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Letter to the Editor

Comparison of several metabolic syndrome definitions with relation to early carotid atherosclerosis in Japanese men

Keywords: Metabolic syndrome; Diagnostic criteria; Carotid atherosclerosis; Japanese; General health screening

To the Editor,

We have read with great interest the recent article by Skilton et al. [1], in which they compared several different definitions of metabolic syndrome in terms of prevalence and strength in predicting increased carotid intima-media thickness, a marker of early atherosclerotic lesions. They found, among the definitions tested, that the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) definition may be more strongly associated with carotid atherosclerosis in men than the other definitions.

We sought to investigate the prevalence of metabolic syndrome as defined by several different diagnostic criteria and their association with carotid atherosclerosis in Japanese individuals. Between October 2005 and August 2006, 1106 males who underwent general health screening that included measurement of waist circumference (WC) and carotid ultrasonography, and were enrolled in the current study. Metabolic syndrome was diagnosed by five different definitions:

- NCEP-ATPIII definition [2]: Presence of three or more of the following: (1) fasting plasma glucose (FPG) $\geq$110 mg/dL; (2) systolic blood pressure (SBP)/diastolic blood pressure (DBP) $\geq$130/85 mmHg; (3) triglycerides (TG) $\geq$150 mg/dL; (4) HDL cholesterol (HDL-C) <40 mg/dL; (5) WC $\geq$102 cm.
- Modified-NCEP-ATPIII definition: The same as NCEP-ATPIII except that body mass index $\geq$25 kg/m^2 is a surrogate for WC.
- Japan definition [3]: WC $\geq$85 cm plus 2 or more of the following: (1) FPG $\geq$110 mg/dL; (2) SBP/DBP $\geq$130/85 mmHg; (3) HDL-C < 40 mg/dL or TG $\geq$150 mg/dL.
- IDF definition [4]: WC $\geq$85 cm plus 2 or more of the following: (1) FPG $\geq$100 mg/dL; (2) SBP/DBP $\geq$130/85 mmHg; (3) TG $\geq$150 mg/dL; (4) HDL-C <40 mg/dL.
- AHA/NHLBI definition [5]: Presence of three or more of the following: (1) FPG $\geq$100 mg/dL; (2) SBP/DBP $\geq$130/85 mmHg; (3) TG $\geq$150 mg/dL; (4) HDL-C <40 mg/dL; (5) WC $\geq$102 cm. In each of these definitions, individuals who were taking anti-hypertensive and anti-diabetic medications were considered to fulfill the blood pressure and glucose criteria. Carotid plaque was defined as a portion of the artery with an intima-media complex thickness of $\geq$1.1 mm [6] with a focal protrusion or point(s) of inflexion.

The prevalence of metabolic syndrome defined by each criterion was as follows: NCEP-ATPIII 108/1106 (9.8%), modified NCEP-ATPIII 211/1106 (19.1%), Japan 223/1106 (21.1%), IDF 321/1106 (29.0%) and AHA/NHLBI 161/1106 (14.6%). The odds ratio of each criterion-defined metabolic syndrome for carotid plaque in logistic regression analysis adjusted for age and smoking status was as follows: NCEP-ATPIII, 1.89 (95% CI 1.17–3.05, $P=0.0088$); modified NCEP-ATPIII, 1.76 (95% CI 1.23–2.51, $P=0.0018$); Japan, 1.74 (95% CI 1.23–2.45, $P=0.0015$); IDF, 1.35 (95% CI 1.00–1.83, $P=0.048$); AHA/NHLBI, 1.66 (95% CI 1.21–2.45, $P=0.011$). Therefore, except for the AHA/NHLBI definition, the higher the prevalence of diagnosis of metabolic syndrome according to a definition, the lower the odds ratio for carotid plaque. The prevalence of metabolic syndrome defined by AHA/NHLBI was lower than that of modified NCEP-ATPIII-defined metabolic syndrome, however, the former was found to be less strongly associated with carotid atherosclerosis than the latter one.

Together with the recent article by Skilton et al. [1], our data suggest that the optimal definition for metabolic syndrome, in terms of the combination of components and the cutoff values, may differ among various racial groups and ethnicities from the viewpoint of predicting early atherosclerosis. It might also differ according to gender and the target population, such as hypertensive and non-hypertensive sub-

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jects. These points should be investigated further in future studies.

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Relationship between smoking, white blood cell count and metabolic syndrome in Japanese women

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Abstract

We found that cigarette smoking increased white blood cell count, and individuals which increased white blood cell count more likely to have metabolic syndrome in Japanese men. We investigated whether similar relationship can be observed also in women. We analyzed the data from 16,383 Japanese women who underwent general health screening. Age-adjusted logistic regression analysis showed that current smoking was positively associated with a highest white blood cell count quartile with an odds ratio of 2.40 (95% CI: 2.16–2.68, $P < 0.0001$). The white blood cell count showed a graded association with metabolic syndrome. On the other hand, the association between current smoking and metabolic syndrome was no longer significant after subdividing the individuals into groups according to the white blood cell quartile. These data collectively suggested that the association between current smoking and metabolic syndrome is heavily confounded by certain factors that increase the circulating white blood cell count in Japanese women, as in men.

