	16 (42)	
Current smokers (%)	563 (26)	57 (22)	14 (37)	

HOMA-IR : Homeostasis model assessment of insulin resistance.

B, where diabetes was defined as either fasting glucose ≥126 mg/dl *or* taking antidiabetic medication, and hypertension was defined as either systolic BP ≥130 mg/dl, diastolic BP ≥85 mg/dl, *or* taking antihypertensive medication. As shown in **Fig. 1B**, individuals with either hypertension or diabetes were more likely to have an AEI ≥30 mg/g (i.e., either micro- or macroalbuminuria) than those with neither of these conditions. In addition, in comparison with individuals who had neither of these conditions, an AEI ≥30 mg/g was more than 7- and 8-times more prevalent in women and men, respectively, when both hypertension and diabetes were present (**Fig. 1B**).

Albuminuria and Insulin Resistance

We can see that AEI increased according to HOMA-IR in both genders (**Figs. 2A** and **B**). After adjusting for age and TC, logistic regression analysis showed that AEI was associated with increased insulin resistance, defined here as HOMA-IR ≥2.0, in a value-dependent manner (**Fig. 2B**). Similar observations could be obtained when micro- and macroalbuminuria group was used in the place of AEI quartiles. As expected, albuminuria was also associated with the prevalence of MetS in a value-dependent manner (**Figs. 3A** and **B**).

Albuminuria and Carotid Atherosclerosis

Next, we investigated whether albuminuria was associated with carotid plaque, which is a marker for early atherosclerosis. The prevalence of carotid plaque increased according to the AEI quartiles in both genders (**Fig. 4A**). After adjusting for age and TC, the highest AEI quartile was found to be associated with a significantly higher prevalence of carotid plaque when the lowest AEI quartile was taken as reference. The association between carotid plaque and microalbuminuria was statistically significant in women, but not in men (**Fig. 4B**). Importantly, even after adjustment for sex, age, TC, *systolic BP, and fasting glucose*, the highest AEI quartile was found to be associated with carotid plaque with an odds ratio of 1.33 (95% CI 1.06–1.67, p=0.013) when the lowest AEI quartile was used as reference. When micro- and macroalbuminuria were put combined (i.e., an AEI ≥30 mg/g), age- and TC-adjusted logistic regression analysis showed that an AEI ≥30 mg/g was statistically significantly associated with carotid plaque in women with an odds ratio of 1.93 (95% CI 1.30–2.86, p=0.0011), but not in men (odds ratio 1.15 [95% CI 0.86–1.53, p=0.87]).

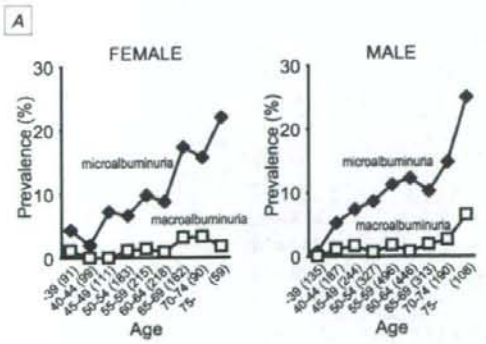

Fig. 1. *Prevalence of micro- and macroalbuminuria according to age (A) and diabetes and smoking status (B)*

Discussion

By analyzing the data of individuals with a mean age of 57 years old, we found that the prevalence of micro- and macroalbuminuria was 9.9% and 1.4%, respectively, in women, and 10.4% and 1.6%, respectively, in men. Albuminuria was value-dependently associated with insulin resistance (defined as a HOMA-IR ≥2.0) and MetS, defined by modified NCEP ATP III criteria. Presence of either hypertension or diabetes increased the prevalence of an AEI ≥30 mg/g (i.e., either micro- or macroalbuminuria), and when compared with those who had neither of these conditions, this prevalence became more than 7-times greater when both hypertension and diabetes were present. Furthermore, we have shown that the highest AEI quartile

