Table 3. Stepwise multiple regression analysis between percent changes in lipid parameters and age, %dWC, and %dBMI

	Women					Men				
	β	95%CI		Standardized β	p value	β	95%CI		Standardized β	p value
Model 1										
Dependent variable, %dLDL										
%dBMI	0.72	0.44	0.99	0.15	<0.001	0.86	0.62	1.10	0.16	<0.001
age						−0.08	−0.15	−0.01	−0.05	0.019
Dependent variable, %dHDL										
%dBMI	−0.23	−0.43	−0.03	−0.07	0.026	−0.70	−0.88	−0.53	−0.17	<0.001
Dependent variable, %TG										
%dBMI	2.08	1.42	2.75	0.18	<0.001	4.47	3.78	5.16	0.28	<0.001
Model 2										
Dependent variable, %dLDL										
%dWC	0.16	0.05	0.26	0.08	0.005	0.25	0.09	0.41	0.07	0.002
age						−0.11	−0.18	−0.04	−0.07	0.003
Dependent variable, %dHDL										
%dWC						−0.24	−0.36	−0.12	−0.09	<0.001
Dependent variable, %TG										
%dWC	0.33	0.06	0.60	0.07	0.015	1.12	0.64	1.60	0.10	<0.001

Model 1. Independent variables include age, %dWC, and %dBMI. For model 2, independent variables included age and %dWC. Standardized β values are the estimates resulting from analysis performed on standardized variables.

heart disease or hypertension[12]. They found that changes in WHR were associated with changes in total cholesterol and triglycerides in men; however, statistical significance was lost after controlling for changes in BMI. On the other hand, after controlling for changes in WHR, changes in BMI remained to be associated with changes in total cholesterol and triglycerides in both genders. Of note, even before controlling for changes in BMI, WC change was not found to be associated with either total cholesterol or triglycerides in women. Wing *et al.* concluded that changes in WHR may not be independently related to changes in cardiovascular risk factors. Pascale *et al.* showed that in subjects participating in a year-long weight loss program, weight loss, but not reductions in WHR, was significantly related with improvements in fasting glucose, fasting insulin, and HbA1, although the magnitude of WHR reduction was strongly related to the amount of weight lost especially in men[13].

Similar to Wing *et al.*'s study, the current study indicated certain gender differences in the association between WC change and lipid parameter change, especially in the model not controlled for BMI. As HDL-C and TG are closely related to insulin sensitivity, and thus visceral fat mass, the closer relationship of %dBMI than %dWC with %dHDL and %dTG was rather unexpected. It is possible that WC measurements may be less reliable than weight and height measurements, which reduced the predictive power of %dWC for lipid changes. The correlation between %dWC and %dBMI was relatively weak, especially in women. This finding may indicate that a loss (gain) of BMI did not necessarily result in a loss (gain) in WC over a one-year period, and that men appear to lose (or gain) weight in their abdominal area more readily than women, which was consistent with previous observations[8, 12]. The finding that %dWC did not predict lipid changes independently of %dBMI may suggest that changes in BMI might be a more reliable goal to avoid adverse lipid changes than changes in WC.

It has recently been demonstrated that measuring both general and abdominal adiposity provides a better assessment of the risk of death[14]; therefore, we cannot lessen the importance of reducing WC and thus control visceral adiposity; in this sense, whether percent changes in abdominal fat demonstrated by computed tomography will have a greater impact on serum lipid data than %dWC should be examined in future studies[15].

The current study has several potential limitations. First, individuals who, for unknown reasons, did not visit our institute in the second year were not enrolled in the current study, which may cause some bias. Second, we do not have sufficient information

on the extent to which modifications of lifestyle and dietary habits affect the observed changes in general/ abdominal obesity[16]. Third, we excluded subjects who were taking lipid-lowering drugs at either visit, and these individuals may, in general, have higher motivation to obtain information on how to improve serum lipid levels effectively as compared with those not taking such drugs. Finally, a longer follow-up should be performed in future studies.

In summary, during a one-year period, percent changes in BMI (%dBMI) were associated positively with percent changes in LDL-C and TG and negatively with those in HDL-C, especially in both genders. Although percent changes in WC (%dWC) also tended to confer adverse changes in lipid parameters, this relationship did not remain significant after controlling for %dBMI.

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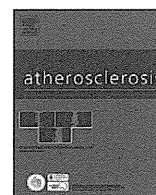

Association between metabolic syndrome and carotid atherosclerosis in individuals without diabetes based on the oral glucose tolerance test

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ABSTRACT

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

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1. Introduction

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

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

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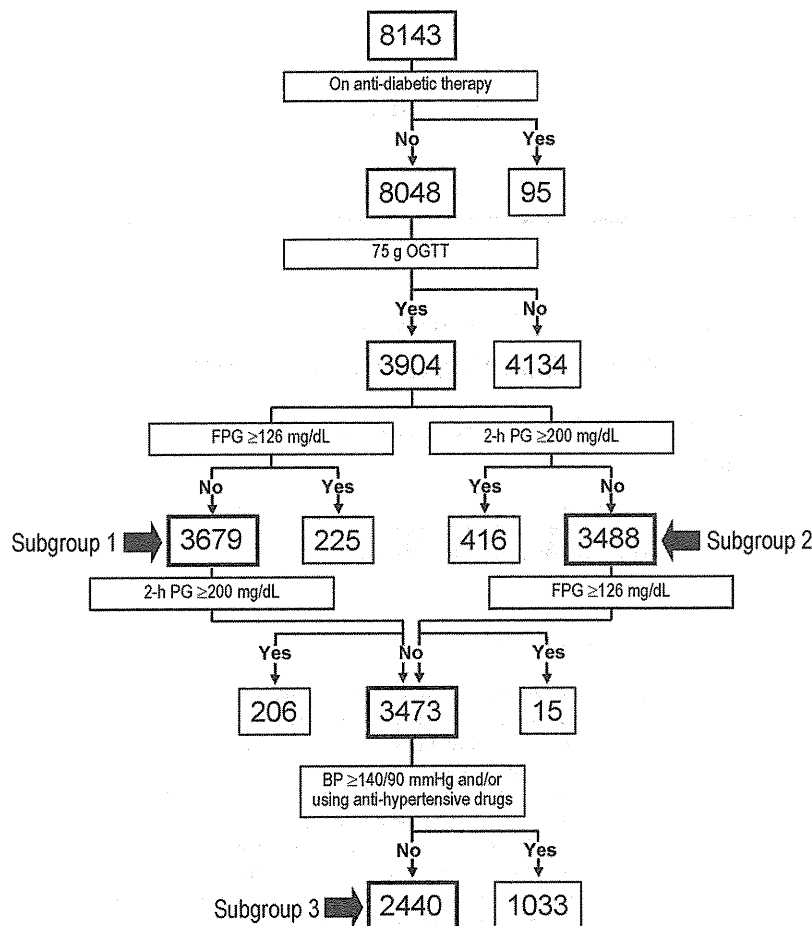

Fig. 1. Flow chart showing selection of the four subgroups.

2. Methods

2.1. Study subjects and selection of subgroups

The study was approved by The Ethical Committee of Mitsui Memorial Hospital and University of Tokyo, Faculty of Medicine. Between September 1994 and December 2003, 8143 subjects underwent general health screening including carotid ultrasonography at the Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital. Of the 8143 subjects, 95 were treated as having diabetes, and of the remaining 8048 individuals, 3904 underwent an OGTT. Among these 3904 individuals, three subgroups were sequentially selected based on various parameters (Fig. 1). Those with a fasting plasma glucose (FPG) value of <126 mg/dL were designated as subgroup 1, and those with a 2-h post-OGTT plasma glucose (2-h PG) value of <200 mg/dL were designated as subgroup 2. Subgroup 3 was comprised of subjects who met all the following conditions: FPG of <126 mg/dL, 2-h PG of <200 mg/dL, and not having hypertension. Hypertension was defined as SBP ≥140 mmHg, DBP ≥90 mmHg, or the use of anti-hypertensive medication. We also selected individuals without impaired glucose tolerance (IGT), i.e., individuals with a 2-h PG value of <140 mg/dL.

At our institute, several types of health screening programs are available, and some general health screening programs include carotid ultrasonography and/or OGTT, while others do not. However, the decision on the type of health screening was made by the individuals and/or their companies and was not decided upon or recommended by any attending physician.

2.2. Definition of MetS

MetS was defined as the presence of three or more of the following: (1) fasting glucose ≥110 mg/dL; (2) SBP/DBP ≥130/85 mmHg or taking anti-hypertensive medication; (3) triglycerides ≥150 mg/dL mmol/L; (4) HDL cholesterol <40 mg/dL in men and <50 mg/dL in women; and (5) body mass index ≥25 kg/m^2 [11].

2.3. Carotid ultrasonography

Carotid artery status was studied using high resolution B-mode ultrasonography (Sonolayer SSA270A, Toshiba, Japan) equipped with a 7.5 MHz transducer as described previously [12]. Plaque was defined to be present when there is one or more clearly isolated focal thickening(s) of the intima-media layer with thickness of ≥1.3 mm at the common or internal carotid artery or the carotid bulb. Carotid wall intima-media thickening was said to be present when intima-media thickness which was measured at the far wall of the distal 10 mm of the common carotid artery was ≥1.0 mm [12].

2.4. Statistical analysis

Logistic regression analysis was used to obtain adjusted odds ratios and their 95% confidence intervals (CIs) to predict the presence of carotid plaque or carotid intima-media thickening. Statistical analyses were carried out by using Dr. SPSS II (SPSS Inc., Chicago, IL). Results are expressed as the mean ± standard deviation (SD). A value of $p < 0.05$ was taken to be statistically significant.

Table 1
Baseline characteristics.