Keywords: Metabolic syndrome; Smoking; White blood cell count

1. Introduction

Many previous studies have demonstrated a positive association between several inflammatory markers, including white blood cell (WBC) count and C-reactive protein, and the prevalence of metabolic syndrome [1–3] or reduced sensitivity to insulin [4]. Cigarette smoking is known to increase the circulating WBC count [5,6] even in individuals who have quit for a substantial period [7]. By analyzing data from individuals who had undergone a general health screening test, we previously demonstrated that cigarette smoking is associated with an increase in the circulating WBC count [8], and with a higher prevalence of metabolic syndrome [9] in men. We also showed that the association between smoking and metabolic syndrome is not significant in those with a high WBC count [10], suggesting that the association between smoking and metabolic syndrome may be confounded by certain factors that increase the circulating WBC count in men. In the current study, we have analyzed the mode of association between smoking status, WBC count, and metabolic syndrome in Japanese women.

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2. Methods

2.1. Study subjects

The study was approved by The Ethical Committee of Mitsui Memorial Hospital. In Japan, regular health check-ups for employees are legally mandated. Therefore, the majority of these subjects did not have serious health problems. Between September 1994 and December 2003, 49,358 subjects, who were older than 20 years old (women 16,877, men 32,481), underwent general health screening including the measurement of hemodynamic and metabolic markers necessary to assess the presence or absence of metabolic syndrome. Subject age and cigarette smoking outcome data were collected in a structured interview. Among 16,877 female subjects, 16,383 answered the questionnaire in full concerning the amount and the duration of smoking, and concerning how long since they had stopped smoking at the time of the general health check when they were former smokers. We were unable to identify any specific reasons why remaining 494 subjects failed to complete the questionnaire about their smoking status. These 16,383 female subjects (14,136 never smokers, 625 former smokers, and 1622 current smokers) were enrolled in the current study. The interquartile cutoff points for the WBC count were 4.2×10^3 cells/μL, 4.9×10^3 cells/μL, and 5.8×10^3 cells/μL.

2.2. Criteria for diagnosing metabolic syndrome

The diagnosis of metabolic syndrome was made by the criteria of the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP-III) [11] using body mass index (BMI) as a surrogate for waist circumference, because data on this parameter were not available in this study sample. The five thresholds used were as follows: triglyceride (TG) levels ≥150 mg/dL; HDL-cholesterol (HDL-C) levels <50 mg/dL fasting plasma glucose (FPG) ≥110 mg/dL or taking an antidiabetic medication; systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg or taking an antihypertensive medication; and BMI >25 kg/m^2. Metabolic syndrome was diagnosed when three or more these components were present.

2.3. Laboratory tests

Blood samples were taken from our subjects after an overnight fasting. Total cholesterol (TC), HDL-C, and TG were determined enzymatically, and haemoglobin A1C was determined using the latex agglutination immunoassay.

2.4. Statistical analysis

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

3. Results

3.1. Baseline characteristics of the enrolled individuals

The age of the subjects enrolled ranged from 20 to 89 years with a median of 52 years. The mean age of the current and former smokers was significantly lower than that of never smokers (Table 1). The WBC count was greater in both current ($P < 0.0001$) and former

Table 1
Baseline characteristics

Variables	All subjects (n = 16,383)	Never smoker (n = 14,136)	Former smoker (n = 625)	Current smoker (n = 1622)	P value
Age (years)	51.5 ± 10.1	52.2 ± 9.9	48.5 ± 10.8	45.8 ± 9.9	<0.0001
Body mass index (kg/m^2)	21.4 ± 2.9	21.5 ± 2.8	21.5 ± 2.9	21.2 ± 3.0	0.0006
Systolic blood pressure (mmHg)	117 ± 19	118 ± 19	116 ± 18	111 ± 17	<0.0001
Blood cell count					
WBC count (×10^3 μL^{-1})	5.0 ± 1.3	4.9 ± 1.2	5.1 ± 1.3	5.7 ± 1.6	<0.0001
Hemoglobin (g/dL)	13.0 ± 1.1	13.0 ± 1.1	13.0 ± 1.1	13.2 ± 1.1	<0.0001
Platelet count (×10^4 μL^{-1})	23.7 ± 5.3	23.5 ± 5.3	24.1 ± 5.6	24.6 ± 5.4	<0.0001
Biochemical data					
Total cholesterol (mg/dL)	209 ± 35	203 ± 35	203 ± 35	197 ± 38	<0.0001
Triglycerides (mg/dL)	89 ± 57	88 ± 49	89 ± 54	96 ± 104	<0.0001
HDL-cholesterol (mg/dL)	71 ± 17	71 ± 17	74 ± 18	67 ± 17	<0.0001
Fasting glucose (mg/dL)	91 ± 13	91 ± 13	90 ± 12	88 ± 13	<0.0001
Hemoglobin A1C (%)	5.1 ± 0.5	5.1 ± 0.5	5.1 ± 0.5	5.1 ± 0.5	<0.0001
CRP > 0.4 mg/dL, n (%)	505 (3.1)	440 (3.1)	14 (2.2)	51 (3.1)	0.46
Erythrocyte sedimentation rate (cm)	17.3 ± 11.1	17.8 ± 11.2	15.9 ± 10.5	13.8 ± 8.7	<0.0001

WBC: white blood cell; CRP: C reactive protein.