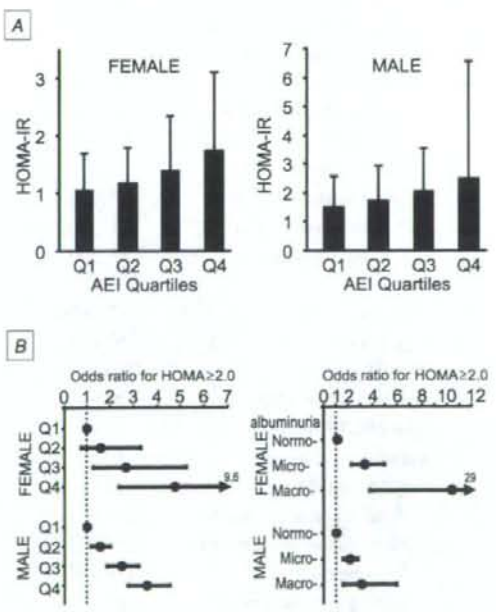

Fig. 2. *A. Homeostasis model assessment of insulin resistance (HOMA-IR) according to albumin excretion index (AEI) quartiles. B. Age-adjusted logistic regression analysis showing the association between urinary albumin excretion and increased insulin resistance, defined as HOMA-IR of ≥2.0.*

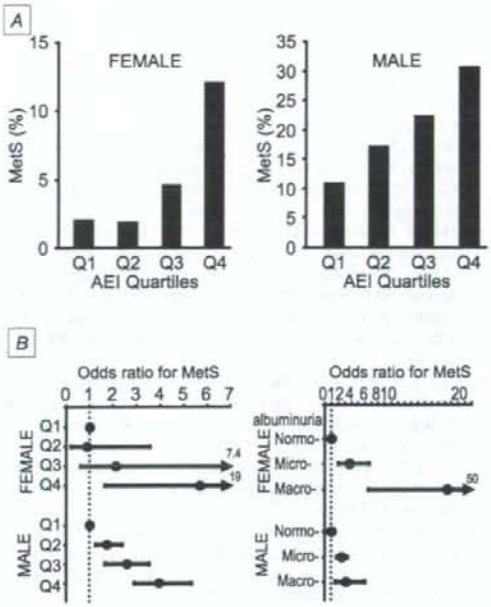

Fig. 3. *A. Prevalence of metabolic syndrome (MetS) according to albumin excretion index (AEI) quartiles. B. Age-adjusted logistic regression analysis showing the association between urinary albumin excretion and MetS.*

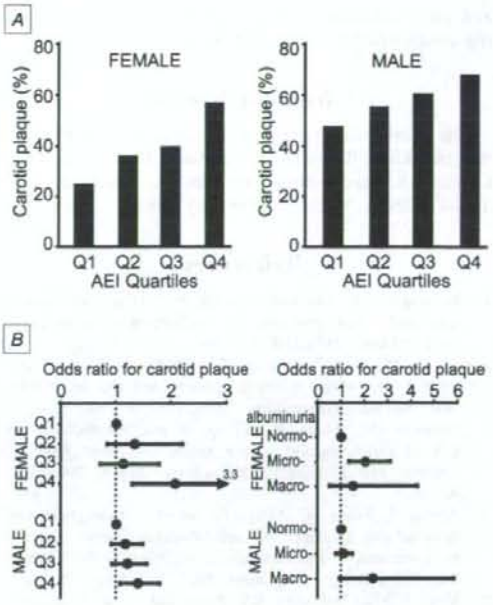

Fig. 4. *A. Prevalence of carotid plaque according to albumin excretion index (AEI) quartiles. B. Age-adjusted logistic regression analysis showing the association between urinary albumin excretion and carotid plaque.*

was significantly associated with carotid plaque after adjustment for age and TC in both genders. We also showed that individuals in the highest AEI quartile (AEI ≥15 mg/g) had a significantly higher prevalence of carotid plaque when compared with those in the lowest AEI quartile (AEI <4.5 mg/g) (**Fig. 4B**).