Variables	Subgroup 1		Subgroup 2		Subgroup 3	
	Men	Women	Men	Women	Men	Women
Number	2548	1131	2386	1102	1588	852
Age, years	58.2 ± 10.6	57.9 ± 10.4	58.0 ± 10.7	57.8 ± 10.3	56.7 ± 10.9	56.6 ± 10.5
Body mass index, kg/m²	24.0 ± 2.8	22.2 ± 3.1	23.9 ± 2.7	22.1 ± 3.1	23.6 ± 2.6	21.7 ± 2.8
Systolic BP, mmHg	127 ± 19	121 ± 21	128 ± 19	120 ± 20	119 ± 12	123 ± 14
Diastolic BP, mmHg	79 ± 12	73 ± 12	79 ± 12	73 ± 12	73 ± 8	69 ± 9
Total cholesterol, mg/dL	206 ± 32	219 ± 35	205 ± 32	219 ± 35	205 ± 32	216 ± 35
HDL-cholesterol, mg/dL	55 ± 16	70 ± 17	55 ± 16	70 ± 17	56 ± 16	71 ± 17
Triglycerides, mg/dL	144 ± 117	96 ± 56	142 ± 98	95 ± 54	141 ± 98	95 ± 54
Uric acid, mg/dL	6.2 ± 1.2	4.7 ± 1.0	6.2 ± 1.2	4.7 ± 1.0	6.2 ± 1.2	4.6 ± 1.0
Fasting glucose, mg/dL	96 ± 10	90 ± 10	95 ± 10	90 ± 9	94 ± 9	88 ± 9
2-h OGTT glucose, mg/dL	132 ± 41	118 ± 32	125 ± 29	115 ± 26	121 ± 29	112 ± 25
Haemoglobin A1C, %	5.2 ± 0.4	5.1 ± 0.4	5.2 ± 0.4	5.1 ± 0.4	5.2 ± 0.4	5.1 ± 0.4
Hypertension, *n* (%)	863 (34)	263 (23)	788 (33)	248 (23)	0	0
Anti-hypertensive drugs, *n* (%)	336(13)	99(9)	307(13)	95(9)	0	0
Metabolic syndrome, *n* (%)	439(17)	84(7)	372(16)	72(7)	131 (8)	25(3)
Smoking status						
Never, *n* (%)	764 (30)	933 (82)	714(30)	909 (82)	465 (29)	689(81)
Former, *n* (%)	799(31)	53(5)	753 (32)	50(5)	464 (29)	44(5)
Current, *n* (%)	985 (39)	145(13)	919(39)	143(13)	659(41)	119(14)

BP indicates blood pressure, OGTT indicates oral glucose tolerance test.

3. Results

3.1. Association between MetS and carotid atherosclerosis in individuals with FPG value of <126 mg/dL (subgroup 1)

Among the 3904 individuals who underwent OGTT, 3679 (94%) had an FPG value of less than 126 mg/dL. Of these, 300 (257 men, 43 women), the FPG value was ≥110 mg/dL, thus impaired fasting glycemia (IFG), and in the remaining 3379 (2291 men, 1088 women) had an FPG value of less than 110 mg/dL (no IFG). Table 1 shows the baseline characteristics of this group according to gender. Carotid plaque was found in 823 (32%) men and 191 (17%) women and carotid intima-media thickening was found in 422 (17%) men and 122 (11%) women (Fig. 2). Age-adjusted logistic regression analysis (Model 2) showed that, in men, MetS was statistically significantly associated with carotid plaque (Table 1) and intima-media thickening (Table 2). In women, MetS tended to be associated with carotid plaque, but not with intima-media thickening after age adjustment. Similar patterns of relationships could be observed after further adjustment for total cholesterol (TC) and smoking status (Model 3). On the other hand, after full adjustment including that for components of MetS (Model 4), MetS was not significantly associated with carotid plaque or intima-media thickening in either men or women.

3.2. Association between metabolic syndrome and carotid atherosclerosis in individuals with 2-h PG value of <200 mg/dL (subgroup 2)

Among 3904 individuals who underwent OGTT, 3488 (89%) had a 2-h PG value of less than 200 mg/dL. Of these 3488 individuals 2644 (1717 men, 927 women) had a 2-h PG value of less than 140 mg/dL (no IGT) and the remaining 844 (669 men, 175 women) had a 2-h PG FPG value of ≥140 mg/dL, and thus IGT. Carotid plaque was found in 761 (32%) men and 182 (17%) women and carotid intima-media thickening was found in 378 (16%) men and 116 (11%) women. Age-adjusted logistic regression analysis (Model 2) showed that, in men, MetS was statistically significantly associated with carotid plaque (Table 2) and intima-media thickening (Table 3). In women, MetS tended to be associated with carotid plaque but not with intima-media thickening. Similar patterns of relationship could be observed after further adjustment for TC and smoking status (Model 3). On the other hand, after full adjustment that included components of MetS (Model 4), MetS was not significantly associated with carotid plaque or intima-media thickening in men or in women. There were only 15 (13 men, 2 women)

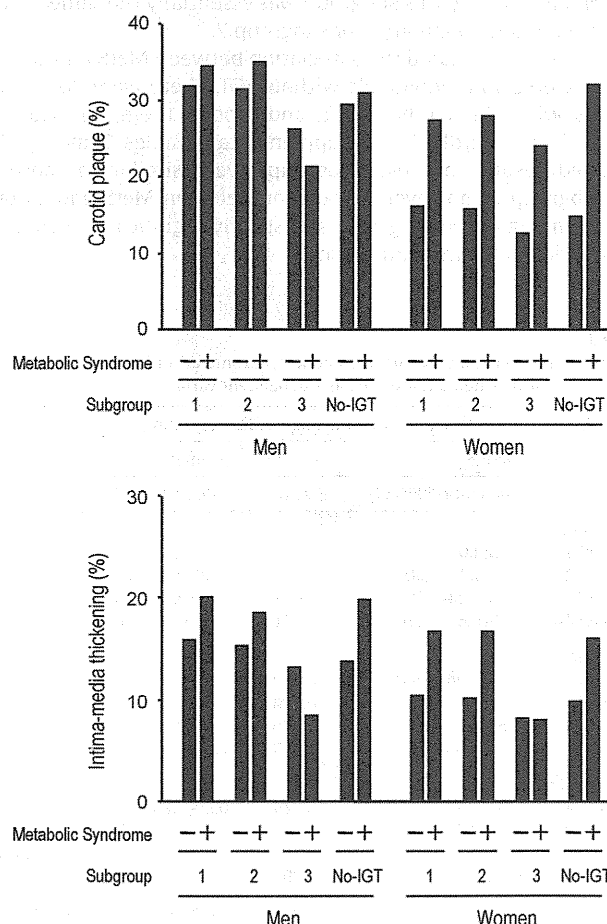

Fig. 2. Prevalence of carotid plaque and carotid intima-media thickening according to the presence or absence of metabolic syndrome in subgroups.

Table 2
Logistic regression analysis with metabolic syndrome as an independent variable and carotid plaque as a dependent variable.

Variables	Odds ratio for carotid plaque			
	Men		Women	
	Odds ratio (95% CI)	P value	Odds ratio (95% CI)	P value
Subgroup 1				
Model 1	1.12(0.90–1.39)	0.302	1.97(1.19–3.28)	0.009
Model 2	1.41(1.11–1.79)	0.005	1.68(0.96–2.95)	0.072
Model 3	1.30(1.03–1.67)	0.030	1.63(0.93–2.88)	0.091
Model 4	1.21(0.90–1.63)	0.209	1.61(0.79–3.29)	0.188
Subgroup 2				
Model 1	1.18(0.93–1.49)	0.170	2.06(1.20–3.55)	0.009
Model 2	1.47(1.14–1.90)	0.003	1.78(0.98–3.24)	0.058
Model 3	1.38(1.07–1.78)	0.014	1.72(0.95–3.14)	0.076
Model 4	1.23(0.90–1.69)	0.202	1.73(0.82–3.63)	0.151
Subgroup 3				
Model 1	0.77(0.50–1.19)	0.232	2.20(0.86–5.62)	0.101
Model 2	0.99(0.62–1.58)	0.971	1.89(0.66–5.43)	0.235
Model 3	0.94(0.59–1.50)	0.796	1.85(0.64–5.33)	0.254
Model 4	0.82(0.48–1.41)	0.479	2.44(0.72–8.29)	0.152

Model 1, unadjusted; Model 2, adjusted for age; Model 3, adjusted for age, total cholesterol and smoking status; Model 4, adjusted for age, body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, triglycerides, fasting plasma glucose, and smoking status.

individuals among the 3488 in subgroup 2 who had an FPG value of <126 mg/dL in addition to a 2-h PG value of <200 mg/dL, and, thus, the mode of association between MetS, carotid plaque, and intima-media thickening in this subgroup was essentially the same as that observed in total population of subgroup 2.

We also investigated the association between MetS and carotid atherosclerosis in individuals without IGT. There were 2644 individuals who did not have IGT, and among them, 61 had FPG value of ≥110 mg/dL (Fig. 2, Supplementary Tables 1 and 2). The obtained results in these subgroups were similar to those in the subgroup 2; however, association between MetS and carotid intima-media thickening was statistically significant even after multivariate adjustment in women.

Table 3
Logistic regression analysis with metabolic syndrome as an independent variable and carotid intima-media thickening as a dependent variable.

Variables	Odds ratio for carotid intima-media thickening			
	Men		Women	
	Odds ratio (95% CI)	P value	Odds ratio (95% CI)	P value
Subgroup 1				
Model 1	1.33(1.03–1.73)	0.031	1.74(0.95–3.19)	0.074
Model 2	1.74(1.31–2.30)	<0.001	1.40(0.72–2.73)	0.324
Model 3	1.65(1.24–2.19)	<0.001	1.38(0.70–2.70)	0.349
Model 4	0.97(0.67–1.39)	0.851	0.70(0.31–1.60)	0.398
Subgroup 2				
Model 1	1.26(0.94–1.68)	0.120	1.78(0.93–3.42)	0.083
Model 2	1.63(1.20–2.22)	0.002	1.47(0.73–2.98)	0.285
Model 3	1.55(1.13–2.11)	0.006	1.44(0.71–2.93)	0.317
Model 4	1.00(0.68–1.48)	0.993	0.71(0.30–1.67)	0.435
Subgroup 3				
Model 1	0.61(0.32–1.15)	0.125	0.99(0.23–4.28)	0.985
Model 2	0.83(0.43–1.61)	0.586	0.71(0.15–3.41)	0.673
Model 3	0.77(0.40–1.50)	0.443	0.70(0.15–3.39)	0.660
Model 4	0.52(0.24–1.11)	0.092	0.56(0.05–1.45)	0.123

Model 1, unadjusted; Model 2, adjusted for age; Model 3, adjusted for age, total cholesterol and smoking status; Model 4, adjusted for age, body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, triglycerides, fasting plasma glucose, and smoking status.

3.3. Association between metabolic syndrome and carotid atherosclerosis in individuals with FPG value of <126 mg/dL, 2-h PG value of <200 mg/dL, and no hypertension (subgroup 3)

Among 3904 individuals who underwent OGTT, 2440 (63%) could be assigned to subgroups 3. Their baseline characteristics according to gender are shown in Table 1. Carotid plaque was found in 409 (26%) men and 110 (13%) women and carotid intima-media thickening was found in 202 (13%) men and 69 (8%) women. Unlike subgroups 1 and 2, MetS was not significantly associated with either carotid plaque or intima-media thickening after age adjustment, or even before any adjustment in either gender (Tables 2 and 3).