($P = 0.0019$) smokers than in never smokers ($P < 0.0001$). Pearson's correlation coefficients for the relationship between age and each variable were as follows: BMI, 0.186; systolic blood pressure, 0.34; WBC count, −0.077; TC, 0.392; triglycerides, 0.195; HDL-C, −0.040; fasting glucose, 0.195; HbA1C, 0.357. A value of $P < 0.0001$ was obtained for all of these correlations.

3.2. Association between WBC count and metabolic syndrome

Prevalence of metabolic syndrome in individuals of the first, second, third, and fourth quartiles of WBC count were 1.7, 3.0, 5.2, and 8.0%, respectively. After adjusting for age and TC, logistic regression analysis showed that odds ratios of the first, second, third, and fourth quartiles of WBC count for metabolic syndrome were 1.0 (reference), 1.76 (95% CI: 1.31–2.37), 3.23 (2.46–4.24), 5.38 (4.14–7.00), respectively.

3.3. Association between smoking status and WBC count

Next, we analyzed the association between various smoking conditions (amount and duration of smoking) and the prevalence of the highest WBC count quartile by logistic regression analysis adjusted for age and TC. Odds ratio for the highest WBC count quartile increased with the amount of daily cigarette smoked in the case of current smoker (Fig. 1A). Similarly, the odds ratios for the highest WBC count quartile showed a greater increase according to the duration of cigarette smoking (Fig. 1B). In the case of former smokers, those who had smoked ≥20 cigarettes per day, but not those who had smoked less than that showed an increased prevalence of the highest WBC count quartile. Former smokers who had last smoked less than 5 years ago, as well as those who had smoked ≥5 years, did not have significantly greater prevalence of metabolic syndrome than never smokers (Fig. 1C).

3.4. Association between smoking status and metabolic syndrome

Logistic regression analysis adjusting for age and TC showed that the odds ratios for metabolic syndrome increased according to the daily number of cigarettes smoked in both former and current smokers (Fig. 2A). Unlike the relationship between smoking duration and high WBC count, the association between current smoking and metabolic syndrome did not seem to be

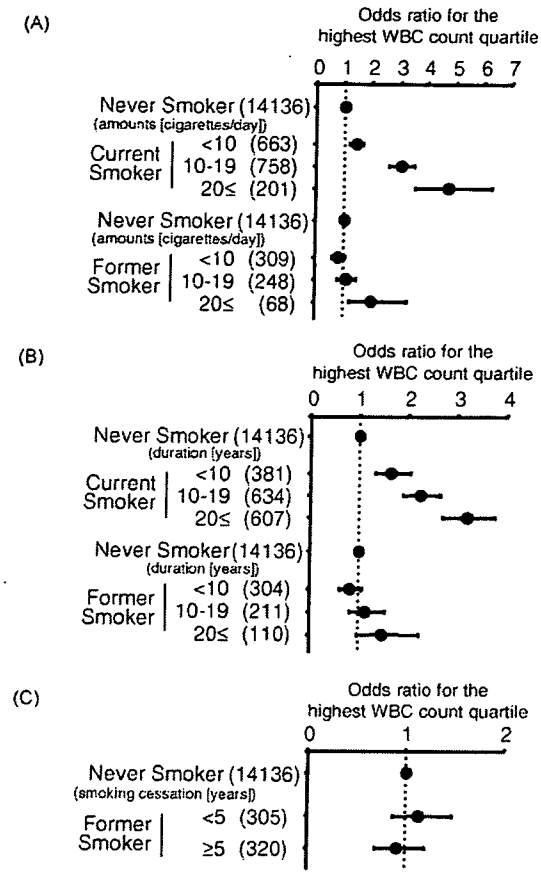

Fig. 1. Multivariate analysis assessing relationship between smoking status and the highest white blood cell count quartile. Data were adjusted for age. (A) Amount of smoking vs. high white blood cell count. (B) Duration of smoking vs. high white blood cell count. (C) Years of smoking cessation vs. high white blood cell count.

increased according to the smoking duration (Fig. 2B). When the former smokers were subdivided into two groups according to the duration of smoking cessation, the prevalence of metabolic syndrome was significantly greater in individuals who had quit smoking <5 years ago, but not in those who had stopped smoking ≥5 years ago (Fig. 2C).

