

**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/m <sup>2</sup>	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  $\geq 110$  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  $\geq 140$  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)

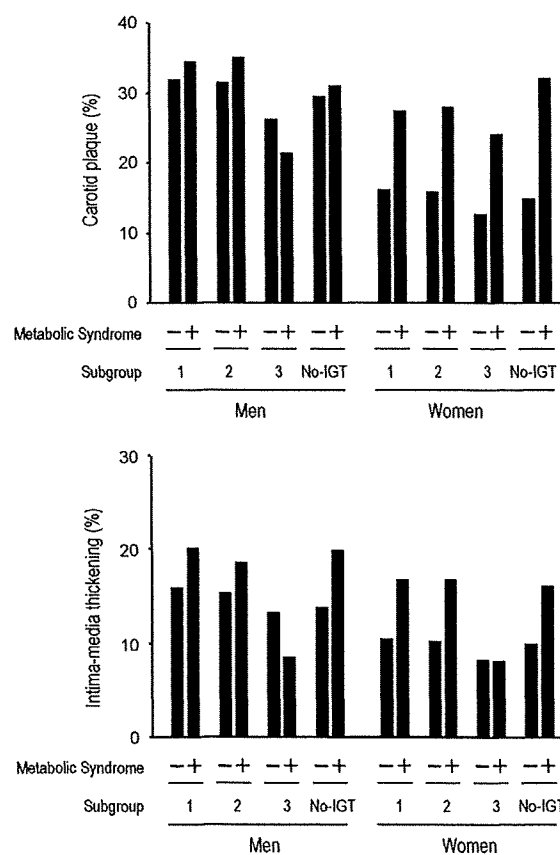


Fig. 2. Prevalence of carotid plaque and carotid intima-media thickening according to the presence or absence of metabolic syndrome in subgroups.

**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
<b>Subgroup 1</b>				
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
<b>Subgroup 2</b>				
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
<b>Subgroup 3</b>				
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  $\geq 110$  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
<b>Subgroup 1</b>				
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
<b>Subgroup 2</b>				
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
<b>Subgroup 3</b>				
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  $\geq 140/90$  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  $\geq 75$ th 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 ( $55 \pm 10$  years versus  $58 \pm 10$  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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## Steatosis, liver injury, and hepatocarcinogenesis in hepatitis C viral infection

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In addition to the link with development of hepatocellular carcinoma (HCC), hepatitis C virus (HCV) infection is associated with several hepatic and extrahepatic manifestations. A role of hepatic steatosis in the pathogenesis of chronic hepatitis C has been shown, implying hepatitis C as a metabolic disease. Furthermore, recent epidemiological studies have suggested a linkage between insulin resistance and chronic HCV infection. In addition to the data indicating the presence of lipid metabolism disturbance and insulin resistance in the cohort of chronic hepatitis C patients, we found evidence showing the association between these two conditions and HCV infection using mice transgenic for the HCV core gene. These mice develop HCC late in life after the phase of hepatic steatosis and insulin resistance. The nonappearance of both steatosis and HCC in HCV core gene transgenic mice that are null for the proteasome activator 28 $\gamma$  implies a close relationship between lipid metabolism disturbance and hepatocarcinogenesis. Also, the core protein is shown to bind with retinoid X receptor (RXR)- $\alpha$ , resulting in the upregulation of some lipid metabolism enzymes, including cellular retinol binding protein II and acyl-CoA oxidase. In addition, the persistent activation of peroxisome proliferator activated receptor (PPAR)- $\alpha$  has recently been found in the liver of HCV core gene transgenic mice, yielding dramatic changes in lipid metabolism and hepatocyte proliferation, including HCC development. These results would provide a clue for further understanding of the role of lipid metabolism in pathogenesis of HCV infection, including liver injury and hepatocarcinogenesis.

**Key words:** lipid metabolism, transgenic mouse, oxidative stress, intracellular signal transduction, peroxisome proliferator activated receptor

### Introduction

Worldwide, approximately 170 million people are persistently infected with hepatitis C virus (HCV), which induces a spectrum of chronic liver diseases from chronic hepatitis to cirrhosis and, eventually, to hepatocellular carcinoma (HCC).<sup>1</sup> HCV has been given increasing attention because of its wide and deep penetration in the community, tied with a very high incidence of HCC in persistent HCV infection. Once liver cirrhosis is established in hosts persistently infected with HCV, HCC develops at a yearly rate of approximately 7%,<sup>2</sup> resulting in the development of HCC in nearly 90% of HCV-associated cirrhotic patients in 15 years. In addition, the outstanding features in the mode of hepatocarcinogenesis in HCV infection, i.e., development of HCC in a multicentric fashion and at a very high incidence, are not common in other malignancies except for hereditary cancers such as familial polyposis of the colon. Knowledge of the mechanism underlying HCC development in persistent HCV infection, therefore, is imminently required for the prevention of HCC.

In addition to the link with development of HCC, HCV infection is associated with several hepatic and extrahepatic manifestations.<sup>3</sup> A role of hepatic steatosis in the pathogenesis of chronic hepatitis C has been shown, implicating hepatitis C as a metabolic disease.<sup>4</sup> Moreover, recent epidemiological studies have suggested a linkage between insulin resistance and chronic HCV infection.<sup>5</sup> In addition to the epidemiological data indicating the presence of lipid metabolism disturbance and insulin resistance in the cohort of chronic hepatitis C patients, detailed analyses on the relationship between



### **Oxidative stress overproduction and intracellular signaling pathway activation are the major pathways in the core-induced liver pathology**

It is difficult to elucidate the mechanism underlying the development of HCC, even for our simple model in which only the core protein is expressed in otherwise normal liver. There is a notable feature in the localization of the core protein in hepatocytes; while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.<sup>10,17</sup> On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were thoroughly investigated.