4. Discussion

Here, we have assessed whether MetS is a risk factor for carotid atherosclerosis in individuals who were determined not to have diabetes mellitus based on results of OGTT. MetS was found to be associated with carotid atherosclerosis especially in men; however, when individuals with hypertension, defined as those having SBP/DBP ≥140/90 mmHg or using anti-hypertensive medication, were excluded, the presence of MetS no longer conferred excess risk when adjustments were made only for age or even when no adjustments were made.

It is known that clustering of certain metabolic abnormalities and hypertension increases the incidence of atherosclerotic diseases [13]. However, whether such clustering of atherogenic risk factors should be separately designated as MetS has been controversial. Whether MetS is independently associated with carotid atherosclerosis has been analyzed in various populations. By analyzing data on a multi-ethnic cohort of apparently healthy individuals in Canada, Paras et al. reported that although MetS was significantly associated with measures of sub-clinical carotid atherosclerosis, this association is mediated entirely through the components of MetS that have been considered as risk factors [14]. Similarly, by analyzing data on individuals recruited from a local community in Italy, Fadini et al. demonstrated that the clustering of MetS components led to a no-more-than additive increase in carotid intima-media thickness [4]. In addition, Vaidya et al. reported that MetS did not have supra-additive association with carotid intima-media thickening [15].

In our previous study that analyzed data on subjects who underwent general health screening, we found that MetS may not be associated with carotid atherosclerosis even after adjustment only for age when individuals did not have hypertension (SBP/DBP <140/90 mmHg and not using anti-hypertensive medication) [8]. In the current study, we expanded this theme to investigate whether MetS increases the risk for carotid atherosclerosis in individuals who had no or only mild (i.e., not in the diabetic range) abnormalities in glucose metabolism. We found that in individuals with FPG values of <126 mg/dL (subgroup 1) or in those with 2-h PG values of <200 mg/dL (subgroup 2), MetS was positively associated with carotid plaque after adjustment for only age (Model 2), although the relationship was only borderline positive in women. In men, the association between MetS and carotid intima-media thickening was also statistically significantly positive after adjustment for only age. These associations lost statistical significance after adjustment for TC, smoking status, and components of MetS (Model 4), suggesting that these associations may not be independent of these factors. Attention should be given to the fact that after excluding individuals with hypertension from the analysis, the association between MetS and carotid plaque or carotid intima-media thickening was no longer statistically significant even after adjustment for only age (subgroup 3), which is in agreement with our previous finding [8].

Several previous cross-sectional and longitudinal studies have investigated whether MetS increases the risk for atherosclerotic diseases in subjects without apparent impairment in glucose metabolism. A prospective population-based study of Finnish men showed that MetS was associated with higher mortality from coronary heart disease in men without impaired fasting glycemia [16]. Wilson et al. reported that MetS was associated with increased risk for cardiovascular disease in those without diabetes [17]. Leoncini et al. reported that MetS was associated with carotid atherosclerosis in non-diabetic hypertensive individuals who attended an outpatient clinic in Italy [18]. Kawamoto et al. analyzed Japanese inpatients and found that MetS increased the risk for carotid intima-media thickening in non-diabetic subjects [19]. Tzou et al. reported that the presence of MetS increased the composite of carotid intima-media thickness of ≥75th percentile of enrolled subjects in non-diabetic young adults [20]. These results support the notion that the presence of MetS will increase the risk for carotid atherosclerosis even in non-diabetic populations; however, caution should be paid in interpreting these results, as these results were not always adjusted for each component of MetS. The present results showed that MetS was associated with carotid plaque and intima-media thickening in men in subgroups 1, and 2 after adjustment for age, TC, and smoking status, although statistically significance would be lost after further adjustment for MetS components.

We found that in the absence of hypertension (subgroup 3), the association between MetS and carotid plaque or intima-media thickening was no more statistically significant after adjustment for only age, or even when no adjustments were made. These data collectively suggested that the presence or absence of hypertension, but not an abnormality in glucose metabolism, is crucial to determine whether the presence of MetS would increase the risk for carotid atherosclerosis. A recent study showed that MetS significantly increased all-cause mortality in hypertensive community-based French individuals with a hazard ratio of 1.40 (95% CI 1.13–1.74), but not in non-hypertensive individuals, during a mean follow-up period of 4.7 years [21], which was consistent with the idea of major role played by hypertension.

This study has several limitations. First, due to the cross-sectional nature of the study, we cannot determine whether there is a causal or resultant relationship between the MetS and presence of atherosclerosis. Second, among 8048 individuals who were not taking anti-diabetic medication, we excluded 4144 individuals who did not undergo OGTT. The mean age of the 3904 individuals who underwent OGTT and those 4144 who did not were significantly different (55 ± 10 years versus 58 ± 10 years, respectively, $P < 0.001$); therefore, it could be said that there had been some selection bias, though, again, the type of health screening was not decided or recommended by the physicians.

In conclusion, we showed that MetS was associated with carotid plaque and carotid intima-media thickening in non-diabetic individuals; although, this relationship did not remain statistically significant after adjustment for MetS components. In non-diabetic non-hypertensive individuals, the association between MetS and carotid plaque or carotid intima-media thickening was not statistically significant when adjustment was made for only age or even when no adjustment were made. These data collectively indicate that presence or absence of hypertension, but not an abnormality in glucose metabolism, is crucial to determine the relationship between MetS and carotid atherosclerosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2008.10.022.

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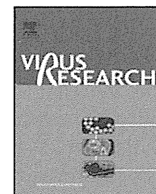

Short communication

Peripheral blood memory B cells are resistant to apoptosis in chronic hepatitis C patients

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ABSTRACT

Our recent study indicated that peripheral B cells in chronic hepatitis C (CHC) patients were infected with hepatitis C virus (HCV). It was also demonstrated that the frequency of $CD27^+$ B cells, i.e. memory phenotype, was significantly reduced in the peripheral blood of CHC patients. An assumption was made by these findings that the $CD27^+$ B cells are susceptible to apoptosis when infected with HCV. Therefore, in this study, the susceptibility of $CD27^+$ B cells to apoptosis in CHC patients was analyzed. Contrary to our assumption, it was found that $CD27^+$ B cells are more resistant to apoptosis than the counterpart subset, i.e. $CD27^-$ B cells. The rationale for this finding is discussed with regard to the possible role for memory B cells as an HCV reservoir for persistent infection in CHC patients.

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Hepatitis C virus (HCV) infection has been recognized as one of the major causes of chronic liver diseases, including chronic hepatitis, cirrhosis and, eventually hepatocellular carcinoma, affecting nearly 200 million people worldwide (Lauer and Walker, 2001). The liver is considered to be the primary and main target of HCV infection. However, extrahepatic manifestations, such as mixed cryoglobulinemia, a systemic immune complex-mediated disorder characterized by B cell proliferation that may evolve into overt B cell non-Hodgkin's lymphoma, have been demonstrated (Agnello et al., 1992; Zuckerman et al., 1997). The occurrence of B cell abnormalities often noticed among patients persistently infected with the HCV has suggested the possibility that HCV infects not only hepatocytes but also peripheral B cells. Recent studies including ours have demonstrated that peripheral B cells are in fact infected with HCV (Inokuchi et al., 2009; Ito et al., 2010), which suggest the unprecedented role for B cells in HCV pathogenesis.

Two major human peripheral B cell subsets have been identified based on the expression of CD27, a member of the tumor necrosis factor receptor family. Functional differences between the two subsets have been extensively investigated and it is now generally accepted that CD27 is a memory B cell marker (Agematsu et al., 2000). Our previous study demonstrated that the frequency of peripheral $CD27^+$ memory B cell subset in chronic hepatitis C (CHC) patients is significantly reduced (Mizuochi et al., 2010). To elucidate the reason of this reduction, in this study, we compared the susceptibility of the peripheral $CD27^+$ and $CD27^-$ B cell subsets to apoptosis in CHC patients. Our results demonstrated that $CD27^+$ memory B cells in CHC patients are more resistant to apoptosis than $CD27^-$ B cells. The rationale for this finding is discussed with regard to the possible role for memory B cells in HCV pathogenesis.

A total of 26 CHC patients were enrolled in this study (male/female: 15/11, mean age: 59.6 ± 6.9 years old, mean serum ALT levels: 65.5 ± 31.7 IU/L, mean serum AST levels: 53.2 ± 24.4 IU/L, HCV genotype: 1b = 23, 2a = 3, mean HCV RNA: 2493 ± 959 KIU/mL). All of them were confirmed to be negative for other viral infections, including hepatitis B virus (HBV) and human immunodeficiency virus (HIV). The study protocols were approved by the Review Board at the National Institute of Infectious Diseases. All donors gave written informed consent. The controls were 15 healthy blood donors at the Tokyo Red Cross Blood Center (Tokyo, Japan), who were confirmed to be negative for HCV, HBV, and HIV. HCV genotype was determined by PCR of the core region with genotype-specific primers (Ohno et al., 1997). HCV RNA was quantified by the Roche Amplicor assay (Roche Diagnostics, Branchberg, NJ), and results were standardized to international units (IU). Determination of serum levels of ALT and AST was performed using standard methods.

The following fluorescence-conjugated antibodies (Abs) were used for flow cytometry: Allophycocyanin-anti-CD19 (Cat. MHCD1905; Invitrogen, Carlsbad, CA); PE-anti-CD27 (Cat. IM2578; Beckman Coulter, Fullerton, CA); and FITC-anti-CD27 (Cat. 555440; BD Biosciences, San Jose, CA). Cells were washed twice with cold PBS containing 0.2% BSA, followed by incubation with an appro-

Abbreviations: CHC, chronic hepatitis C patients; HCV, hepatitis C virus.