The prevalence of microalbuminuria has been compared in various races and ethnicities. Metcalf *et al*. discussed how the prevalence of microalbuminuria may be higher in non-Europeans (between 8% and 28%) than in Europeans (between 2% and 10%), although several different criteria may have been used for the diagnosis of microalbuminuria[9]. In Japan, the prevalence of microalbuminuria has been reported to be 13.2%[10] and 13.7%[11] in the general population, which were slightly greater than that found in the current study. The reported prevalence of microalbuminuria in non-diabetic subjects in our country seems to be similar to that in general population[12,13]. In the non-diabetic population, the prevalence of microalbuminuria in Japan was comparable to figures reported in UK[14], USA[15], and Korea[16], whereas Aborigines have exceedingly high rates of albuminuria (36% in men and 39% in women)[17].

Several studies have shown that the presence of albuminuria is a risk factor for atherosclerotic diseases[18–20]. Importantly, microalbuminuria may be associated with atherosclerotic diseases even in the general population[21] and in non-diabetic subjects[22]. In the current study, we also found that the highest AEI quartile was significantly positively associated with carotid plaque in both genders (**Fig. 4B**) after adjusting for age and TC, and, when both genders were analyzed together, this association remained statistically significant with an odds ratio of 1.33, even after further adjustment for systolic BP and fasting glucose. These observations indicate that an association between microalbuminuria and the early stages of atherosclerosis might already be present in relatively low-risk Japanese subjects, such as health-screening participants, and this association might be independent of other atherogenic risk factors in such a population. These observations might provide a clinical basis for deciding on the usefulness and/or necessity of measuring urinary albumin excretion as part of general health screening or Ningen Dock programs, although cost-effectiveness is another issue that needs to be discussed. It should also be stressed that the clinical importance of measuring albuminuria has recently received international attention[23]. Researchers are now focusing on trying to establish the true normal range of urinary albumin excretion[4,24], and whether or not microalbuminuria itself might be a meaningful therapeutic target[25].

In conclusion, in general health-screening participants, albuminuria was found to be value-dependently associated with insulin resistance and MetS. The highest AEI quartile (AEI ≥15 mg/g) was associated with carotid plaque in both genders. Based on these findings, it is obviously of value to keep on evaluating and discussing the importance of measuring albuminuria in the setting of the general population for the purpose of estimating the risk for insulin resistance

and atherosclerosis, with the ultimate goal of establishing guidelines for a healthier lifestyle.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Association between Cigarette Smoking, White Blood Cell Count, and Metabolic Syndrome as Defined by the Japanese Criteria

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Abstract

Objective Cigarette smoking increases the circulating white blood cell (WBC) count and the prevalence of metabolic syndrome. We investigated the association between cigarette smoking, WBC count, and metabolic syndrome as defined by the Japanese criteria.

Method Cross-sectional data from 3,687 men undergoing general health screening between 2005 and 2006 were analyzed.

Results After adjustment for age and total cholesterol, former and current smoking were associated with the highest WBC quartile ($\geq 6.3 \times 10^3$ cells/μL) with an odds ratio of 1.35 (95% CI 1.09-1.66, P=0.0055) and 4.45 (95% CI 3.69-5.37, P<0.0001), respectively. It was found that increased WBC count was a risk factor for metabolic syndrome; on the other hand, the current smoking was not found to be a predictor for metabolic syndrome, when each WBC count quartile was separately analyzed.

Conclusions Our data suggest that the risk for MetS, defined by Japanese criteria, might be estimated by the WBC count in Japanese men irrespective of their smoking status, although it should also be noted that the cigarette smoking increases the number of circulating WBC count.

Key words: metabolic syndrome, cigarette smoking, white blood cell count, risk stratification

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Introduction

Several previous studies have shown that cigarette smoking may decrease insulin sensitivity (1, 2) and increases the prevalence of metabolic syndrome (MetS) (3-6). By analyzing the data from individuals undergoing general health screening, we reported that smoking increases the prevalence of MetS in a manner that was dependent on the daily number of cigarettes smoked and the duration of smoking; in addition, this increased prevalence is reversed after quitting, although only partially (7). In that study, MetS was defined by the criteria of the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP-III) (3), but body mass index (BMI) was used as a surrogate for the waist circumference (WC) criterion. This was because data on this parameter were not available in that study sample. In the current study, we have re-evaluated the association between cigarette smoking and MetS as defined by the Japanese criteria after subdividing individuals according to their WBC count.