One effect of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver. This finding reflects a state of overproduction of reactive oxygen species (ROS) in the liver,<sup>18</sup> or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.<sup>19,20</sup> The overproduction of oxidative stress results in the generation of deletions in mitochondrial and nuclear DNA, an indicator of genetic damage. In addition, analysis of antioxidant system revealed that some antioxidative molecules are not increased despite the overproduction of ROS in the liver of core gene transgenic mice: hemeoxygenase-1 and glutathione peroxidase are not augmented whereas catalase and glutathione S-transferase levels are increased and enhanced by iron overloading (Moriya et al., manuscript in preparation). These results suggest that HCV core protein not only induces overproduction of ROS but also attenuates some of the antioxidant systems, which may explain the mechanism underlying the production of a strong oxidative stress in HCV infection compared to other forms of hepatitis.

In the absence of inflammation, thus, the core protein induces oxidative stress overproduction, which may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation were added to the liver with the HCV core protein, the production of oxidative stress would be escalated to an extent that can no longer be scavenged by a physiological antagonistic system. This idea suggests that the inflammation in chronic HCV infection would have a characteristic difference in its quality from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to the mitochondrial dysfunction.<sup>10,19</sup> The dysfunction of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.<sup>21</sup>

Other pathways in hepatocarcinogenesis would be the alteration of the expression of cellular genes and modulation of intracellular signaling pathways. For example, tumor necrosis factor (TNF)- $\alpha$  and interleukin-1 $\beta$  have been found to be transcriptionally activated.<sup>22</sup> The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model before HCC development, only the JNK route is activated. Downstream of JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced.<sup>20,21</sup> At far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives an advantage for cell proliferation to the hepatocytes. Interestingly, we found recently that a protein interacting with the core protein, proteasome activator 28 $\gamma$  (PA28 $\gamma$ ), is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance, and HCC.<sup>23,24</sup>

### **Lipid metabolism and HCV infection**

Steatosis is frequently observed in chronic hepatitis C patients and is significantly associated with increased fibrosis and progression rate of fibrosis of the liver.<sup>6</sup> A comprehensive analysis of gene expression in the liver of core gene transgenic mice, in which steatosis develops from early in life, revealed that a number of genes related to lipid metabolism are significantly upregulated or downregulated (Table 1).

The composition of fatty acids that are accumulated in the liver of core gene transgenic mice is different from that in fatty liver resulting from simple obesity. Carbon-18 monounsaturated fatty acids (C18:1) such as oleic or vaccenic acids are significantly increased; this is also the case in the comparison of liver tissues from hepatitis C patients and patients with simple fatty liver due to obesity.<sup>20</sup> The mechanism of steatogenesis in hepatitis C was investigated using this mouse model. There are at least three pathways for the development of steatosis. One is the frequent presence of insulin resistance in hepatitis C patients as well as in the core gene transgenic mice, which occurs through the inhibition of tyrosine phosphorylation of insulin receptor substrate (IRS)-1.<sup>25</sup> Insulin resistance increases the peripheral release and hepatic uptake of fatty acids, resulting in an accumulation of lipid in the liver. The second pathway is the suppression of the activity of

**Table 1.** Cellular genes differentially expressed in hepatitis C virus (HCV) core transgenic mouse liver

	Upregulated	Downregulated
Lipid metabolism	NPC1 Catalase Very long chain acyl-CoA dehydrogenase Carboxylesterase selenoprotein P Carbonic anhydrase Adipose differentiation-related protein Bilirubin/phenol family UDP glucuronosyltransferase	Stearoyl-CoA desaturase Sterol-carrier protein X Alpha-enolase carnitine acetyltransferase Gal beta 1,4(3) GlcNAc alpha 2,3-sialyltransferase Very long chain acyl-CoA synthetase Liver transferrin 4-Hydroxyphenylpyruvate dioxygenase LAF1 transketolase s-Adenosylmethionine synthetase Apolipoprotein A-II Human guanine nucleotide regulatory protein Alpha-fetoprotein Retinol binding protein
Transcription and cell proliferation	Int-6 GCN5L1 <i>H. sapiens</i> 8.2k-Da differentiation factor USF1 Initiation factor eIF-4A1 Human elongation factor-1-delta Sui1	
Inflammation	Alpha-1 protease inhibitor 3 Hemopexin	Alpha-2-macroglobulin LMW prekininogen Complement component C3 AHSG(alpha 2 HS-glycoprotein) homologue Vitronectin Epithelin 1 and 2 Murnoglobulin
Others	Microvascular endothelial differentiation gene 1 Diazepam-binding inhibitor Argininosuccinate synthetase Skeletal muscle alpha-tropomyosin Ampd3 gene DNA-binding protein	

microsomal triglyceride transfer protein (MTP) by HCV core protein<sup>26</sup>; this inhibits the secretion of very low density protein (VLDL) from the liver, yielding an increase of triglycerides in the liver. The last pathway involves sterol regulatory element-binding protein (SREBP)-1c, which regulates the production of triglycerides and phospholipids. In HCV core gene transgenic mice, SREBP-1c is activated, whereas neither SREBP-2 nor SREBP-1a is upregulated.<sup>27</sup>

In relation to lipid metabolism, the core protein has also been found to interact with retinoid X receptor (RXR)- $\alpha$ .<sup>28</sup> RXR- $\alpha$  is one of the nuclear receptors, which forms a homodimer or heterodimers with other nuclear receptors, including PPAR (peroxisome proliferator-activated receptor)- $\alpha$ , and plays a pivotal role in the regulation of the expression of genes relating to lipid metabolism, cell differentiation, and proliferation. In fact, the core protein of HCV activates genes that have an RXR- $\alpha$ -responsive element as well as those with a PPAR- $\alpha$ -responsive element, both in mice and in cultured cells.<sup>28</sup> Based on these results, we, then, examined the expression and function of PPAR- $\alpha$  in the liver of core gene transgenic mice.