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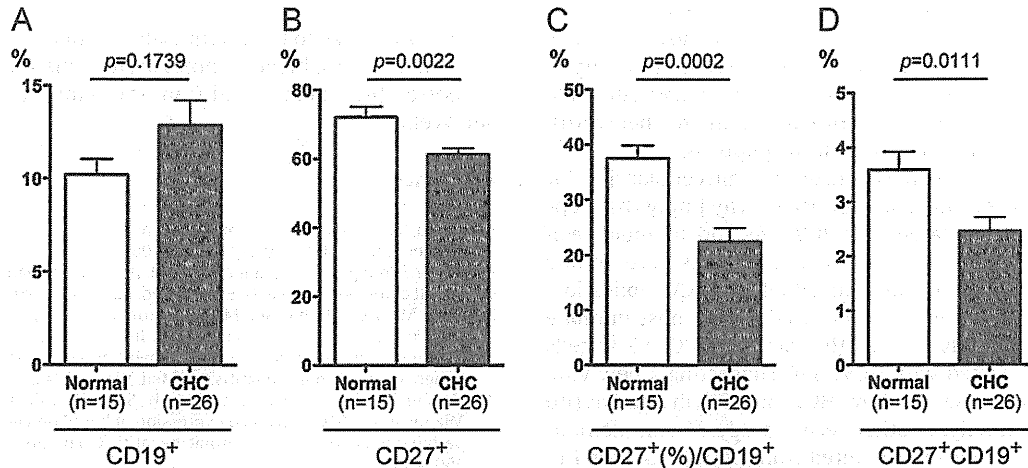

Fig. 1. Flow cytometric analysis of PBMC from normal subjects and CHC patients. Lymphocytes were identified by forward and orthogonal light scatter characteristics. Percentages of $CD19^+$ cells (A), $CD27^+$ cells (B), $CD27^+$ (%) in $CD19^+$ cells (C) and $CD27^+CD19^+$ cells (D) in normal ($n = 15$) and CHC patients ($n = 26$) are shown with SEM bars and *p*-values.

priate combination of directly conjugated Abs for 30 min on ice. Stained cells were analyzed by FACSCallibur (Becton Dickinson, San Jose, CA). Data were collected using CellQuest software (Becton Dickinson, San Jose, CA) and were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Levels of Annexin V binding to both $CD27^+$ and $CD27^-$ B cells were assessed with a commercially available Annexin V apoptosis detection kit Annexin V-FITC (PN IM3546, Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions.

Unpaired (two-tailed) Student's *t*-test was applied at the 95% confidence level ($p < 0.05$) using Prism ver.4 (GraphPad Software, Inc., San Diego, CA) in all cases.

We first analyzed the frequencies of peripheral blood $CD19^+$ cells, i.e. B cells. They were not statistically different ($p = 0.1739$) between normal subjects and CHC patients as shown in Fig. 1A. When the percentages of peripheral $CD27^+$ cells were analyzed, a statistically significant ($p = 0.0022$) decrease was noticed in CHC patients when compared to normal subjects (Fig. 1B). The percentages of peripheral $CD27^+$ cells in $CD19^+$ cells were then analyzed. A significant ($p = 0.0002$) decrease was noticed in CHC patients when compared to normal subjects (Fig. 1C). It was also verified that the frequencies of peripheral $CD27^+CD19^+$ cells were significantly ($p = 0.01111$) reduced in CHC patients (Fig. 1D). These results are in good agreement with those of Racanelli et al. (2006). In their report, patients with higher plasma HCV loads had lower percentages of $CD27^+$ B cells, thus suggesting that high HCV replication is associated with a reduction in $CD27^+$ B cells. They hypothesized that, under conditions of persisting HCV antigenemia, memory B cells not receiving specific B cell receptor triggering before having T-cell help would be pushed to enhance immunoglobulin production and prone to apoptosis (Racanelli et al., 2006), which may explain the reduction of $CD27^+$ memory B cells in HCV-infected patients. We next examined this possibility by analyzing apoptosis in both peripheral blood $CD27^+$ and $CD27^-$ B cells.

The levels of spontaneous apoptosis among peripheral blood $CD27^+$ and $CD27^-$ B cells in both normal subjects and CHC patients were analyzed using three-color flow cytometry by staining with allophycocyanin-anti-CD19, PE-anti-CD27 and Annexin V-FITC. As shown in Fig. 2A, $CD27^-$ B cells bound to much larger amounts of Annexin V than $CD27^+$ B cells in CHC patients. In contrast, the pat-

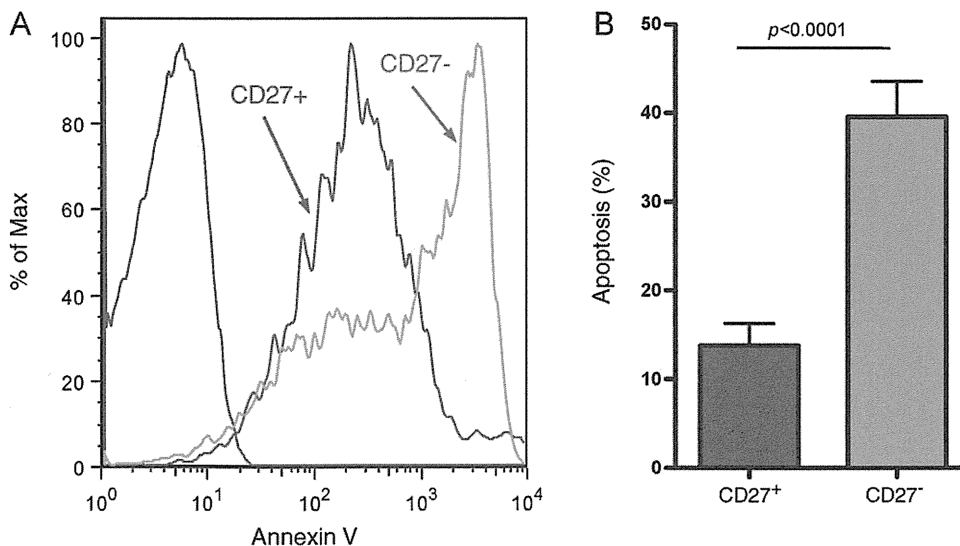

Fig. 2. Annexin V binding to $CD27^+$ and $CD27^-$ B cells. Representative staining patterns for Annexin V binding to $CD27^+$ (red line) and $CD27^-$ (green line) B cells are shown in CHC patients (A). Blue lines indicate background (bkg) staining in negative controls. Summary of data on Annexin V binding to $CD27^+$ (red bar) and $CD27^-$ (green bar) B cells in normal subjects ($n = 8$) and CHC patients ($n = 9$) are shown with SEM bars and *p*-value (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

terns of Annexin V binding were similar between $CD27^+$ and $CD27^-$ B cells in normal subjects (data not shown). The percentages of each cell subset bound to large amounts of Annexin V are shown in Fig. 2B (the cut-off point was tentatively set at a fluorescence intensity of 2000). It was concluded that, $CD27^-$ B cells were more vulnerable to apoptosis than $CD27^+$ B cells upon HCV infection; in other words, $CD27^+$ B cells were apparently resistant to apoptosis.

Hepatocytes have long been recognized as main cellular sites for HCV infection. However, this does not necessarily imply that hepatocytes are the exclusive targets for HCV infection. It would be of benefit for HCV to seek other cellular compartments as reservoirs in the event that the liver becomes unsuitable for HCV replication, possibly due to cellular destruction caused by the host immune response. Our recent study verified that peripheral $CD19^+$ B cells in CHC are in fact infected with HCV, thus suggesting a new viral reservoir during the course of natural HCV infection in humans (Ito et al., 2010). Interestingly, another recent study of ours demonstrated that $CD27^+$ B cells are recruited from peripheral blood to the inflammatory site of the liver of CHC patients (Mizuochi et al., 2010). The present study thus may offer new insights into the role of memory B cells in HCV pathogenesis. We assume that memory B cells are the main extrahepatic reservoir of HCV infection because of their long life span which may be correlated with their apparent resistance to apoptosis. This would be a robust strategy for HCV in order to secure sites for persistent infection.

In conclusion, the present study demonstrated that $CD27^+$ B cell subsets in CHC patients are resistant to apoptosis. The long-life of memory $CD27^+$ B cells may be suitable for persistent infection of HCV. Therefore, elimination of peripheral $CD27^+$ B cells in CHC patients with anti-B cell monoclonal antibodies, such as rituximab, would be effective for HCV clearance in CHC patients. Additional study with large sample number and infection with distinct HCV genotype may offer further information.

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Peripheral B Cells May Serve as a Reservoir for Persistent Hepatitis C Virus Infection

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Key Words

Hepatitis C virus · B cells · Retinoic acid-inducible gene-I · Interferon promoter-stimulator-1 · Interferon regulatory factor-3 · Interferon β

Abstract

A recent study by our group indicated that peripheral B cells in chronic hepatitis C (CHC) patients are infected with hepatitis C virus (HCV). This raised the logical question of how HCV circumvents the antiviral immune responses of B cells. Because type I interferon (IFN) plays a critical role in the innate antiviral immune response, IFNβ expression levels in peripheral B cells from CHC patients were analyzed, and these levels were found to be comparable to those in normal B cells, which suggested that HCV infection failed to trigger antiviral immune responses in B cells. Sensing mechanisms for invading viruses in host immune cells involve Toll-like receptor-mediated and retinoic acid-inducible gene-I (RIG-I)-mediated pathways. Both pathways culminate in IFN regulatory factor-3 (IRF-3) translocation into the nucleus for IFNβ gene transcription. Although the expression levels of RIG-I and its adaptor molecule, IFN promoter-stimulator-1, were substantially enhanced in CHC B cells, dimerization and subsequent nuclear translocation of IRF-3 were not detectable. TANK-binding kinase-1 (TBK1) and IκB kinase ε (IKKε) are essential for IRF-3 phosphorylation. Constitutive expression of both kinases was markedly enhanced in CHC B cells. However, reduced expression of heat shock protein of 90 kDa, a TBK1 stabilizer, and enhanced expression of SIKE, an IKKε suppressor, were observed in CHC B cells, which might suppress the kinase activity of TBK1/IKKε for IRF-3 phosphorylation. In addition, the expression of vesicle-associated membrane protein-associated protein-C, a putative inhibitor of HCV replication, was negligible in B cells. These results strongly suggest that HCV utilizes B cells as a reservoir for persistent infection.

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Introduction

Hepatitis C virus (HCV) is an enveloped positive-stranded RNA virus that belongs to the *Flaviviridae* family [1]. It is responsible for public health problems worldwide and affects nearly 200 million people [2]. The liver is regarded as the primary target of HCV infection; however, HCV infection is also associated with B cell lymphoproliferative disorders such as mixed cryoglobulinemia and B cell non-Hodgkin lymphoma [3, 4]. In fact, epidemiological evidence suggests a close link between chronic HCV infection and B cell non-Hodgkin lymphoma [5,

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6]. A pathogenic role for HCV in B cell disorders has been further demonstrated by reports showing clinical resolution of the above-mentioned B cell disorders after successful anti-HCV treatment with interferons (IFNs) [3, 7]. Based on this circumstantial evidence, a possible role for B cells in HCV pathogenesis has been postulated, although this has not been conclusively demonstrated.