3.5. Association between current smoking and metabolic syndrome after subdivision into WBC quartile groups

When the analysis was done for each WBC quartile separately, logistic regression analysis showed that the current smoking was not statistically significantly associated with metabolic syndrome; the odds ratios

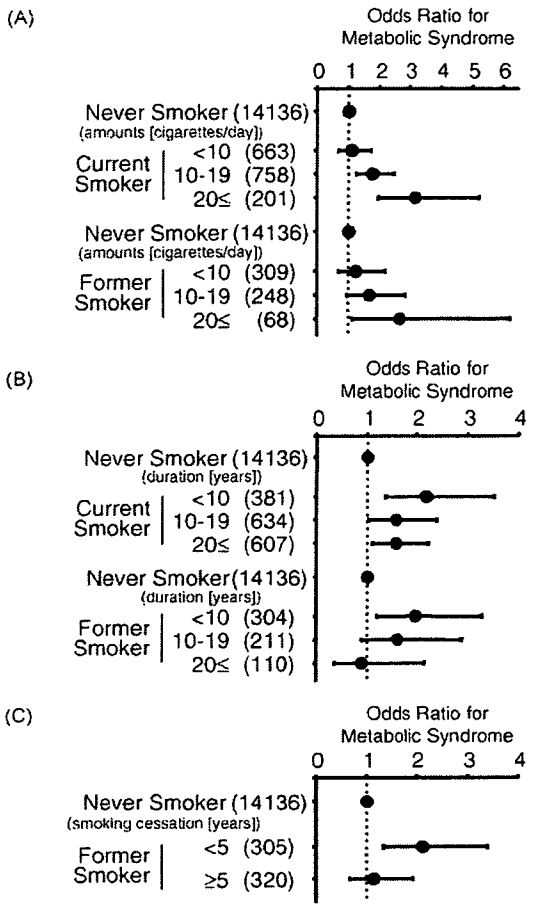

Fig. 2. Multivariate analysis assessing relationship between smoking status and metabolic syndrome. Data were adjusted for age and total cholesterol levels. (A) Amount of smoking vs. metabolic syndrome. (B) Duration of smoking vs. metabolic syndrome. (C) Years of smoking cessation vs. metabolic syndrome. The numbers of subjects with various smoking status are described in the parenthesis.

was 0.87 (95% CI: 0.21–3.61, $P = 0.84$) in the first WBC quartile, 1.85 (95% CI: 0.91–3.77, $P = 0088$) in the second WBC quartile, 0.92 (95% CI: 0.53–1.59, $P = 0.75$) in the third WBC quartile, and 1.30 (95% CI: 0.94–1.79, $P = 0.12$) in the fourth WBC quartile.

4. Discussion

In the current study, we found that current smoking is associated with an increase in the WBC count according to the amount of cigarettes smoked and the duration of smoking, and that the WBC count showed a graded association with the prevalence of metabolic syndrome. In addition, current smoking was not statistically associated with metabolic syndrome after subdividing individuals according to WBC count quartiles. These data collectively indicate that association between current smoking and metabolic syndrome might be heavily confounded certain factors that also increase the circulating WBC count in Japanese women.

In previous studies, we have reported that cigarette smoking shows a graded association with metabolic syndrome according to the amount and duration of smoking in Japanese men [9]. We have also shown that current smoking is associated with an increased WBC count in men [8,10]. In the current study, we aimed to extend these investigations to a female population. In essence, the observations of the current study were similar to those of our previous studies targeted to the male population, although there were several differences. For example, in women, current smoking, but not former smoking, was significantly associated with an increased prevalence of metabolic syndrome. In addition, the prevalence of metabolic syndrome was greater in individuals with longer smoking history in men [9], but this mode of association was not present in women (current study). Some of the different observations between the women and men may be due to the relatively small numbers of women smokers, which may weaken the power of the statistical assessment.

Several previous studies have shown a possible relationship between WBC count and individual components of metabolic syndrome or subsets of these components. For example, Nieto et al. have reported in the ARIC study that the WBC count is associated positively with blood pressure, fasting insulin, and triglycerides and inversely with HDL cholesterol [5]. Similarly, Targher et al. have reported that the WBC count correlates positively with BMI blood pressure and plasma triglycerides, and negatively with HDL cholesterol [12]. Vozarova et al. showed an association between high WBC count and worsening insulin sensitivity [13]. Together with the current study, these studies suggest that increased circulating WBC count, presumably reflecting the chronic inflammation, may exacerbate insulin resistance, leading to metabolic syndrome. Potential candidates for factors that increase WBC count and exacerbate insulin resistance may include proinflammatory cytokines [14–17] and adipocytokines [18–21]. Level of such proinflammatory cytokines and/or adipocytokines in smokers with or without metabolic syndrome should be investigated in health screening participants in future studies. We may also have to investigate why either former or current smoking was not associated with increased CRP value in the current study population.

In summary, in Japanese women, there are positive dose-dependent associations between current smoking and WBC count, and between current smoking and metabolic syndrome. The finding that current smoking was no more statistically significant predictor for metabolic syndrome after subdivision of the individuals according to the WBC quartiles collectively suggests that certain factors that increases circulating WBC count may be one of the factors that promotes development of metabolic syndrome in current smokers. Our data also suggest that increased levels of circulating WBC count might help identify the subjects at higher risk for metabolic syndrome in Japanese women who currently smoke.

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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 non-smokers. 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.