#### PPAR- $\alpha$ activation in HCV-associated hepatocarcinogenesis

PPAR- $\alpha$ , one of the PPAR genes, plays a central role as a heterodimer with RXR- $\alpha$  in regulating fatty acid transport and catabolism. It is also known as a molecular target for lipid-lowering fibrate drugs.<sup>29</sup> On the other hand, prolonged administration of PPAR- $\alpha$  agonists causes HCC in rodents. Currently, there is little evidence that the low-affinity fibrate ligands are associated with human cancers, but it is possible that chronic activation of high-affinity ligands could be carcinogenic in humans.<sup>29</sup>

The level of PPAR- $\alpha$  protein was increased in the liver of core gene transgenic mice as early as 9 months of age. PPAR- $\alpha$  protein is accumulated with age in the nuclei of hepatocytes together with cyclin D1 protein. However, the level of PPAR- $\alpha$  mRNA was not increased at any age. By pulse-chase experiment, the stability of nuclear PPAR- $\alpha$  was increased in the presence of the core protein. In line with the increase of PPAR- $\alpha$  protein, target genes of PPAR- $\alpha$  were activated in the liver of core gene transgenic mice; these genes include

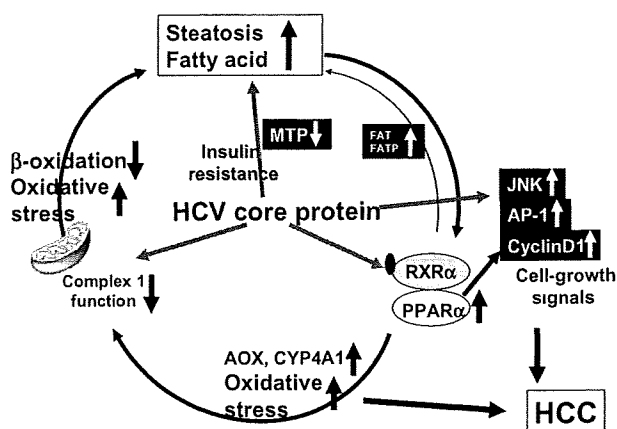
cyclin D1, cyclin-dependent kinase (CDK)-4, acy-CoA oxidase, and peroxisome thiolase.<sup>30</sup> However, in general, the activation of PPAR- $\alpha$  leads to improvement but not aggravation of steatosis. Then, what is the function of PPAR- $\alpha$  activation that is observed in the core gene transgenic mice?

To clarify the role of PPAR- $\alpha$  activation in pathogenesis of steatosis and HCC, we mated a core gene transgenic mouse with a PPAR- $\alpha$  knockout (KO) mouse and studied the phenotype. PPAR- $\alpha$  KO mice have reduced expression of target genes of PPAR- $\alpha$ , and have mild steatosis in the liver, as expected.<sup>31</sup> It was unanticipated, however, that steatosis was absent in PPAR- $\alpha$ -null or -heterozygous core gene transgenic mice but present in PPAR- $\alpha$ -intact core gene transgenic mice at the age of 9 or 24 months.<sup>30</sup> 8-Hydroxy deoxyguanosine (8-OHdG) and peroxy lipids, both of which are markers for oxidative stress, were decreased in PPAR- $\alpha$  KO core gene transgenic mice. Mitochondrial dysfunction in the core gene transgenic mice, which contributes to overproduction of oxidative stress,<sup>19</sup> was also improved in PPAR- $\alpha$  KO core gene transgenic mice.

Finally, PPAR- $\alpha$  KO core gene transgenic mice did not develop HCC at the age of 24 months, whereas about one-third of PPAR- $\alpha$ -intact core gene transgenic mice did. It should be noted that core gene transgenic mice that are heterozygous for the PPAR- $\alpha$  gene also did not develop HCC.<sup>32</sup> When clofibrate, a peroxisome proliferator, was administered for 24 months to PPAR- $\alpha$ -heterozygous mice, either with or without the core gene, HCC developed in a higher rate in the core gene (+) mice with greater PPAR- $\alpha$  activation. It should be noted that steatosis was present only in core gene (+) PPAR- $\alpha$ -heterozygous mice. In summary, steatosis and HCC developed in PPAR- $\alpha$ -intact but not in PPAR- $\alpha$ -heterozygous or PPAR- $\alpha$ -null core gene transgenic mice, indicating that not the presence but the persistent activation of PPAR- $\alpha$  would be important in hepatocarcinogenesis by HCV core protein. In general, PPAR- $\alpha$  acts to ameliorate steatosis, but with the presence of mitochondrial dysfunction, which is also provoked by the core protein, the core-activated PPAR- $\alpha$  may exacerbate steatosis. Persistent activation of PPAR- $\alpha$  with "strong" ligands such as the core protein of HCV could be carcinogenic in humans, although the low-affinity fibrate ligands are not likely associated with human cancers.

### HCV core protein causes "fatty acid spiral"

Figure 2 illustrates our current hypothesis for the role of lipid metabolism in HCV-associated hepatocarcinogenesis. Immune-mediated inflammation should also play a pivotal role in hepatocarcinogenesis in HCV



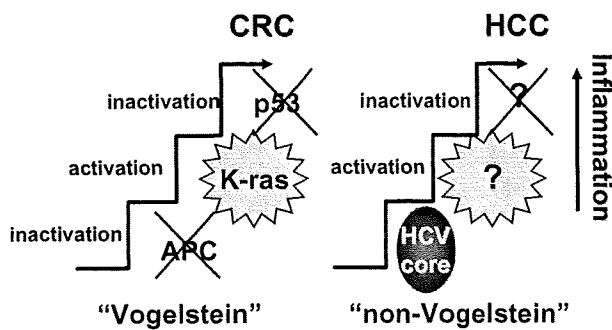
**Fig. 2.** "Fatty acid spiral" by HCV core protein. In HCV infection, the core protein induces steatosis via several pathways, leading to "fatty acid spiral" in the presence of the mitochondrial complex 1 dysfunction and PPAR- $\alpha$  activation, both of which are also caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling. *HCV*, hepatitis C virus; *HCC*, hepatocellular carcinoma; *ROS*, reactive oxygen species; *JNK*, c-Jun N-terminal kinase; *ERK*, extracellular signal-regulated kinase; *AP-1*, activating protein-1; *RXR- $\alpha$* , retinoid X receptor- $\alpha$ ; *PPAR- $\alpha$* , peroxisome proliferator activated receptor- $\alpha$ ; *AOX*, acyl-CoA oxidase; *CYP*, cytochrome P450; *MTP*, microsomal triglyceride transfer protein; *FAT*, fatty acid translocase; fatty acid transport protein

infection. However, in HCV infection, the core protein induces steatosis through the aforementioned pathways, leading to "fatty acid spiral" in the presence of the mitochondrial complex 1 dysfunction and PPAR- $\alpha$  activation, both of which are caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical-type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling.