A body of evidence suggests that HCV RNA replication occurs in a variety of extrahepatic cells, including peripheral dendritic cells, monocytes and macrophages [8–10]. It has also been suggested that HCV preferentially infects B cells that express CD81, a putative HCV receptor molecule [11–14]. A recent study by our group verified that peripheral CD19+ B cells in chronic hepatitis C (CHC) patients were infected with HCV, which suggested a new viral reservoir during the course of natural HCV infection in humans [15]. Thus, we assume that HCV has an escape strategy for persistent infection of B cells.

Foy et al. [16] found that nonstructural (NS) HCV proteins could inhibit the activation of early signaling pathways, such as Toll-like receptor 3 (TLR3)- and retinoic acid-inducible gene-I (RIG-I)-mediated pathways, which lead to IFNβ production. These results indicated that HCV NS3/4A serine protease blocked IFN regulatory factor-3 (IRF-3) activation upon HCV infection in the human hepatoma cell line HuH-7. Subsequent studies have shown that NS3/4A blocks IFN promoter-stimulator-1 (IPS-1)-mediated signaling pathways by cleaving the IPS-1 molecule and impeding downstream IRF-3 activation [17]. Thus, HCV apparently has a strategy to evade host innate immunity. However, recent studies by Dansako et al. [18, 19] found that the effects of HCV NS3/4A protease on IFN production depended on the cell lines used, because a non-neoplastic human hepatocyte cell line, PH5CH8 [20], retained both TLR3- and RIG-I-mediated pathways, in contrast to HuH-7 cells, which lack the former pathway [21]. However, no studies have examined the effects of HCV infection on IFN responses of nonhepatic cell lines.

In this study, we aimed to understand the mechanisms by which HCV evades innate immune responses in CHC B cells. We found that the antiviral immune response, represented by IFNβ induction, was severely impaired in B cells of CHC patients. Our results strongly suggest that the IRF-3 activation cascade is impeded in B cells upon HCV infection. Thus, IFNβ gene transcription is not augmented, which may result in failed IFNβ-inducible antiviral responses in CHC B cells. Furthermore, the expression of vesicle-associated membrane protein-associated protein-C (VAP-C), a putative inhibitor of HCV replication, was negligible in B cells. These results support the notion that HCV can successfully reside in B cells, resulting in persistent infection. This is the first study describing analysis of the suppressive effects of HCV infection on antiviral innate immunity in peripheral B cells. Thus, this study offers new insights into the role of B cells in the pathogenesis of HCV.

Methods

Patients and Samples

A total of 24 CHC patients were enrolled in this study, with the following characteristics: 14 males and 10 females; mean age 62.4 ± 7.4 years; mean serum ALT levels 67.5 ± 36.0 IU/l; mean serum AST levels 66.7 ± 34.3 IU/l; 21 patients with HCV genotype 1b and 3 with HCV genotype 2a, and mean HCV RNA 1,752 ± 1,188 KIU/ml. All cases were confirmed to be negative for other viral infections, including hepatitis B virus and human immunodeficiency virus. The study protocols were approved by the Review Board of the National Institute of Infectious Diseases. All donors gave written informed consent. Controls were healthy blood donors at the Tokyo Red Cross Blood Center (Tokyo, Japan) who were confirmed to be negative for HCV, hepatitis B virus and human immunodeficiency virus.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia Biotech, Quebec, Que., Canada) density gradient centrifugation. CD19+ B lymphocytes were isolated from PBMCs by negative selection (B Cell Isolation Kit II, human; Miltenyi, Auburn, Calif., USA). The purity of isolated B cells was generally >95%, as assessed by flow cytometry.

Semiquantitative Real-Time PCR

Total RNA was extracted from lymphoid cells using Isogen (Nippon Gene Co. Ltd., Tokyo, Japan). cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, Calif., USA) with oligo(dT)12–18 primer (Invitrogen). PCR amplification was performed using SYBR Premix Ex Taq™ II (Takara Shuzo, Kyoto, Japan) with gene-specific primers (Bex Co. Ltd., Tokyo, Japan) available in the public database RTPrimerDB [22] under the codes 3542 for IFNβ and 3539 for GAPDH, and the Universal Probe Library Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp; Roche Applied Science) as follows: IPS-1 (No. 19, 04686926001), TIR domain-containing adaptor inducing IFN (TRIF; No. 37, 04687957001), suppressor of IκB kinase ε (IKKε) (SIKE; No. 56, 04688538001), heat-shock protein of 90 kDa (Hsp90; No. 25, 04686993001) and DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked (DDX3X; No. 69, 04688686001). The primer sequences for RIG-I were 5′-GTG CAA AGC CTT GGC ATG T-3′ (forward) and 5′-TGG CTT GGG ATG TGG TCT ACT C-3′ (reverse) [23], and for TLR3 they were 5′-GTT ACG AAG AGG CTG GAA TGG T-3′ (forward) and 5′-GCC AGG AAT GGA GAG GTC TAG A-3′ (reverse) [24].

Real-time PCR was carried out for 45 cycles at 94°C for 1 min and at 60°C for 25 s (two-step PCR) using a Light Cycler (Roche Diagnostics, Basel, Switzerland). Amplification of predicted fragments was confirmed by melt curve analysis and gel electropho-

resis. Standard curves were established with 10-fold serial dilutions of amplified products. Measured amounts of each mRNA were normalized to GAPDH mRNA. mRNA expression levels in normal B cells were arbitrarily defined as 1.0.

Immunoblot Analysis

To extract whole-cell proteins, cell pellets were suspended in modified RIPA buffer [150 mM NaCl, 50 mM Tris-Cl (pH 7.4), 1 mM EDTA, 1.0% NP-40, 0.5% sodium deoxycholic acid and 0.1% SDS] containing Halt protease inhibitor cocktail (Pierce, Rockford, Ill., USA) and Halt phosphatase inhibitor cocktail (Pierce; 2×10^7 cells/ml). After 20 min of incubation on ice, cell extracts were centrifuged at 12,000 *g* for 10 min at 4°C, transferred to other tubes and stored at –80°C. Nuclear and cytoplasmic proteins were separated using a Nuclear Extraction Kit (Active Motif, Carlsbad, Calif., USA) according to the manufacturer's protocol. Protein concentration was determined using the BCA™ Protein Assay Kit – Reducing Agent Compatible (Pierce). Samples (whole-cell extract, 1 g; fractionated extract, 2×10^5 cells) were loaded onto 7.5 or 12.5% SDS acrylamide gels (Real Gel Plate; Bio Craft, Tokyo, Japan), followed by transfer to polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature using Block Ace™ (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan). They were then sequentially probed with primary and secondary antibodies at 4°C overnight and for 1 h at room temperature, respectively.

For primary antibodies, we used anti-IFN rabbit polyclonal antibody (ab9662, 1/1,000 dilution; Abcam Inc., Cambridge, Mass., USA), anti-ACTB (β-actin) rabbit polyclonal antibody (4967, 1/1,000 dilution; Cell Signaling Technology, Danvers, Mass., USA), anti-TLR3 rabbit polyclonal antibody (ab62566, 1/1,000 dilution; Abcam), anti-TRIF rabbit polyclonal antibody (4596, 1/1,000 dilution; Cell Signaling Technology), anti-RIG-I rabbit polyclonal antibody (29010, 1/100 dilution; Immuno-Biological Laboratories Co. Ltd., Gunma, Japan), anti-IPS-1 rabbit polyclonal antibody (AT107, 1/2,000 dilution; Alexis Biochemicals, Farmingdale, N.Y., USA), anti-IRF-3 rabbit polyclonal antibody (18781, 1/100 dilution; Immuno-Biological Laboratories), anti-GAPDH mouse monoclonal antibody [5G4(6C5), 1/9,000 dilution; HyTest Ltd., Turku, Finland], anti-PARP-1 mouse monoclonal antibody (AM30, 1/500 dilution; Calbiochem, San Diego, Calif., USA), anti-TANK-binding kinase-1 (TBK1) rabbit polyclonal antibody (3504, 1/1,000 dilution; Cell Signaling Technology) and anti-IKKε rabbit polyclonal antibody (ab7891, 1/500 dilution; Abcam). Anti-VAP-C rabbit polyclonal antibody (2.66 g/ml) was produced by a group of the authors (H.K., K. Moriishi and Y.M.).

The secondary antibodies used were horseradish peroxidase-coupled donkey anti-rabbit Ig (NA934, 1/10,000 dilution; GE Healthcare Ltd.; UK, Buckinghamshire, UK) and horseradish peroxidase-coupled sheep anti-mouse Ig (NA931, 1/10,000 dilution; GE Healthcare UK). Protein bands were detected using ECL Plus™ Western Blotting Detection Reagents (GE Healthcare UK) and a LAS-3000 Image Analyzer (Fuji Film, Tokyo, Japan). Densitometric analysis was performed within a linear range using Image Gauge (Fuji Film). The density of each band (the amount of protein) was normalized against that of the corresponding β-actin.

Native PAGE for IRF-3 Dimer Detection

Native PAGE was performed using 7.5% SDS acrylamide gels (Real Gel Plate; Bio Craft). Gels were prerun with 25 mM Tris-Cl (pH 8.4) and 192 mM glycine with and without 0.2% deoxycholate in the cathode and anode chambers, respectively, for 30 min at 40 mA. Samples were extracted in lysis buffer (3×10^7 cells/ml; 50 mM Tris-Cl, pH 8.0, 1% NP40, 150 mM NaCl) containing Halt protease inhibitor cocktail and Halt phosphatase inhibitor cocktail, mixed with equal volumes of Tris-glycine native sample buffer (2×; Invitrogen), applied to the gel and electrophoresed for 60 min at 25 mA.

Immunoblotting was performed as described above. As a positive control for IRF-3 dimerization, HeLa cells were added with 100 g/ml polyriboinosinic-polyribocytidylic acid (poly I:C; kindly provided by Toray Co. Ltd., Tokyo, Japan). Three hours after incubation, cells were harvested and cell lysates were prepared as described above.

Poly I:C Transfection

CD19+ B lymphocytes isolated from PBMCs were cultured in RPMI-1640 medium containing 10% FCS, 2 mM L-glutamine, 1 mM HEPES, 0.05 mM β-mercaptoethanol, penicillin and streptomycin in a flat-bottom 96-well plate for 3 h (2.5×10^6 cells/well). To activate the RIG-I-mediated pathway, cells were transfected with 10 μg/ml poly I:C using Poly(I:C)/LyoVec (Invivogen, San Diego, Calif., USA). After 18 h of culture, transfected or non-transfected cells were dissolved in Isogen (Nippon Gene) for semi-quantitative real-time PCR assay. Three independent triplicate transfection experiments were performed in order to verify the reproducibility of the results.