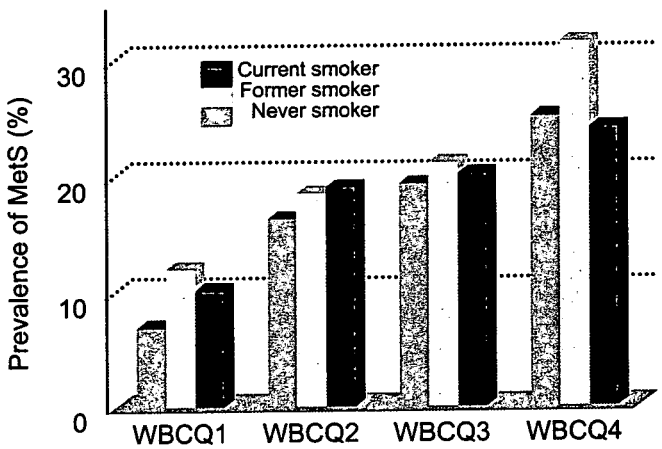

Figure 1. Bar graph illustrating the prevalence of metabolic syndrome (MetS).

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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Original Article

Angiotensin II–Induced Regulation of the Expression and Localization of Iron Metabolism–Related Genes in the Rat Kidney

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Due to recent discoveries of novel genes involved in iron metabolism, our understanding of the molecular mechanisms underlying iron metabolism has dramatically increased. We have previously shown that the administration of angiotensin II alters iron homeostasis in the rat kidney, which may in turn aggravate angiotensin II–induced renal damage. Here we have investigated the effect of angiotensin II administration on the localization and expression of transferrin receptor (TfR), divalent metal transporter 1 (DMT1), ferroportin 1 (FPN), and hepcidin mRNA in the rat kidney. Weak expression of TfR, DMT1, FPN, and hepcidin mRNA was observed in the kidneys of control rats. In contrast, after 7 days of angiotensin II infusion by osmotic minipump, the expression of these mRNAs was more widely distributed. Staining of serial sections revealed that some, but not all, of the renal tubular cells positive for these genes contained iron deposits in the kidney of angiotensin II–infused animals. Real-time polymerase chain reaction (PCR) showed that the mRNA expression of TfR, iron-responsive element–negative DMT1, FPN, and hepcidin mRNA increased ~1.9-fold, ~1.7-fold, ~2.3-fold, and ~4.7-fold, respectively, after angiotensin II infusion as compared with that of untreated controls, and that these increases could be suppressed by the concomitant administration of losartan. Our data demonstrate that these genes were unequivocally expressed in the kidney and could be regulated by angiotensin II infusion. The relative contribution, if any, of these genes to renal and/or whole-body iron homeostasis in various disorders in which the renin angiotensin system is activated should be investigated in future studies. (*Hypertens Res* 2007; 30: 195–202)

Key Words: **angiotensin II, iron metabolism, hypertension, gene regulation**

Introduction

Iron is an essential element that is required for fundamental cell functions in all living organisms. On the other hand, excess body iron is potentially harmful because of its ability to catalyze the conversion of hydrogen peroxide to toxic free radicals. Thus, maintaining an appropriate balance of iron in the body is important. Recently, our understanding of the mechanisms underlying iron metabolism has dramatically increased due to discoveries of novel genes related to iron metabolism. Divalent metal transporter 1 (DMT1), also referred to as natural resistance–associated macrophage protein 2 (Nramp2) or divalent cation transporter (DCT1), is a

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12-segment transmembrane-spanning integral membrane protein (*1*) that is expressed in the duodenum, where it transports divalent metals across the apical membrane of enterocytes. A G185R point mutation of this gene causes microcytic hypochromic anemia in rodents (*2, 3*), thus indicating the fundamental role played by this gene in iron homeostasis. Ferroportin 1 (FPN), also referred to as metal transporter protein (MTP1) or iron-regulated protein 1 (Ireg1), is a multiple transmembrane-spanning protein that transports iron out of cells. FPN is expressed strongly in the basolateral region of absorptive duodenal enterocytes and in tissue macrophages in the liver, *i.e.*, Kupffer cells (*4, 5*). Hepcidin (hepc) is a recently discovered cysteine-rich 25-amino acid peptide that has antimicrobial properties and also acts as a negative regulator of intestinal iron absorption and macrophage iron release, and its overproduction may contribute to the anemia associated with inflammation (*6*). Hepc was initially isolated from human urine (*6*); however, it is mainly produced in the liver, from where it is released into the systemic circulation. Binding of hepc to the iron exporter FPN leads to the internalization and degradation of FPN (*7, 8*); it is presumably by this mechanism that hepc functions as a negative regulator of intestinal iron absorption.

In general, the kidney is not considered to be the major expression site of these newly discovered iron metabolism–related genes. Although expression of the transferrin receptor (TfR) (*9, 10*), DMT1 (*11, 12*), FPN (*13*), and hepc (*14*) has been demonstrated in the kidney, information about the physiological importance and the regulation of these genes has been limited to date. We previously reported that the administration of angiotensin II to rats causes prominent iron deposition in the kidney, which occurs primarily in the proximal tubular epithelial cells; this deposition is thought to be associated with increased proteinuria and the upregulation of fibrosis-related genes (*15, 16*). Here, we have characterized the renal expression patterns of these iron metabolism–related genes and investigated how their expression might be regulated by angiotensin II in the kidney.