### The HCV protein may allow some steps in multistep hepatocarcinogenesis to be skipped

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein in vivo; thus, HCV would be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the theory outlined by Kinzler and Vogelstein<sup>33</sup> has gained wide popularity. They have proposed that the





**Fig. 3.** Mechanism of HCV-associated hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of HCC in the presence of the core protein. The overall effects achieved by the expression of the core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations required for carcinogenesis. By considering such a “non-Vogelstein-type” process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers

development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They have deduced that mutations in the APC gene for inactivation, those in *K-ras* for activation, and those in the *p53* gene for inactivation accumulate, which cooperate toward the development of colorectal cancer.<sup>33</sup> Their theory has been extended to the carcinogenesis of other cancers as well, called “Vogelstein-type” carcinogenesis (Fig. 3).

On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a different mechanism for hepatocarcinogenesis in HCV infection. We do allow multistages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute (see Fig. 3). The overall effect achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations required for carcinogenesis.

By considering such a “non-Vogelstein-type” process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers.<sup>34</sup> Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence. Our theory may also give an account of the nonmetastatic and multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

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

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

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

## Introduction

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

2006; Huang *et al.*, 2005; Linke *et al.*, 2007; Liu *et al.*, 2006; Qian *et al.*, 2006).

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

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

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

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

## Experimental

### Materials and Methods

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

### Affinity Columns

The prepacked IgY-R7 Spin Column and Seppro®-IgY12 were obtained from Beckman Coluter (Fullerton, CA, USA) and GenWay Biotech (San Diego, CA, USA), respectively. The IgY-R7 Spin Column (1.2 mL spin column) removes seven high-abundance proteins in rodent plasma (albumin, IgG,  $\alpha$ 2-antitrypsin, IgM, transferrin, haptoglobin and fibrinogen) and utilizes centrifugation as the force for affinity separation. The column is said to be reusable 100 times under proper conditions. The Seppro®-IgY12 column is optimized for human plasma and removes 12 high-abundance proteins (IgA,  $\alpha$ 1-acid glycoprotein,  $\alpha$ 2-macroglobin, apolipoproteins A-I and apolipoproteins A-II besides the above seven proteins). This column is used with the high-throughput automated proteomic sample processing instrument (Magtration System SA-1; Precision System Science, Chiba, Japan) and is said to be able to be used 30 times. Both companies are corporate partners for the exclusive marketing of the IgY microbeads technology, and both column materials are the same except for recognized animal species to the IgY.

### Plasma Samples

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

### Treatment of Mouse Plasma with the IgY-R7 Spin Column

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

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

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

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

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

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

to 15  $\mu\text{L}$  with a Microcon YM-3 device. A series cycle including the sample loading–washing–eluting–neutralization finished in 65 min.

#### FD-LC-MS/MS Conditions

Each FD condition was optimized for the protein standards and for plasma samples in order to obtain the highest peak on the chromatograms. For the protein standards, a 2.5  $\mu\text{L}$  aliquot of the flow-through fraction was mixed with 30  $\mu\text{L}$  of a mixture of 0.83 mM TCEP, 3.3 mM  $\text{Na}_2\text{EDTA}$  and 16.6 mM CHAPS in the pH 8.7 buffer solution, 12.5  $\mu\text{L}$  of the buffer solution and 5.0  $\mu\text{L}$  of 8.0 mM DAABD-Cl in acetonitrile. The mixture was reacted at 50°C for 5.0 min, and the reaction was stopped with 1.5  $\mu\text{L}$  of 20% TFA. A 20  $\mu\text{L}$  aliquot of the above reaction mixture was injected to the HPLC system (Hitachi L-2000 series; Hitachi Instruments, Tokyo, Japan) using a column of Intradra WP-RP (30 nm pore size, 250  $\times$  4.6 mm i.d., Imtakt Co, Kyoto, Japan) at 60°C with a flow rate of 0.55 mL/min. The eluent (A) and eluent (B) were water–acetonitrile–TFA (90:10:0.15, v/v/v) and water–acetonitrile–TFA (30:70:0.05, v/v/v), respectively. The gradient condition was established from 5 to 100% eluent (B) over a period of 60 min. For mouse and control plasma samples, a 6.0  $\mu\text{L}$  aliquot of the flow-through fraction was mixed with 30  $\mu\text{L}$  of the above mixture of TCEP,  $\text{Na}_2\text{EDTA}$  and CHAPS, 10  $\mu\text{L}$  of the buffer solution and 4.0  $\mu\text{L}$  of 825 mM DAABD-Cl in dioxane. After the FD reaction (50°C for 5.0 min), the reaction was stopped with 2.0  $\mu\text{L}$  of 20% TFA. An aliquot (10  $\mu\text{L}$ ) of the reaction mixture was injected, and the longer column (Intradra WP-RP 30 nm pore size, 500  $\times$  4.6 mm i.d., Imtakt Co) together with a precolumn (Intradra WP-RP 30 nm pore size, 5.0  $\times$  2.0 mm i.d., Imtakt Co) at 60°C was adopted with a flow rate of 0.55 mL/min on the HPLC system. The mobile phases consisted of water–acetonitrile–TFA (A) 90:10:0.15 and (B) 30:70:0.05. Mobile phase (C) was the same as (A), except with 0.05% TFA. The gradient condition is described in Fig. 1. Fluorescence detection was carried out at 395 and 505 nm for the excitation and emission wavelengths, respectively. The peak height of each protein peak obtained from the HPLC chromatograms was calculated by HITACHI EZChrom Elite™ Chromatography Data System (Hitachi Instruments) and the identification of the standard proteins was accomplished according to the previous report (Ichibangase *et al.*, 2007).