Statistics

Unpaired (two-tailed) Student's t tests were applied at the 95% confidence level ($p < 0.05$) using Prism (version 4; GraphPad Software Inc., San Diego, Calif., USA) in all cases.

Results

Impaired IFN Responses in Peripheral B Cells of CHC Patients

We recently demonstrated that HCV infected and may have replicated in peripheral B cells of CHC patients [15]. This implied that HCV may have evaded the immune response by B cells, resulting in persistent infection. Because the induction of type I IFNs, including IFNβ, is crucial for host defense against invading viruses, we first examined constitutive IFNβ expression levels in peripheral B cells of CHC patients. As shown in figure 1a, IFNβ mRNA expression levels were not augmented in CHC B cells compared with normal B cells. The results of Western blotting analysis (fig. 1b) indicated that constitutive IFNβ protein expression levels were not enhanced in B cells of CHC patients, which supported the finding of unaltered IFNβ mRNA expression.

We then stimulated normal and CHC B cells using poly I:C transfection, which triggers RIG-I- and melanoma differentiation-associated gene-5-mediated IFNβ path-

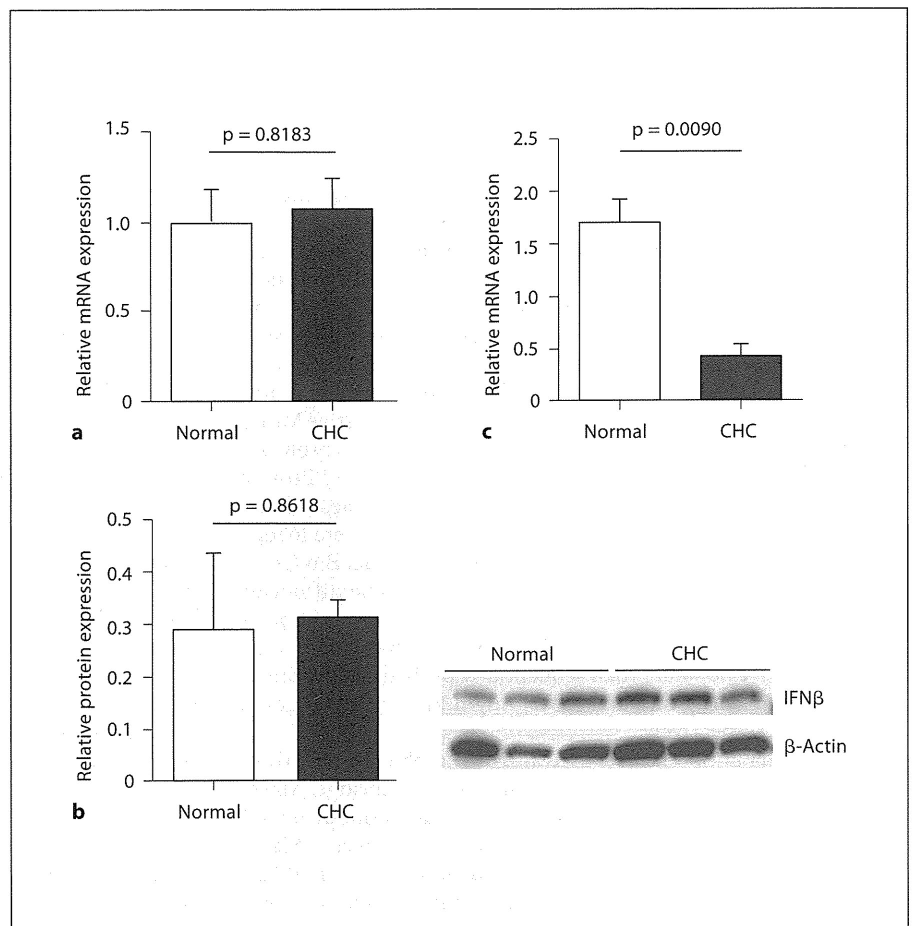

Fig. 1. IFNβ expression in CHC B cells. Fractionation of CD19+ B cells from PBMCs was performed as described in Methods. **a** IFNβ mRNA expression levels in CD19+ B cells isolated from normal individuals (n = 4) and CHC patients (n = 7) were measured in duplicate by quantitative real-time RT-PCR and normalized against those of the housekeeping gene GAPDH. mRNA expression levels in normal B cells were arbitrarily defined as 1.0. **b** Whole-cell extracts prepared from CD19+ B cells isolated from normal individuals (n = 3) and CHC patients (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-IFNβ and anti-ACTB antibodies. Relative IFNβ protein expression levels normalized against β-actin expression are shown. **c** CD19+ B cells isolated from normal individuals (n = 3) or CHC patients (n = 3) were transfected with poly I:C (10 g/ml). Eighteen hours after transfection, cells were harvested and total RNA was isolated. IFNβ mRNA expression levels were measured in duplicate using quantitative real-time RT-PCR and normalized against those of the housekeeping gene GAPDH. mRNA expression levels in untransfected normal or CHC B cells were arbitrarily defined as 1.0. Representative results from at least 2 independent experiments with similar results are shown.

ways. As shown in figure 1c, IFNβ mRNA expression levels in CHC B cells were much lower than those in normal B cells, suggesting that CHC B cells are defective with regard to IFNβ production upon stimulation with the intracellular delivery of poly I:C. In addition, the expression levels of IFN-stimulated genes, such as ISG-15 and ISG-56, in CHC B cells were also much lower than those in normal B cells upon poly I:C stimulation (data not shown).

Taken together, these results indicate that chronic HCV infection fails to induce an IFNβ response in CHC B cells. Subsequent experiments were designed to elucidate the underlying mechanism(s) by which HCV interrupted the IFN responses in CHC B cells.

Expression Levels of HCV Sensor Molecules in Peripheral B Cells of CHC Patients

We next examined the gene expression levels in peripheral B cells of two major viral sensors, TLR3 and RIG-I, as well as their corresponding adaptor molecules, TRIF and IPS-1, which are indispensable for initiating innate immune responses [25]. As shown in figure 2, TLR3, TRIF, RIG-I and IPS-1 expression levels were significantly enhanced in peripheral B cells of CHC patients. Expression of another cytoplasmic sensor molecule, melanoma differentiation-associated gene-5, was also enhanced (data not shown). These results demonstrate that the expression levels of cytoplasmic virus sensors as well as their adaptors are constitutively augmented in CHC B cells.

Expression, Dimerization and Nuclear Translocation of IRF-3 in CHC B Cells

The IRF-3 activation cascade, including phosphorylation, dimerization and nuclear translocation, is essential for IFNβ gene transcription [26]. We found that constitutive IRF-3 expression levels in CHC B cells were significantly lower than those in normal B cells ($p = 0.0018$) as assessed by Western blotting (fig. 3a). Furthermore, IRF-

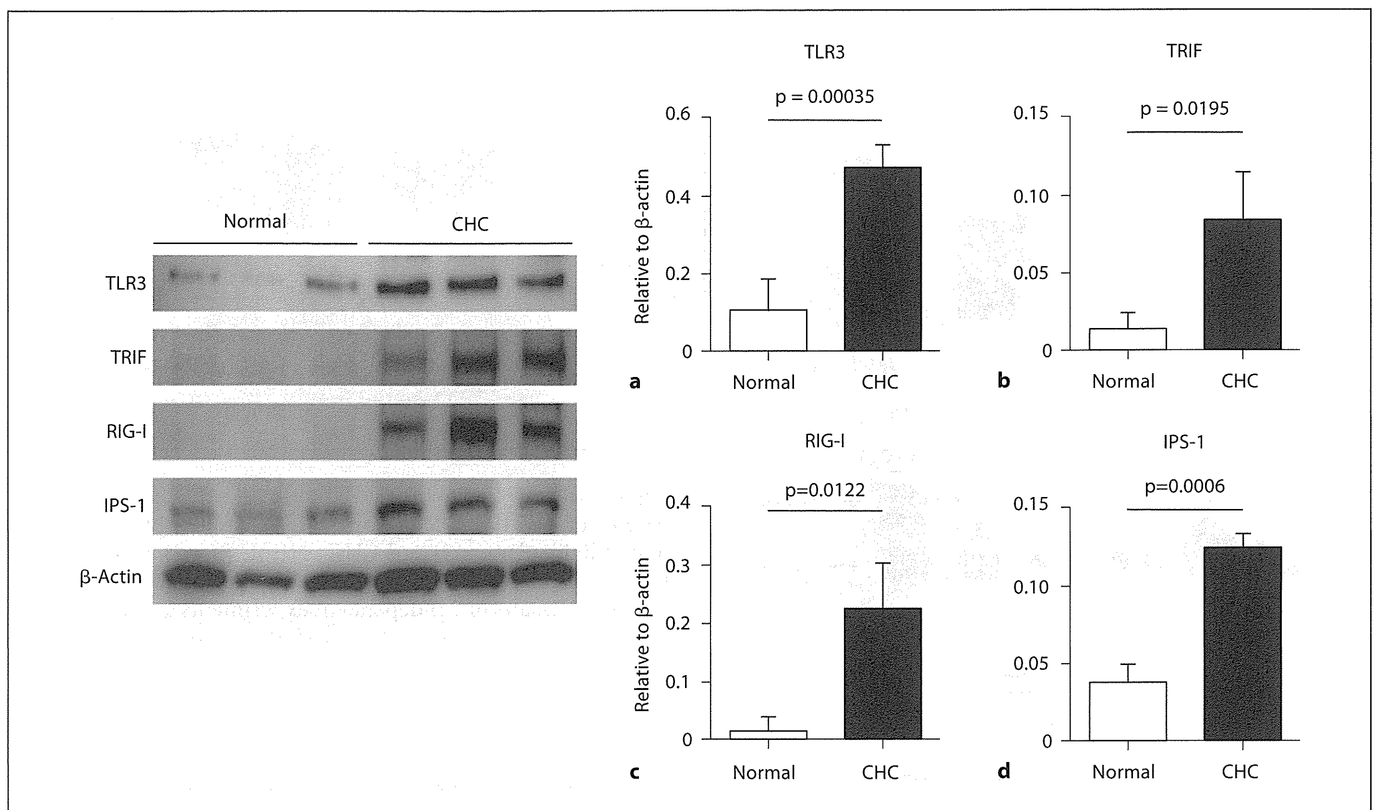

Fig. 2. Expression levels of HCV sensor and adaptor molecules in CHC B cells. Whole-cell extracts prepared from CD19+ B cells isolated from normal individuals (n = 3) and CHC patients (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-TLR3, anti-TRIF, anti-RIG-I, anti-IPS-1 and control anti-ACTB antibodies. Relative protein expression levels normalized against β-actin expression are shown.