Methods

Generation of Animal Models

The experiments were performed in accordance with the guidelines for animal experimentation approved by the Animal Center for Biomedical Research, Faculty of Medicine, University of Tokyo. Angiotensin II was continuously infused into male Sprague-Dawley rats by subcutaneous implantation of an osmotic minipump (Alzet model 2001; Alza Pharmaceutical, Mountain View, USA) as described previously. In brief, Val5-angiotensin II was infused at doses of 0.7 mg/kg/day for 7 days by subcutaneously implanted osmotic minipumps (Alza Pharmaceutical) which exerted hypertensive effects (192 ! 5 mmHg [n=12], p<0.01 *vs.* control rats, 131 ! 3 mmHg [n=6]). In some experiments, the selective angiotensin type 1 (AT_1) receptor antagonist, losartan (25 mg/kg/day) or the nonspecific vasodilator, hydralazine (15 mg/kg/day) (Sigma Chemical, St. Louis, USA) was given in the drinking water, beginning 2 days before pump implantation and throughout angiotensin II infusion (angiotensin II+losartan, 126 ! 5 mmHg [n=7]; angiotensin II+hydralazine 126 ! 3 mmHg [n=7]). In some experiments, norepinephrine was infused at a dose (2.8 mg/kg/day) which exerted hypertensive effects (192 ! 4 mmHg) comparable to those of angiotensin II. Systolic blood pressure was measured in conscious rats by tail-cuff plethysmography (Ueda Seisakusho, Tokyo, Japan).

In Situ Hybridization, Histological Analyses

Rat cDNAs corresponding to rat sequences of TfR, DMT1, FPN1, and hepc were obtained by subcloning the reverse-transcription (RT)–polymerase chain reaction (PCR) product using rat kidney mRNA. Sense and antisense primers were as follows: 5 ¦TACCTGTGCAGACGATCTCAAGAG-3 ! and 5 ¦AGGACGACTTTATCCAGATTAAT-3 ¦ respectively, for TfR; 5 ¦CTACCTGGATCCAGGAAACATT-3 ! and 5 ¦AAGTACTTATTGGCTTCTCGAA-3 ¦ respectively, for DMT1; 5 ¦AGACCCCTGCTCTGGCT GTA-3 ! and 5 ¦AGA CACATTAGCATAAGCAT-3 ¦ respectively, for FPN; and 5 ¦GGCAGGACAGAAGGCAAGAT-3 ! and 5 ¦GGTAGG ACAGGAATAAATAAT-3 ¦ respectively, for hepc. The sequence targeted for the amplification of DMT1 was a common region of DMT1 with or without iron-responsive element (IRE), which was designated here as IRE(+)DMT1 and IRE(!)DMT1, respectively. Rat cDNA corresponding to these iron metabolism–related genes were subcloned into a pGEM-T vector, and then *in situ* hybridization was performed as described previously (*17*). After digestion with a restriction enzyme and linearization of the plasmid, antisense and sense cRNA riboprobes were transcribed *in vitro* using the DIG RNA labeling Kit SP6/T7 (Roche Diagnostics, Basel, Switzerland). Hybridization was performed using *In Situ* Hybridization Reagents (Nippon Gene, Tokyo, Japan). *In situ* hybridization was performed on either formalin-fixed specimens or un-fixed frozen specimens. Prussian blue staining was used for iron staining, and Oil red O staining was used to detect the accumulation of lipid in unfixed frozen tissue sections.

RNA Extraction, Northern Blot Analysis, and Real Time RT-PCR

Total RNA was isolated from homogenized aorta by the acid guanidinium thiocyanate–phenol chloroform method as described previously (*18*). To investigate the mRNA expression by quantitative PCR with gene-specific HybriProbes was performed by LightCycler (Roche Diagnostics). The following respective sense and antisense primers were used: 5 ¦AAGTCCTGCTGAGCGAAGAT-3 ! and 5 ¦TGGTCCCTA