Methods

Study subjects

The study was approved by The Ethical Committee of Mitsui Memorial Hospital and University of Tokyo Graduate School of Medicine. In Japan, regular health check-ups for employees are legally mandated. Therefore, the majority of these subjects did not have serious health problems. Cigarette smoking status data were collected in a self-reported questionnaire. The available data were limited to the classification of smoking to three categories; never, former, or cur-

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Table 1. Clinical Characteristics of the Study Subjects

	Never smoker (n=1399)	Former smoker (n=1130)	Current smoker (n=1158)	P value
Age, years	53.1±11.7	56.4±10.1	51.1±9.7	<0.01
Body mass index, kg/m^2	23.5±2.9	24.0±2.7	23.7±2.9	<0.01
Waist circumferrence, cm	84.8±7.9	86.5±7.3	85.7±7.7	<0.01
Systolic blood pressure, mmHg	126±19	128±18	123±18	<0.01
Diastolic blood pressure, mmHg	80±11	81±11	78+±11	<0.01
Laboratory data				
WBC count, x10^3/μL	5.1±1.1	5.3±1.3	6.3±1.6	<0.01
Hemoglobin, g/dL	15.4±1.0	15.4±1.0	15.7±1.1	<0.01
Platelet count, x10^4/μL	22.2±4.8	22.3±4.7	23.7±5.2	<0.01
Total cholesterol, mg/dL	208±32	211±31	207±33	<0.01
HDL-cholesterol, mg/dL	57±13	57±13	53±13	<0.01
Triglycerides, mg/dL	116±67	124±74	155±106	<0.01
Uric acid, mg/dL	6.1±1.2	6.2±1.2	6.2±1.2	0.07
CRP, mg/dL	0.11±0.31	0.13±0.32	0.15±0.33	<0.01
Fasting glucose, mg/dL	97±16	101±20	100±24	<0.01
Haemoglobin A1C, %	5.3±0.6	5.4±0.7	5.5±0.9	<0.01
Fasting insulin, μU/mL	6.6±4.0	7.1±4.1	7.0±4.5	<0.05
HOMA-IR	1.63±1.14	1.80±1.26	1.77±1.30	<0.01

One way ANOVA analysis was performed for continuous variables, and χ^2 test for categorical variables.

rent. The majority of current and former smokers were able to provide information on the number of cigarettes they smoked (four categories; <10, 10-19, 20-39, or ≥40 cigarettes/day) and the duration of smoking (three categories; < 10, 10-19, or ≥20 years). Former smokers, but again not all of them, provided information on how long since they had stopped smoking at the time of the general health check (three categories; <1, 1-4, ≥5, years).

Between October 2005 and July 2006, 6,649 subjects (women 2,464, men 4,185) aged between 22 and 89 underwent general health screening including the measurement of blood pressure and metabolic markers necessary to assess the presence or absence of MetS. Of the 4,185 male subjects, the percentages of never, former, and current smokers were 1,399 (33%), 1,509 (36%), and 1,277 (31%), respectively. Among these 4,185 male subjects, 3,687 (88%) answered the questionnaire in full concerning the amount and the duration of smoking, and, if they were former smokers concerning how long since they had stopped smoking at the time of the general health check, and these subjects were enrolled in the current study. We were not able to identify any specific reasons as to the reason the remaining 498 subjects failed to complete the questionnaire about their smoking status.

The upper limits of the fist, second, and third quartiles of WBC count were set at 4.6 × 10^3 cells/μL, 5.3 × 10^3 cells/μL, and 6.2 × 10^3 cells/μL, respectively, which were slightly different from the cutoff values that had been used previously (8), which was because different target populations were studied between these studies.

Diagnostic criteria for metabolic syndrome

We used the Japanese criteria for the diagnose of MetS (9), in which MetS was diagnosed when WC ≥85 cm plus two or more of the following were present: HDL cholesterol (HDL-C) <40 mg/dL or triglycerides (TG) ≥150 mg/dL; systolic blood pressure (SBP) ≥130 mmHg, diastolic blood pressure (DBP) ≥85 mmHg, or on therapy; fasting plasma glucose (FPG) ≥110 mg/dL or on therapy. Of the 3,687 study subjects, 113 subjects (3%) were taking anti-diabetic medicine, and were considered to fulfill the FPG criterion. Of these 113 subjects, 102 were found to have FPG levels of ≥110 mg/dL.