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

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

For SEM analysis, the column materials were diluted with phosphate buffer (300 mOsm) and fixed with fixative (2.5% glutaraldehyde in PBS, pH 7.0) for 10 min. The fixed sample was captured on the filter (SEM-Pore: 0.6  $\mu\text{m}$  i.d.; 10  $\mu\text{m}$ ) and washed with the buffer. After the osmium-fix (2%  $\text{OsO}_4$ ) and a brief rinse with the fixative solutions, specimens were dehydrated in a series of graded ethanol (30–100%). The immersed specimens in ethanol were replaced with isoamyl phenylacetate and subjected to critical point drying. The dried samples were coated with osmium using a plasma coater (OPC80N, Jeol). Images were acquired using a Jeol JSM-7401F in normal SEM mode. For the low power

microscope images for the whole picture of a material, a lower electron image (LEI) was applied.

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

## Results and Discussion

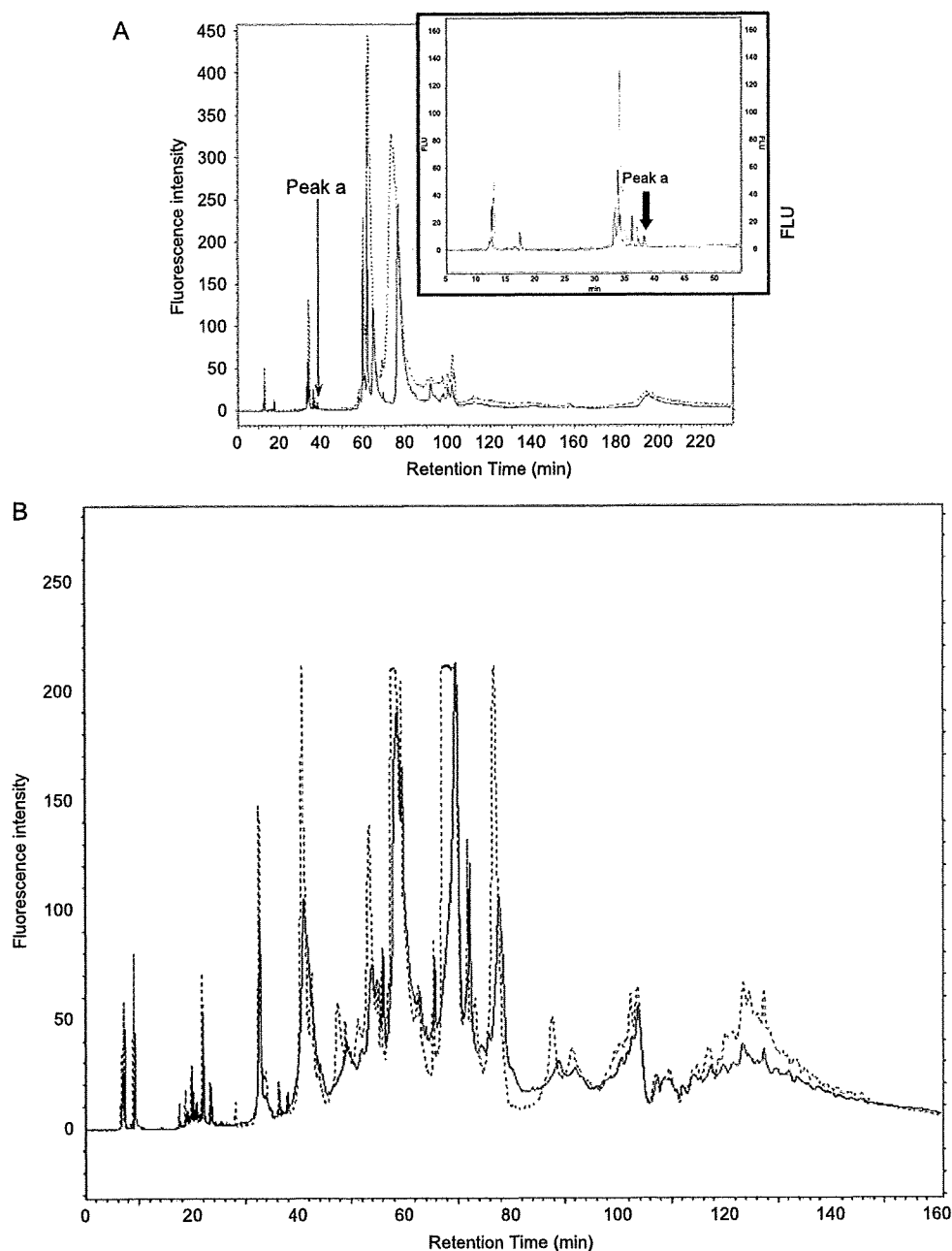
#### Quantitative Functional Changes of the IgY-R7 Spin Column after a Number of Treatments with Mouse Plasma

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

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

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

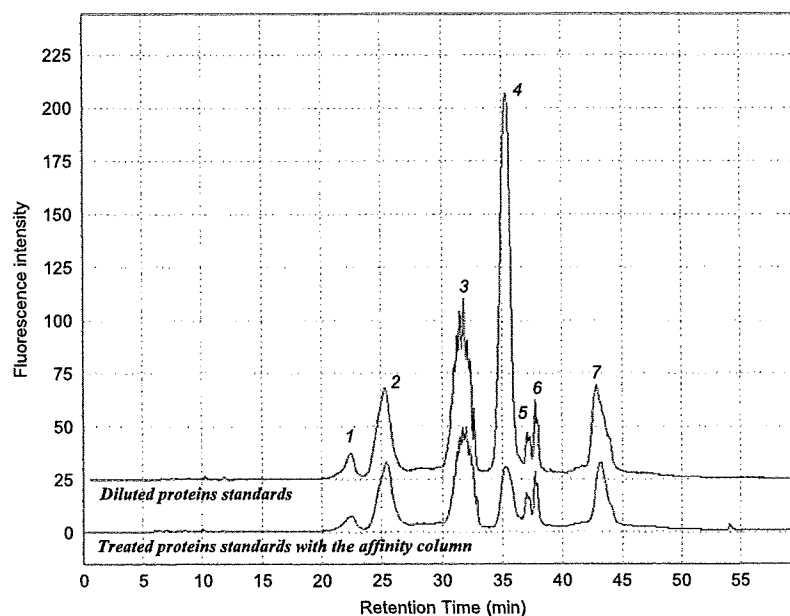
At first, to eliminate the non-specific adsorption to the affinity column, the protein standards mixture was divided into two parts and one was subjected into the untreated affinity column, and the flow-through fraction was derivatized with the fluorogenic reagent, DAABD-Cl, and separated by the HPLC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