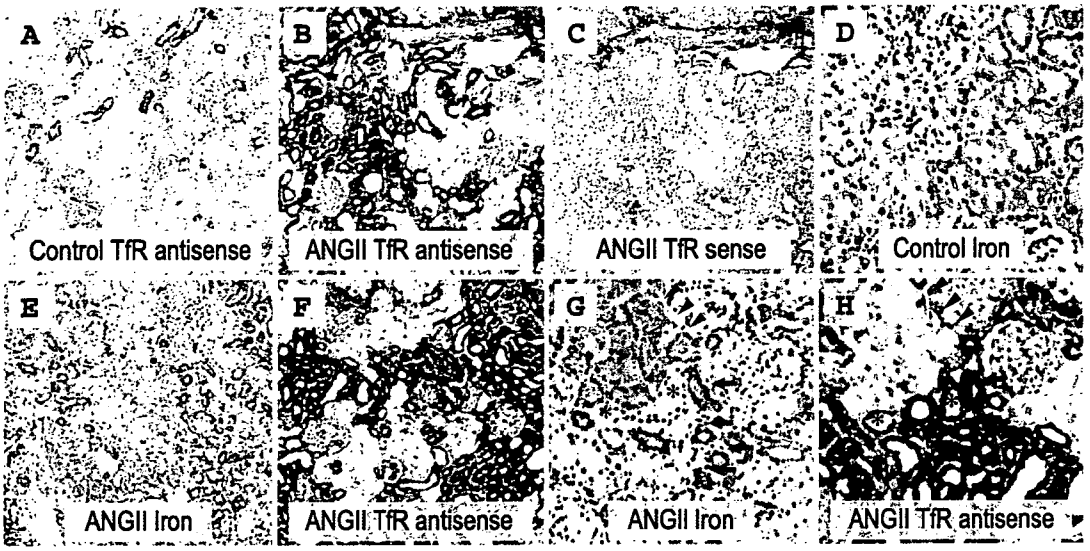

Fig. 1. *Expression and localization of TfR mRNA. Samples were from the kidney of control (A, D) and angiotensin II (ANGII)–infused (B, C, E–H) rats. B-C, E-F, and G-H are serial specimens. A, B, F, H:* In situ *hybridization using the TfR antisense probe. C:* In situ *hybridization using the TfR sense 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)

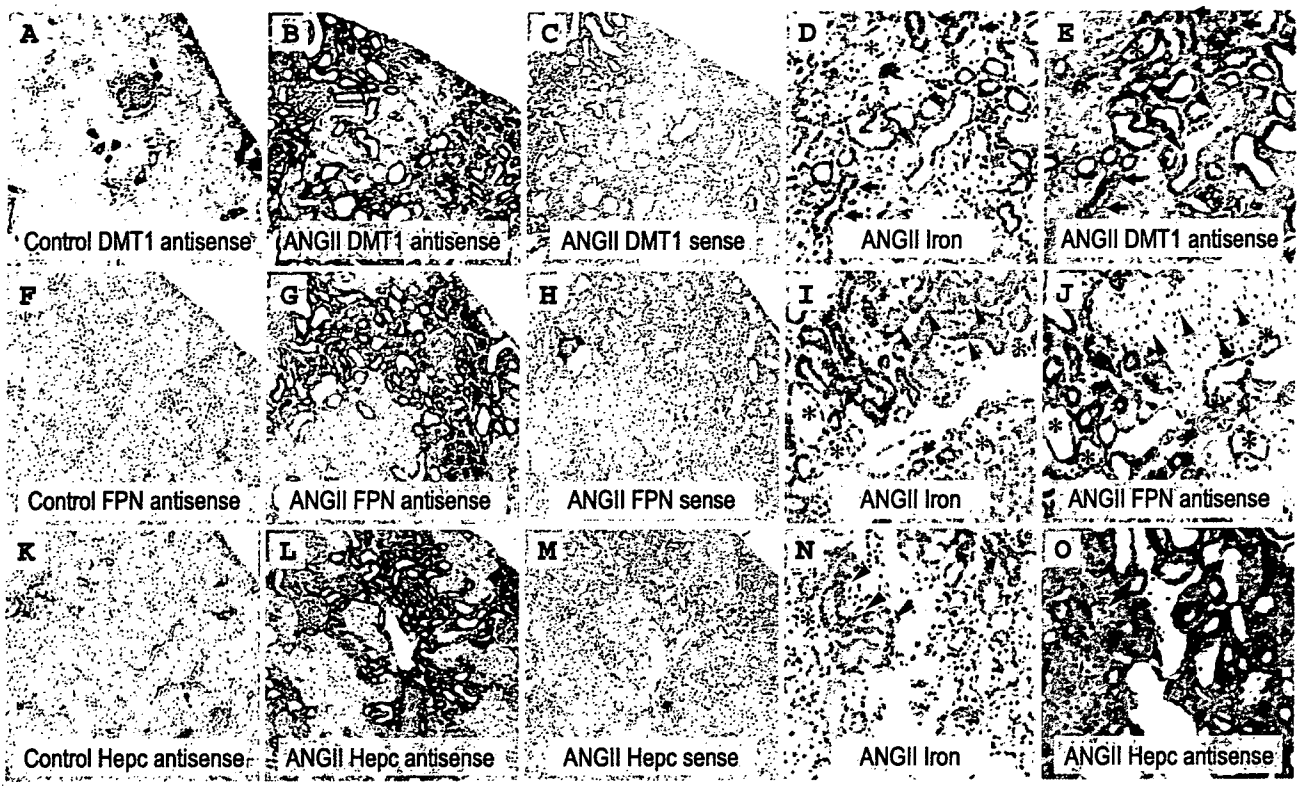

Fig. 2. *Expression and localization of DMT1, FPN, and hepc mRNA and iron deposits. Samples were from the kidney of control (A, F, K) and angiotensin II (ANGII)–infused (B–E, G–J, L–O) rats. B-C, D-E, G-H, I-J, L-M, and N-O are serial specimens. A, B, E:* In situ *hybridization using the DMT1 antisense 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-!1 (TGF-!1) 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-