Laboratory tests

Blood samples were taken from our subjects after an overnight fasting. Total cholesterol (TC), HDL-C, and TG were determined enzymatically, and hemoglobin A_{1C} was determined using the latex agglutination immunoassay. Homeostasis model assessment insulin resistance (HOMA-IR) was calculated according to the following formula: HOMA-IR=[fasting immunoreactive insulin (IRI; μU/mL) × FPG (mg/dL)]/405. HOMA has been previously validated and used in cross-sectional population studies (10), such as ours.

Statistical analysis

The data in this study were analyzed by one-way ANOVA with Bonferroni post-hoc test, χ^2 test, Pearson correlation, and univariate and multivariate logistic regression analysis using 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 ± SD unless stated otherwise.

Results

The age of the subjects enrolled ranged from 22 to 89 years with the mean of 53.5 ± 10.8 years. The current and former smokers were significantly younger and older, respectively, than the never smokers (P<0.0001, by ANOVA with a Bonferroni post-hoc analysis, Table 1). C-reactive protein (CRP) was significantly greater in the current smokers (P=0.0017), but not in the former smokers (P=0.072), than in the never smokers. In 3,687 enrolled subjects, 154 individuals had a FPG level of ≥140 mg/dL, 113 were taking antidiabetic medications, and 70 had both of these con-

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Table 2. Logistic Regression Analysis with Smoking Status as An Independent Variable and the MetS as Dependent Variable

Smoking status	yes/no	Odds ratio (95% CI) Unadjusted	P	Odds ratio (95% CI) Adjusted for age and TC	P
Amount of smoking					
Never smoking	208/1191	1.00	-	1.00	-
Former smoking§					
<10 (cigarettes/d)	20/131	0.87 (0.53-1.43)	0.59	0.87 (0.53-1.42)	0.57
10-19	74/387	1.10 (0.82-1.46)	0.54	1.07 (0.80-1.44)	0.63
20-39	95/300	1.81 (1.38-2.38)	<0.0001	1.77 (1.34-2.33)	<0.0001
40≤	31/92	1.93 (1.25-2.98)	0.0029	1.87 (1.21-2.90)	0.0052
Current smoking§					
<10	34/144	1.35 (0.91-2.02)	0.14	1.36 (0.91-2.04)	0.14
10-19	83/435	1.09 (0.83-1.44)	0.53	1.17 (0.89-1.56)	0.26
20-39	104/309	1.93 (1.48-2.52)	<0.0001	2.04 (1.57-2.67)	<0.0001
40≤	16/33	2.78 (1.50-5.14)	0.0011	2.81 (1.51-5.24)	0.0011
Duration of smoking					
Never smoking	208/1191	1.00	-	1.00	-
Former smoking§					
<10y	35/252	0.80 (0.54-1.17)	0.24	0.78 (0.53-1.15)	0.21
10-19	78/277	1.61 (1.21-2.16)	0.0013	1.60 (1.19-2.14)	0.0017
20≤	107/381	1.61 (1.24-2.09)	0.0003	1.57 (1.20-2.05)	0.0110
Current smoking§					
<10y	11/47	1.34 (0.68-2.63)	0.39	1.52 (0.77-3.01)	0.23
10-19	30/167	1.03 (0.67-1.56)	0.89	1.29 (0.84-1.99)	0.25
20≤	196/707	1.59 (1.28-1.97)	<0.0001	1.60 (1.29-2.00)	<0.0001
Years of cessation					
Never smoking	208/1191	1.00	-	1.00	-
Former smoking§					
Last smoked <1y ago	17/81	1.20 (0.70-2.07)	0.51	1.21 (0.70-2.09)	0.49
Last smoked 1-4y ago	42/150	1.60 (1.11-2.33)	0.013	1.58 (1.09-2.30)	0.016
Last smoked ≥5y ago	161/679	1.36 (1.83-1.70)	0.020	1.31 (1.04-1.65)	0.021

§Never smoking was used as reference.

ditions. Therefore, 3,490 (95%) of 3,687 enrolled subjects were free from anti-diabetic medication and had a FPG level of <140 mg/dL. In these 3,490 subjects, Pearson's correlation coefficient for the relationship between age and HOMA-IR was 0.004 (P=0.14).