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

database-searching algorithm. Table 2 shows the adsorption ratio of the proteins to the untreated column and the identified protein names. The protein names of peak 1 and 2 could not be identified since these peaks were peptides coexisting in the protein standards. Also, carbonic anhydrase and ovalbumin could not be detected. Since carbonic anhydrase has only two cysteine

residue for labeling with DAABD-Cl, its detection might be difficult. The reason for the undetected ovalbumin was not clear. Since this study was aimed at investigating the changes in the adsorption of the specific and non-specific proteins using the column, this issue was not examined further. Consequently, although the affinity column was able to efficiently remove bovine serum



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

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

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

<sup>a</sup>3, <sup>b</sup>5: Most highest peak

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

Next, the time series changes of the specific and non-specific adsorption of proteins to the column were investigated. The protein standards mixture was treated with the column periodically after 10 and 20 cycles of treatment of the control plasma sample. The relation of the changes of the protein standards adsorption to the number of uses of the affinity column is shown in Fig. 3(A). The specific adsorption of BSA decreased with an increase in the number of times the column was used. However, the non-specific adsorption for lysozyme, trypsin inhibitor and phosphorylase B reached a maximum at 11 cycles and decreased at 21 cycles. Since the affinity column was optimized for human plasma, the absorption of BSA for the column might be weaker than for plasma albumin. However, the adsorp-

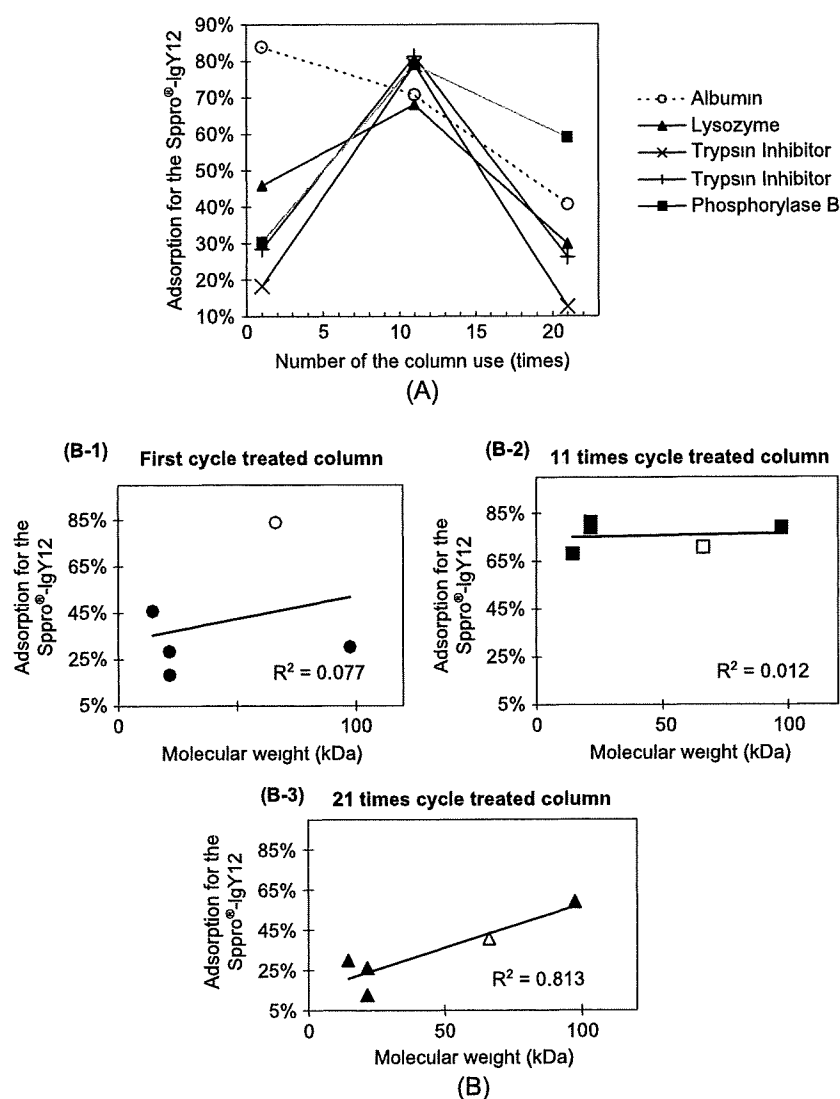
tion of albumin in control plasma also decreased with an increase in the number of times the column was used (data not shown). Moreover, since the slopes of decrease differed among the protein standards, the correlation of each adsorption with molecular weight of each protein was calculated [Fig. 3(B)]. The open dots show the value of BSA in Fig. 3(B). The correlation coefficient value was the closest to 1.00 ( $R^2 = 0.813$ ) for 21 cycles [Fig. 3(B-3)], demonstrating that the adsorption ability of the column does not depend any longer on the affinity of the antibody but on the hydrophobicity of the protein. In contrast, the correlation of the values obtained from the first cycle [Fig. 3(B-1);  $R^2 = 0.077$ ] was not fairly observed between the adsorption ability and the hydrophobicity, and the value for BSA was apart from those for other proteins. Therefore, BSA was specifically removed as compared with the other protein standards by the immunoaffinity adsorption. Also, as shown in Fig. 3(B-2), the result obtained from 11 cycles ( $R^2 = 0.012$ ) demonstrated that all proteins bound to the surface of the affinity column materials equally. Therefore, the present data demonstrates that the quantitative changes of the adsorption for the affinity column appear before the limited use of the column (30 times in the manufacturer's instructions).