3 dimerization assessed by native PAGE was not observed in CHC B cells (fig. 3b). Consequently, IRF-3 nuclear translocation did not occur in CHC B cells (fig. 3c). Thus, these results indicate that the IRF-3 activation cascade does not proceed in CHC B cells, which may explain the lack of IFNβ responses to HCV infection.

Cleavage of IPS-1 in CHC B Cells

Several lines of evidence have indicated an essential role for the adaptor molecule IPS-1, also called Cardif, MAVS or VISA, which acts downstream of RIG-I in the IRF-3 signaling pathway [27]. Recent studies have confirmed that HCV can cleave IPS-1 via its NS3/4A protease activity [28]. As a result of this proteolytic cleavage, IPS-1 is dislodged from the mitochondria and becomes an inactive cytosolic fragment. This causes the failure of downstream signaling for IRF-3 activation. Therefore, we examined IPS-1 cleavage in CHC B cells by native PAGE. As shown in figure 4, IPS-1 cleavage was incomplete, and a substantial amount of uncleaved (intact) IPS-1 was detected. Thus, we concluded that impaired IRF-3 activation cannot be solely explained by IPS-1 cleavage in CHC B cells. These results suggest either that the NS3/4A protease is not expressed in CHC B cells or that IPS-1 in B cells is resistant to this protease. Because NS3 molecules were detected in CHC B cells by Western blotting [15], the latter seems to be more likely.

Stabilization or Inhibition of TBK1/IKKε Kinase Activity in CHC B Cells

Two protein kinases, TBK1 and IKKε, which are both located downstream of IPS-1, are essential for IFNβ production via IRF-3 phosphorylation [29]. Interestingly, the constitutive expression levels of both kinases were markedly enhanced in CHC B cells compared with those in normal B cells (fig. 5), although the downstream IRF-3 activation cascade was severely impaired, as shown in figure 3.

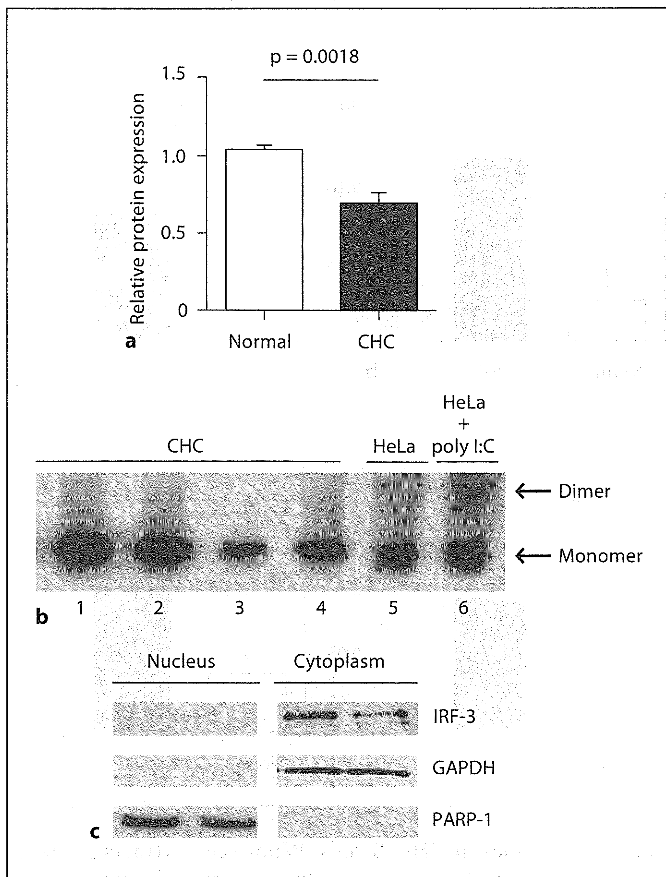

Fig. 3. Expression, dimerization and nuclear translocation of IRF-3 in CHC B cells. **a** Whole-cell extracts prepared from CD19+ B cells isolated from randomly selected normal individuals (n = 3) and CHC patients (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-IRF-3 and anti-ACTB antibodies. Relative protein expression levels of IRF-3 normalized against β-actin expression are shown. **b** Dimerization of IRF-3 in CD19+ B cells isolated from CHC patients (n = 4) were analyzed by native PAGE as described in Methods. As a positive control to detect IRF-3 dimer formation, cell extracts from HeLa cells stimulated with poly I:C were applied (lane 6). **c** Nuclear and cytoplasmic proteins were isolated from CD19+ B cells of CHC patients (n = 2) as described in Methods. Samples were subjected to SDS-PAGE and analyzed by immunoblotting using anti-IRF-3, anti-GAPDH (cytoplasmic marker protein) and anti-PARP-1 (nuclear marker protein) antibodies.

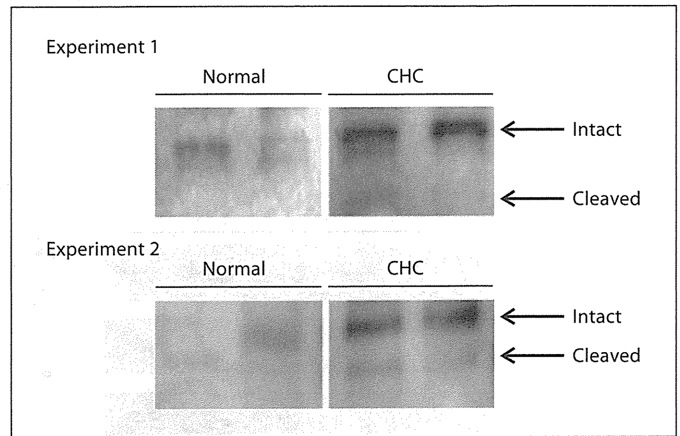

Fig. 4. Cleavage of IPS-1 in CHC B cells. Whole-cell extracts prepared from CD19+ B cells isolated from randomly selected normal individuals (n = 2) and CHC patients (n = 2) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-IPS-1 antibody. Results of 2 independent experiments are shown.

Huang et al. [30] identified a protein called SIKE that interacts with IKKε and TBK1 and has an inhibitory effect on the IRF-3 activation pathway. Yang et al. [31] demonstrated that Hsp90 was important for stabilizing TBK1 and promoting IRF-3 phosphorylation by TBK1 in response to viral infection. The gene expression levels of these two molecules in B cells were analyzed by real-time PCR, as shown in figure 6a. SIKE expression levels were significantly enhanced in CHC B cells ($p = 0.0059$), while those of Hsp90 were significantly reduced ($p = 0.001$). These results strongly suggest that the kinase activities of TBK1 and IKKε are downregulated in CHC B cells, which may be responsible for the failure in IRF-3 activation and subsequent IFNβ transcription.

The DEAD box helicase DDX3X [32] is a critical component of TBK1-dependent type I IFN induction [33, 34]. As shown in figure 6a, DDX3X expression levels were significantly reduced in CHC B cells ($p = 0.0043$). This could be just a concomitant observation; however, this result is of interest assuming that HCV has an additional mechanism by which it interferes with IRF-3 activation.

VAP-C Expression in B Cells

Human VAP subtype A (VAP-A) and subtype B (VAP-B) are essential host factors for HCV replication because they bind to both NS5A and NS5B [35]. VAP-C is a splicing variant of VAP-B that lacks two thirds of the C terminus [36, 37]; therefore, it cannot interact with VAP-A, VAP-B or NS5A. A physiological role of VAP-C was recently demonstrated by Kukihara et al. [38], who found that VAP-C inhibited the association between VAP-A/B and NS5B, thereby reducing HCV replication efficiency. Interestingly, VAP-C expression in hepatocytes was found to be negligible, which may be advantageous for

HCV replication in the liver [38]. These results prompted us to examine VAP-C expression in B cells. As shown in figure 6b, VAP-C was expressed in CD19– cells (i.e. non-B cells), but not in CD19+ B cells. Together with the defect in antiviral immune responses of CHC B cells described above, this observation further supports the notion that HCV utilizes B cells as a reservoir for persistent infection.

Discussion

HCV infection of hepatocytes has long been an implicit assumption. However, this does not necessarily imply that hepatocytes are the exclusive target for HCV infection. HCV may seek other cellular compartments as reservoirs in the event that the liver becomes unsuitable for replication, possibly due to cellular destruction caused by the host immune response and/or the development of conditions such as cirrhosis and hepatocellular carcinoma. Our previous study suggested the possibility that HCV persistently infects peripheral B cells [15]. Consequently, we were extremely interested in how HCV is able to escape host innate immunity and persistently remain in peripheral B cells. The results of this study may provide plausible answers to these questions.

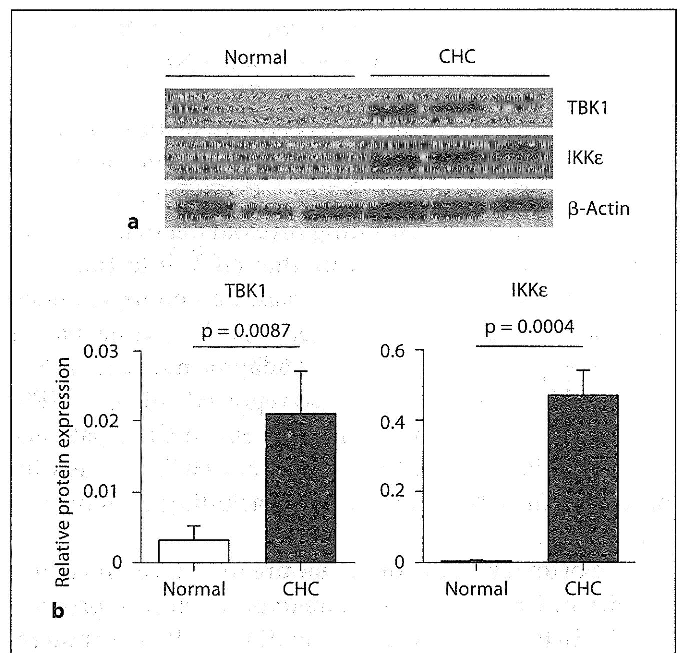

Fig. 5. Expression of TBK1 and IKKε in CD19+ B cells. **a** Whole-cell extracts prepared from CD19+ B cells isolated from normal individuals (n = 3) and CHC patients (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-TBK1, anti-IKKε and anti-ACTB antibodies. **b** Relative protein expression levels of each protein normalized against β-actin expression are shown.