The overall prevalence of MetS was 665 (18%), and the prevalence of MetS was 15%, 19%, and 20% in the never, former, and current smokers. Logistic regression analysis after adjusting for age and TC showed that former and current smoking were associated with MetS with an odds ratio of 1.31 (95% CI 1.07-1.62, P=0.011) and 1.53 (95% CI 1.25-1.89, P<0.0001), respectively.

In the current smokers, the risk for MetS was found to increase along with the daily number of cigarettes smoked (Table 2). A statistically significant positive association between smoking and MetS was observed when the duration of smoking was ≥10 years in the case of former smokers, and ≥20 years in the case of current smokers.

Next, we investigated the association between various types of smoking status and WBC count. The WBC count in never, former, and current smokers was 5.1 ± 1.1 cells/μL, 5.3 ± 1.4 cells/μL, and 6.3 ± 1.6 cells/μL (P<0.0001), respectively. Pearson's correlation coefficient for the relationship between age and WBC count was -0.04. Logistic regression analysis showed that the odds ratio of former and current smoking for the highest (i.e., fourth) WBC quartile was 1.34 (95% CI 1.09-1.66, P=0.0056) and 4.45 (95% CI 3.69-5.37, P<0.0001), respectively, after adjusting for age and TC. The odds ratio for the highest WBC quartile showed a graded increase according to the amount and duration of smoking in the current smokers, and according to the duration of smoking in former smokers (Table 3). The association between former smoking and the highest WBC quartile was significant when the duration of cessation was <1 year, whereas it was not statistically significant when more than 1 year had passed since smoking had stopped.

The prevalence of MetS in the first, second, third, and fourth WBC quartile was 93/1,015 (9%), 155/877 (18%), 172/855 (20%), and 245/940 (26%), respectively. Age and TC-adjusted logistic regression analysis showed that the odds ratio of the highest WBC quartile for MetS was 1.99 (95% CI 1.66-2.38, P<0.0001). When plotted the prevalence of MetS according to the WBC count quartile and smoking status (Fig. 1), the prevalence of MetS showed graded increase according to the WBC count regardless of the smoking status. It was rather unexpected that the prevalence of metabolic syndrome did not differ much according to smoking status.

After subdivision according to the WBC quartile, neither former nor current smoking was an independent predictor for MetS in individuals who were in the second, third, or fourth quartile of the WBC count (Table 4). In individuals in the lowest WBC quartile, the association between former smoking, but not current smoking, and MetS was statistically significant (Table 4).

Discussion

In this study, we showed that cigarette smoking was associated with both MetS and WBC count in a manner dependent on the daily number of cigarettes smoked and on the duration of smoking. However, the association between current smoking and MetS was no more statistically significant after subdividing individuals according to WBC quartiles. These data suggested that the risk for MetS defined by the Japanese criteria might be estimated by stratifying the circulating WBC levels in Japanese male smokers as well as nonsmokers. In our previous study, we already found similar

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Table 3. Logistic Regression Analysis with Smoking Status as an Independent Variable and the Highest WBC Count Quartile as Dependent Variable