#### Understanding the State of the Plasma-treated Column Materials

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

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

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



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

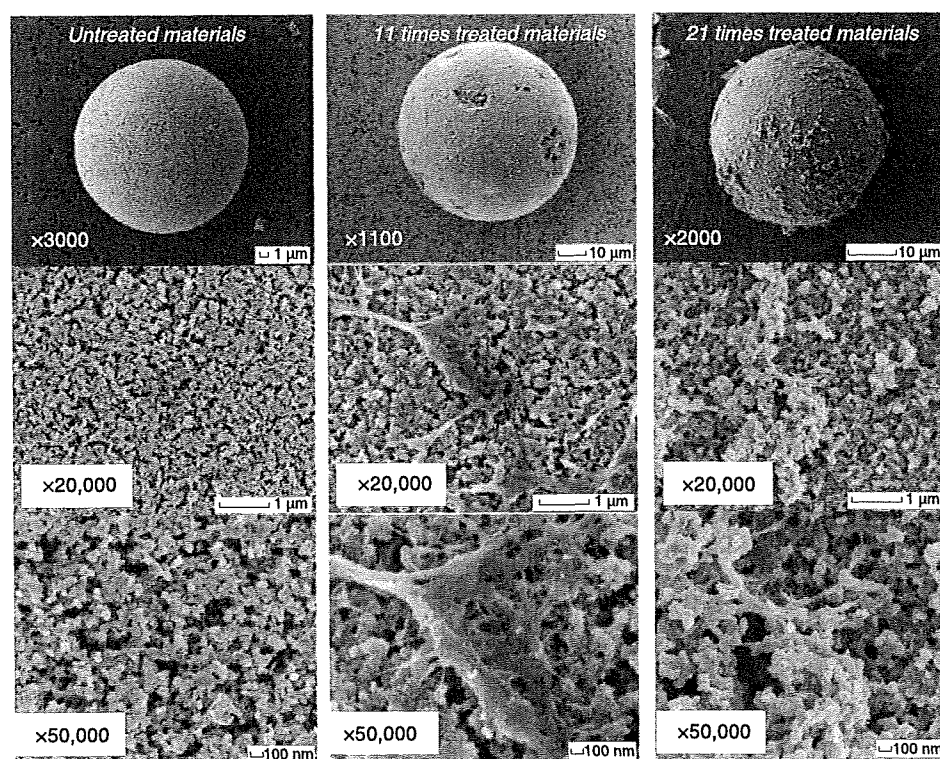
direct laser irradiation of the materials could affect the instrument, the positions to be irradiated were the points of the existence of many matrices on a few column materials. Although several peaks existed of less than 70,000  $m/z$  in each mass spectrum, the higher molecular weight peaks (7266,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

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

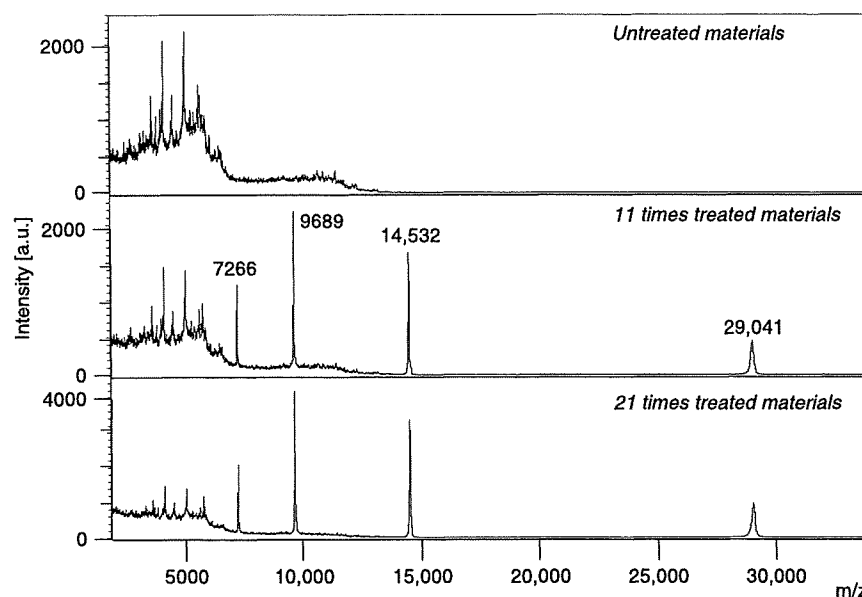
## Conclusions

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





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



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

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

of the compounds might lead to the improvement of the affinity column technique and contribute to progress in quantitative plasma proteomics.

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

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

### Acknowledgements

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# ヘルペスウイルス学のエッセンス

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現在8つのヒトヘルペスウイルスが同定されているが、最初に分離された単純疱疹ヘルペスウイルスから考えると、その研究の歴史は90年に及ぶ。その結果、慢性感染症に関わる本ウイルスの実態が少しずつ分かりつつある。ひとたびヒトに侵入すると、潜伏感染状態を樹立し、感染細胞内でゲノムの一部として振る舞い、子孫存続の機会を待ち続ける。その過程で宿主との相互作用の結果、多彩な病態に関与している。どのような機構でそれぞれの病態が惹起されるのか、いまだ不明の点も多く、今後の研究展開が期待される。そして研究成果に基づいた、より効果的なウイルス制御法が開発されることを願う。

**Key Words** : ヘルペスウイルス, 潜伏感染,  $\alpha$ ヘルペスウイルス,  $\beta$ ヘルペスウイルス,  $\gamma$ ヘルペスウイルス

## I はじめに

“Herpes”とはギリシャ語で“這う”を意味する言葉で、這うようにして広がる皮膚疾患に用いられていたと思われる。口唇ヘルペスの疾患としての認識は紀元前100年くらいにはあったと思われるが、20世紀初期に口唇ヘルペスからウイルスが分離され、ヘルペスウイルス属の確立に至った。この時点で研究が開始されたと考え、ヘルペスウイルス研究は90年の歴史を持つ。