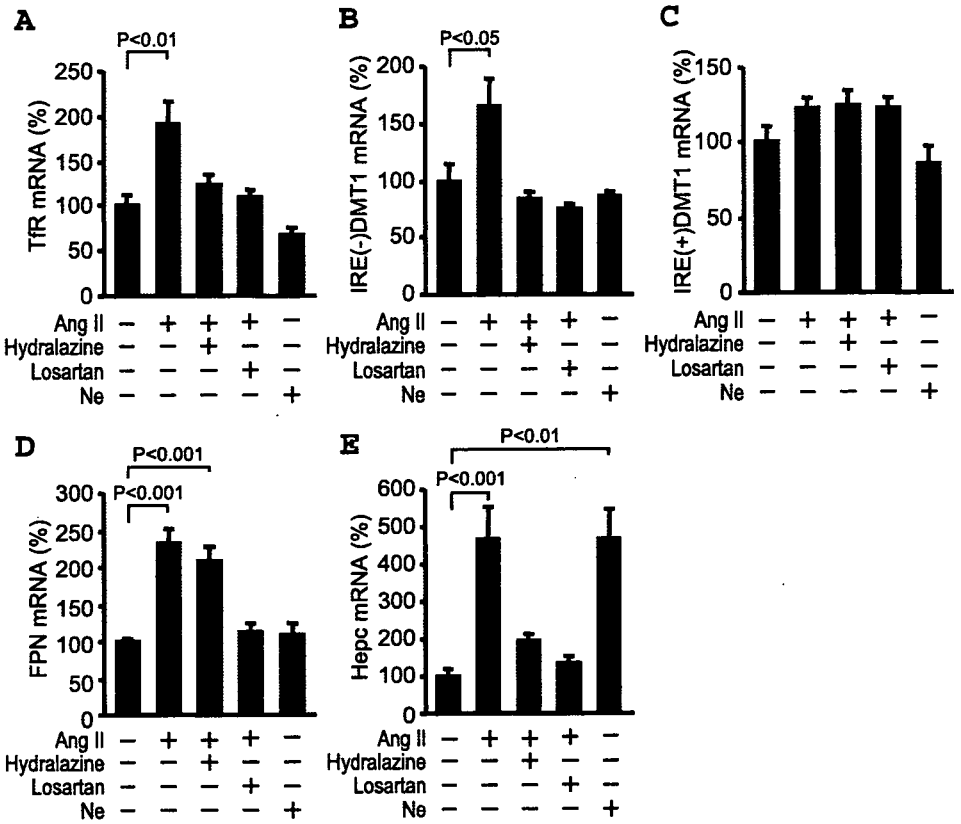

Fig. 3. *Quantification of the expression of iron metabolism–related genes. Expression of TfR (A), IRE(!)DMT1 (B), IRE(!)DMT1 (C), FPN (D), and hepc (E) mRNA was analyzed by quantitative PCR using the HibriProbe system and a LightCycler (Roche). Shown is a summary of the results from quantitative RT-PCR 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-!1 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

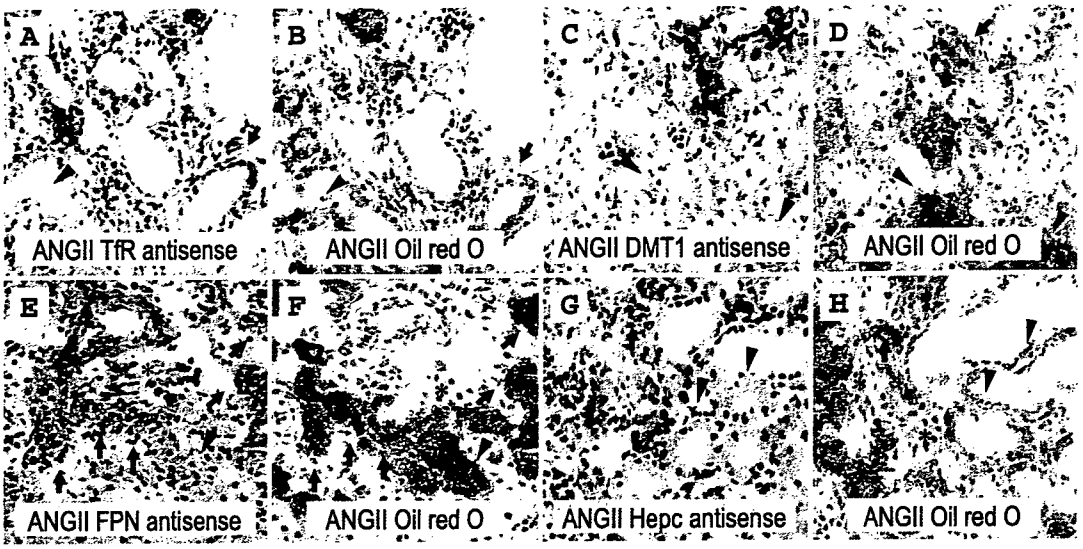

Fig. 4. *Localization of DMT1, FPN, and hepc mRNA and lipid deposition. Unfixed, frozen samples from the kidneys of angiotensin II–infused rats were used. A-B, C-D, E-F, and G-H are serial specimens. A:* In situ *hybridization using the TfR antisense probe. B, D, F, H: Oil red O staining. C:* In situ *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