Smoking status	WBC quartiles (Q1/Q2/Q3/Q4)	Odds ratio (95% CI) Unadjusted	P	Odds ratio (95% CI) Adjusted for age and TC	P
Amount of smoking					
Never smoking	507/375/306/211	1.00	-	1.00	-
Former smoking§					
<10 (cigarettes/d)	55/42/33/21	0.91 (0.56-1.48)	0.70	0.91 (0.56-1.48)	0.70
10-19	148/107/113/93	1.42 (1.09-1.87)	0.011	1.42 (1.08-1.89)	0.011
20-39	129/99/83/84	1.52 (1.14-2.02)	0.0036	1.52 (1.14-2.02)	0.0040
40≤	37/38/27/21	1.16 (0.71-1.90)	0.56	1.16 (0.70-1.90)	0.57
Current smoking§					
<10	38/47/44/49	2.14 (1.05-3.07)	<0.0001	2.15 (1.05-3.09)	<0.0001
10-19	69/101/134/214	4.00 (3.16-4.98)	<0.0001	4.01 (3.19-5.04)	<0.0001
20-39	30/60/107/216	6.17 (4.85-7.87)	<0.0001	6.21 (4.87-7.92)	<0.0001
40≤	2/8/8/31	9.70 (5.33-17.65)	<0.0001	9.79 (5.38-17.83)	<0.0001
Duration of smoking					
Never smoking	507/375/306/211	1.00	-	1.00	-
Former smoking§					
<10y	108/67/63/49	1.16 (0.83-1.63)	0.40	1.16 (0.82-1.63)	0.40
10-19	122/83/79/71	1.41 (1.04-1.90)	0.025	1.41 (1.05-1.90)	0.025
20≤	139/136/114/99	1.43 (1.10-1.87)	0.0078	1.44 (1.09-1.89)	0.0094
Current smoking§					
<10y	15/12/14/17	2.34 (1.30-4.19)	0.0044	2.32 (1.29-4.17)	0.0052
10-19	35/41/52/69	3.04 (2.19-4.21)	<0.0001	3.00 (2.13-4.22)	<0.0001
20≤	89/163/227/424	4.98 (4.10-6.07)	<0.0001	4.99 (4.10-6.07)	<0.0001
Years of cessation					
Never smoking	507/375/306/211	1.00	-	1.00	-
Former smoking§					
Last smoked <1y ago	19/25/19/35	3.13 (2.02-4.85)	<0.0001	3.15 (2.03-4.89)	<0.0001
Last smoked 1-4y ago	67/41/50/34	1.21 (0.81-1.81)	0.35	1.21 (0.81-1.80)	0.35
Last smoked ≥5y ago	283/220/187/150	1.22 (0.97-1.54)	0.084	1.21 (0.96-1.53)	0.11

§Never smoking was used as reference.

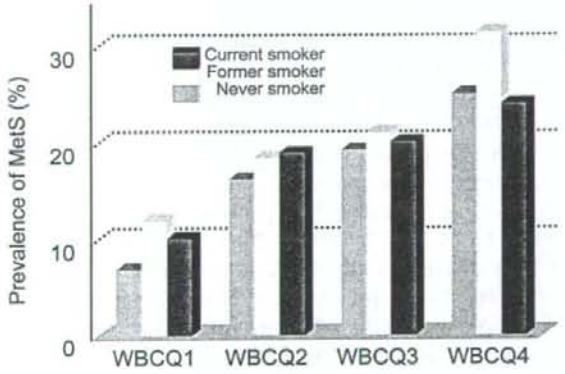

Figure 1. Bar graph illustrating the prevalence of metabolic syndrome (MetS).

observations (8); in that study, however, NCEP ATPIII criteria, with BMI criteria as a surrogate for WC criteria, were used for the diagnosis of MetS, because WC data were not available. On the other hand, the criteria of MetS in Japan have been advocated on 2005, in which WC of ≥85 cm is a mandatory requirement for the diagnosis. Hence, here we re-evaluated the association between cigarette smoking, WBC count, and MetS as defined by the Japanese criteria, in which WC data is required for the diagnosis of MetS.

We showed here that increased WBC count was a risk factor for MetS defined by the Japanese criteria. The association between inflammatory markers, such as CRP and WBC count, and MetS has been demonstrated in several previous studies (11-15). It is postulated that the link between subclinical inflammation and MetS might be mediated by certain inflammatory cytokines, such as tumor necrosis factor-α (16-18).

We also showed that cigarette smoking increases the circulating WBC count, which was in agreement with several previous studies (19, 20). In the previous study, we have investigated whether the prevalence of MetS, that had been defined by modified NCEP ATPIII criteria, was increased in smokers even after subdividing them according to the WBC count. We found that the association between current smoking and metabolic syndrome was statistically significant in the first or second quartiles of the WBC count quartiles, but not in the higher quartiles (8). In the current study, we set out to re-evaluate this observation by using the Japanese criteria, instead of modified NCEP-criteria, for the diagnosis of MetS (9).