ヘルペスウイルスはヒトに潜伏感染するDNAウイルスの代表格である。ヒト染色体には組み込まれないepisome(ウイルスに特化してviromeという言い方も使われ始めている)として機能し、水平感染によってヒト界に伝播・維持されている。本稿では8つのヘルペスウイルス<sup>1)</sup>について、その特徴を総論的に解説したい。

## II 疫学

ヒトに感染するヘルペスウイルスの感染率は地域性・民族により若干の差があるものと考えられ

るが、本ウイルスの感染率は群を抜いて高い。水疱瘡ウイルス(varicella zoster virus: VZV)<sup>2)</sup>、ヒトヘルペスウイルス6(human herpesvirus 6: HHV-6)<sup>3)</sup>、ヒトヘルペスウイルス7(human herpesvirus 7: HHV-7)<sup>3)</sup>、サイトメガロウイルス(cytomegalovirus: CMV)<sup>4)</sup>やエプスタイン・バーウイルス(Epstein-Barr virus: EBV)<sup>5)</sup>は、ヒト成人での感染率は80%以上であると考えられている。単純疱疹ヘルペスウイルス1型(herpes simplex virus: HSV-1)<sup>6)</sup>で50~70%、単純疱疹ヘルペスウイルス2型(herpes simplex virus 2: HSV-2)<sup>6)</sup>が20~50%、カポジ肉腫関連ヘルペスウイルス(Kaposi's sarcoma-associated herpesvirus: KSHV)<sup>7)</sup>が2~60%と言われている。KSHVの感染率は地域差が大きいと思われるが、ポリオーマBK(polyoma virus BK, 72~98%)、ポリオーマJC(polyoma JC, 72~98%)、アデノ随伴ウイルス(adenovirus-associated virus: AAV, 60~90%)を除くと、ヘルペスウイルスはヒト界に広く蔓延し潜伏感染状態を樹立する代表格で、潜伏感染した細胞

The essence of herpesvirology

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においてゲノムの一部として振る舞うことになる<sup>8)</sup>。

### III 感染経路・様式

8つのヘルペスウイルスの感染様式にはそれ程違いはなく、基本的には salivary shedding (唾液拡散)、感染局所との粘膜接触感染がおもな感染様式であると思われる。HSV-1, CMV, EBV, HHV-6 および HHV-7, KSHV は唾液発散による感染が主流で、幼少期に、特に両親との緊密な接触により感染が成立するものと思われる。この時期の感染はほとんど不顕性で、そのまま潜伏感染状態となりウイルス保因者となる。

一方、VZV は一般に幼児期の流行時期に感染を受ける。HSV-2 はウイルス学的には HSV-1 に酷似するが、感染組織は性器を中心とすることが多く、また KSHV も男性同性愛者にカポジ肉腫 (Kaposi's sarcoma : KS) が多くみられることから、性行為感染症としての側面も持つ。

### IV 潜伏感染と溶解感染

ヘルペスウイルスの最大の特徴のひとつは潜伏感染を樹立することであり、慢性感染症を惹き起こす多くのウイルスが持続感染状態になることと対比される。潜伏感染と持続感染の違いは、ウイルスを産生し続けているかどうかである。個体の中でウイルスを産生し続けながら感染状態を維持する持続感染では、免疫監視機構をいかに調節して回避するかが感染存続の鍵となる。一方、潜伏感染はウイルス自体がその遺伝子発現を極限状態まで落として、いわばステルス (stealth) 状態にな

ることで免疫監視機構を回避し、娘ウイルスを産生して子孫を残す機会を待っていると考えられる。

潜伏感染を樹立する組織・細胞は各ヘルペスウイルスにより異なる。一般的には、 $\alpha$ ヘルペスウイルス (HSV-1 および HSV-2, VZV) は感覚神経系の神経節神経細胞に、 $\beta$ ヘルペスウイルス (CMV, HHV-6 および HHV-7) は血球系に、 $\gamma$ ヘルペスウイルス (EBV, KSHV) はBリンパ球に潜伏感染するとされている。

娘ウイルスを産生する生活環を溶解感染と呼ぶ。本生活環では100個前後存在するウイルス遺伝子すべてが順序よく発現して、最終的には爆発的な娘ウイルス産生に至る。潜伏感染状態に何らかの刺激が入ると、溶解感染が誘導 (再活性化) されることがある。HSV-1 では日光 (UV) など、EBV では IgG (免疫グロブリンG) の結合、KSHV ではある種のサイトカインやフォルボールエステルなど、薬剤が誘導の起因になる。

### V ウイルス粒子構造・ウイルスゲノム・遺伝子

ヘルペスウイルス粒子は、外側に脂質二重膜からなる膜粒子 (envelope : 120nm 前後)、内部にキャプシッド (capsid : 70nm 前後) を内包し、その内部にゲノムがある。粒子としての最大の特徴は、膜粒子とキャプシッドのあいだにテグメント (tegument) という構造があることである。

ヘルペスウイルスは120～230kbの直鎖状二本鎖DNAゲノムを持つ。直鎖状のゲノムは感染後に末端で結合し、環状ゲノムとなる。基本的なエレメントとしては、ユニーク領域、反復配列領

VZV (varicella zoster virus)

HHV-6 (human herpesvirus 6, ヒトヘルペスウイルス6)

HHV-7 (human herpesvirus 7, ヒトヘルペスウイルス7)

CMV (cytomegalovirus, サイトメガトウイルス)

EBV (Epstein-Barr virus, エプスタイン・バーウイルス)

HSV-1 (herpes simplex virus, 単純疱疹ヘルペスウイルス1型)

HSV-2 (herpes simplex virus 2; 単純疱疹ヘルペスウイルス2型)

KSHV (Kaposi's sarcoma-associated herpesvirus, カポジ肉腫関連ヘルペスウイルス)

AAV (ade-no-associated virus, アデノ随伴ウイルス) KS (Kaposi's sarcoma, カポジ肉腫)

IgG (免疫グロブリンG)