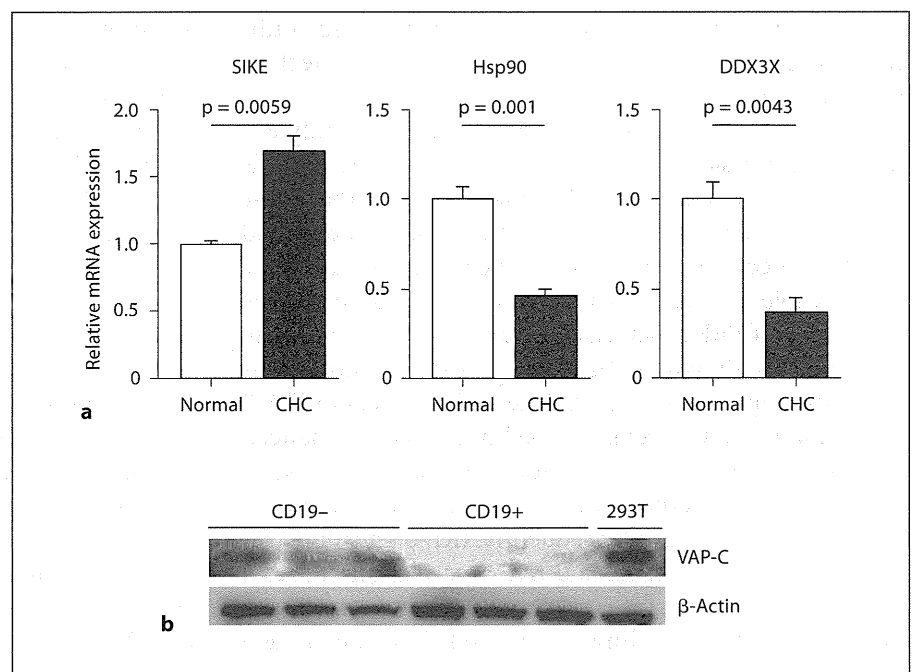

Fig. 6. Expression of SIKE, Hsp90, DDX3X and VAP-C in CD19+ B cells. **a** mRNA expression levels of SIKE, Hsp90 and DDX3X in CD19+ B cells isolated from normal individuals (n = 3–7) and CHC patients (n = 3–5) were measured in duplicate by quantitative real-time RT-PCR and normalized against those of the housekeeping gene GAPDH. mRNA expression levels in normal B cells were arbitrarily defined as 1.0. **b** Whole-cell extracts prepared from CD19– and CD19+ B cells isolated from normal individuals (n = 3) were subjected to SDS-PAGE and analyzed by immunoblotting using anti-VAP-C antibody. Whole-cell extracts from 293T cells were used as a positive control.

In this study, we observed augmented expression of RIG-I, a cytoplasmic double-stranded RNA sensor molecule, and its adaptor molecule IPS-1 in CHC B cells (fig. 2). To our knowledge, this is the first study describing the enhanced expression of a cytoplasmic HCV sensor in CHC B cells. Miyazaki et al. [39] recently reported similar findings on examining myeloid dendritic cells in CHC patients, thus suggesting that HCV infection augments the expression of a cytoplasmic double-stranded RNA sensor regardless of the cell type. The same may be true for expression of the RIG-I adaptor molecule IPS-1, because Miyazaki et al. [39] also reported enhanced IPS-1 expression in myeloid dendritic cells in CHC patients. These results support the notion that HCV triggers innate immunity by immune cells, including peripheral B cells.

The primary cause for the failure to induce innate immunity in CHC B cells appears to be a defect in promoting the IRF-3 activation cascade (fig. 3). HCV is thought to evade an innate antiviral response by the actions of the viral NS3/4A protease-helicase complex, which inhibits RIG-I signaling via proteolytic cleavage and IPS-1 inactivation [28], thereby preventing its downstream activation (i.e. IRF-3 activation). This hypothesis was essentially derived from in vitro experiments using human hepatoma cell lines such as HuH-7. In the present study, IPS-1 cleavage was analyzed for the first time in peripheral B cells of CHC patients. The results shown in figure 4 indicate only marginal cleavage of IPS-1 in CHC B cells, such that this could not be the main cause of the defect in downstream IRF-3 activation. We therefore sought additional explanations for the IRF-3 activation defect in CHC B cells.

The results shown in figure 5 indicate markedly enhanced expression of TBK1 and IKKε in CHC B cells compared with normal B cells, thus suggesting that IPS-1 signaling is mainly intact and not completely abolished by HCV infection as expected. In the present study, it was not possible to identify the mechanism underlying the expression of TBK1 and IKKε induced by HCV infection. However, as shown in figure 3, IRF-3 activation was markedly suppressed in CHC B cells. The defect in IRF-3 activation in CHC B cells may be linked to the enhanced expression of SIKE and the reduced expression of Hsp90 (fig. 6a), which together may affect the kinase activities of both TBK1 and IKKε. Consequently, IRF-3 phosphorylation (i.e. the first step of the IRF-3 activation cascade) would not be successfully executed. However, the possibilities that IPS-1 signaling is disrupted along the cascade despite the existence of the signal molecule and/or that IRF-3 activation is directly blocked by HCV proteins should also be taken into consideration.

It was recently demonstrated that the expression of DDX3X, a DEAD box RNA helicase [32], enhanced IFNβ promoter induction by TBK1/IKKε, whereas its knockdown inhibited IRF3 activation [33]. The results shown in figure 6a confirm that DDX3X expression levels were significantly downregulated in CHC B cells, which is in agreement with previous studies. However, Ariumi et al. [40] reported that DDX3X was required for HCV RNA replication, which seems contradictory to our results. We currently do not know the reasons for this discrepancy; however, the fact that in their study they used the HuH-7 hepatoma cell line, while we examined naturally occurring HCV-infected B cells, may account for these conflicting results.

The role of VAP proteins in HCV replication is another interesting aspect. HCV NS5A is associated with a range of cellular proteins, including VAP-A [41], which are involved in cell signaling pathways. VAP-B has been identified as another NS5A-binding protein by screening human libraries using the yeast two-hybrid system with NS5A as bait [35]. Both VAP-A and VAP-B are involved in HCV replication via interactions with NS5A and NS5B [35], while VAP-C inhibits the association between VAP-A/B and NS5B, which results in reduced HCV replication efficiency [38]. Therefore, the absence of VAP-C expression in B cells, similar to hepatocytes, may be favorable for HCV replication. This could be another strategy for maintaining persistent HCV infection in B cells. In fact, our recent study demonstrated that a high copy number of HCV RNA was detected in CHC B cells, but not in CHC non-B cells [15], suggesting that the absence of VAP-C expression in B cells is, at least in part, responsible for the HCV replication. Further analyses are required to examine if the forced expression of VAP-C could inhibit HCV replication in B cells.

Several lines of evidence indicate that chronic infection with HCV can induce molecular alterations in lymphocytes that may subsequently play a role in the multistep process of malignant lymphocyte transformation with the induction of clonal B cell expansion. Lymphoid cells from patients with chronic HCV overexpress the antiapoptotic protein Bcl-2 with a high incidence of *t* (14;18) translocations involving the *bcl-2* gene [42, 43]. Our recent study revealed that the expression level of activation-induced cytidine deaminase, which promotes B cell lymphomagenesis by its overexpession [44, 45], in CHC B cells was significantly increased [15]. Furthermore, enhanced expression of putative lymphomagenesis-related

genes such as cyclin D1, cyclin D2, B aggressive lymphoma gene, serine/threonine kinase 15 and galectin-3 was observed in CHC B cells [15]. In addition, expression of HCV core protein and NS3 was detected in CD19+ B cells of CHC patients [15]. HCV core protein has been demonstrated to promote immortalization in different cell lines as well as being capable of blocking c-myc-induced apoptosis [46]. NS3 has also been shown to promote oncogenic transformation and to interact with p53 and interfere with apoptosis [47]. Thus, persistent infection with HCV via the suppression of innate immunity responses in CHC B cells would cause functional disorders and lead to B cell lymphoma.

Interestingly, it has been shown that interaction between HCV E2 and CD81 on B cells triggers enhanced expression of activation-induced cytidine deaminase, which induces double-strand DNA breaks and hypermutation, specifically in the VH gene of B cells [48]. Stamataki et al. [49] demonstrated that peripheral blood B cells could bind infectious HCV in the cell strain JFH-1. Accordingly, it seems likely that mere interaction between envelope proteins of HCV and signaling receptors on the surface of B cells could generate lymphoproliferative disorders.

The difficulty in collecting an adequate number of purified B cells from CHC patients prevented us from analyzing a large sample size in each experiment, which may weaken the impact of these results. However, in the light of the fact that potentially heterogeneous patient samples were examined in this study, we believe that our considerably homogeneous results do have a certain biological impact. In addition, it may be worth noting that most CHC patients enrolled in this study (21 of 24) were infected with HCV of genotype 1b, which is prevalent in Asia. It would have been ideal to perform these experiments with different genotypes to observe if this phenomenon is noted across all genotypes or if it is specific only to genotype 1b. A study enrolling CHC patients infected with other HCV genotypes will be required in order to draw more robust conclusions, although it is very difficult to enroll such CHC subjects in Japan.

Taken together, the present results strongly suggest that HCV utilizes B cells as an extrahepatic reservoir for persistent infection. Whether the apparent suppression of innate immune responses in B cells is restricted to HCV infection or if this is a phenomenon seen in other B cell tropic viruses such as Epstein-Barr virus is currently unknown. This intriguing question could be answered by further elucidating the suppression mechanisms in CHC B cells as well as by investigating innate immune responses in Epstein-Barr virus-infected B cells in future studies.

We assume that memory B cells are the main reservoir of HCV infection because of their long life span. In support of this proposal, one of our current studies indicated that CD19+CD27+ cells (i.e. memory B cells) are recruited to the livers of CHC patients via interactions between CXCR3 expressed on CD19+CD27+ cells and IP-10 produced in the liver [Mizuochi et al., in press]. This would be a robust strategy for HCV in order to secure sites for long-lasting infection. Interestingly, a recent study by Stamataki et al. [49] indicated that HCV associated with B cells had the potential to transfect HuH-7.5 in vitro. Our results strongly suggest that such HCV transfection may occur in vivo under physiological conditions. This would offer new therapeutic insights for HCV clearance by eliminating peripheral B cells with anti-B cell antibodies and drugs such as rituximab in conjunction with a combination therapy using peginterferon and ribavirin.

In conclusion, we propose that peripheral B cells serve as a reservoir for persistent HCV infection. Based on this proposal and from a therapeutic perspective, it may be beneficial to eliminate peripheral B cells in CHC patients. Together with antiviral treatment to eliminate circulating HCV in the blood, this could lead to a synergistic effect for HCV clearance in CHC patients.

Acknowledgments

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