It is of note that, even when the Japanese criteria were used, the prevalence of MetS was not much different in never, former, and current smokers when they belong to the same WBC quartile (Fig. 1). This was also demonstrated by a multivariate logistic regression analysis that current smoking was not a predictor for MetS in any WBC quartile after adjusting for age and TC (Table 4). These data are compatible to the idea that the development of subclinical inflammation may play a crucial role in the development of MetS in smokers as well as non-smokers, or in reverse, the presence of metabolic syndrome may increase inflammatory stress (21). Whichever the case is, it seems to be important that we may be able to estimate the risk for MetS in smokers as well as non-smokers by simply stratifying the circulating WBC count. Investigation whether the decrease in WBC count and the cancellation of MetS would be the parallel events in individuals who are quitting smoking by future longitudinal studies will provide further important information.

There are several limitations in the current study. First, 187 of the 3,687 enrolled subjects self-reported to be taking anti-hyperlipidemic drug(s). As it could not be specified

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Table 4. Odds Ratios of Smoking Status for MetS, Stratified by WBC Counts

	yes/no	Odds ratio (95% CI) Unadjusted	P value	Odds ratio (95% CI) Adjusted for age and TC	P value
Indivisuals in the WBC-Q1					
Never smoking	35/472	1.00	-	1.00	-
Former smoking	44/325	1.83 (1.15-2.91)	0.011	1.75 (1.09-2.80)	0.020
Current smoking	14/125	1.51 (0.79-2.90)	0.21	1.65 (0.85-3.18)	0.14
Indivisuals in the WBC-Q2					
Never smoking	61/314	1.00	-	1.00	-
Former smoking	53/233	1.17 (0.78-1.76)	0.45	1.08 (0.72-1.63)	0.71
Current smoking	41/175	1.21 (0.78-1.87)	0.40	1.24 (0.80-1.93)	0.33
Indivisuals in the WBC-Q3					
Never smoking	59/247	1.00	-	1.00	-
Former smoking	54/202	1.12 (0.74-1.69)	0.59	1.12 (0.74-1.71)	0.59
Current smoking	59/234	1.06 (0.71-1.58)	0.79	1.10 (0.73-1.65)	0.66
Indivisuals in the WBC-Q4					
Never smoking	53/158	1.00	-	1.00	-
Former smoking	69/150	1.37 (0.90-2.09)	0.14	1.27 (0.83-1.95)	0.27
Current smoking	123/387	0.95 (0.65-1.37)	0.78	0.97 (0.66-1.41)	0.87

whether such subjects were taking TC lowering drug(s) or TG lowering drug(s) could not be specified in the current study, however, these subjects were not judged to fulfill the TG criteria of metabolic syndrome just because they were taking anti-hyperlipidemic drugs. Thus, it is possible that some of the individuals who were taking TG lowering drug(s) should have been categorized as not fulfilling the TG criteria. Second, we analyzed data from only male subjects. Although during the study period, 2,419 female subjects answered the questionnaire in full concerning smoking status, the prevalence of smokers was much smaller [309/2,419 (13%)] in women than in men (62%). For example, there were only two female current smokers and two former smokers who had smoked more than 40 cigarettes per day, therefore statistical analysis may not be applicable. Third, due to the cross-sectional nature of the current study, we could not conclude whether stratification of WBC count would also be useful in predicting the *future* development of atherosclerotic diseases among smokers.

In conclusion, we found that cigarette smoking was associated with MetS defined by the Japanese criteria, which was dependent on the daily amount of cigarettes smoked and the duration of smoking, and that elevated circulating WBC count was associated with MetS even within the normal range. These data collectively suggested that the risk for MetS might be estimated by stratifying the circulating WBC levels in Japanese irrespective of the smoking status, although it should also be noted that the cigarette smoking increases the number of circulating WBC count in a dose- and duration-dependent manner.

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