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A Proteomics Method Revealing Disease-Related Proteins in Livers of Hepatitis-Infected Mouse Model

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In this post-genome era, a sensitive quantitative method is required for differential profiling analyses of clinical proteomes to understand the disease progress. Here, we adopt the FD-LC−MS/MS method, consisting of fluorogenic derivatization (FD), separation by liquid chromatography (LC), and identification by LC-tandem mass spectrometry (MS/MS), to reveal disease-related proteins in livers of hepatocarcinogenesis in transgenic (Tg) and non-transgenic (NTg) mice at three developmental stages. After 6 months, the expression of apoptosis-related proteins is suppressed. After 12 months, proteins related to respiration, the electron-transfer system, and anti-oxidation are significantly up-regulated. After 16 months, proteins related to defense, β-oxidation, and apoptosis are significantly suppressed. This fluctuating expression of proteins could explain the progression of hepatocarcinogenesis. The method would be useful for clinical proteomics analysis because of its high resolution, sensitivity, and reproducibility.

Keywords: DAABD-Cl • FD-LC−MS/MS method • core protein • hepatitis C • liver protein • fluorogenic derivatization

Introduction

Recent work in clinical proteomics has involved quantitative and comparative studies of mixture composition and/or the relative abundance of proteins under differing physiologically relevant conditions and differing experimental approaches, commonly referred to as differential profiling. Numerous approaches have been employed for protein quantification, including a one- or two-dimensional gel electrophoretic and liquid chromatographic (LC) method, followed by mass spectrometry (MS).[1−4] Each of the technical approaches has advantages and limitations. For example, gel-based methods are based on the densitometric quantification of proteins visualized using dyes on gel, followed by in-gel enzymatic digestion of the subject protein spots, with the resulting peptides then subjected to MS analysis. This approach has been widely practiced in proteomics studies because of its high resolution, which enables separating the protein isoforms and post-translational modifications. However, this method suffers from a lack of reproducibility, low sensitivity, low dynamic range, and difficulty in resolving proteins with extreme hydrophobicity or isoelectric points, among other issues.[2,3,5,6] In recent years, the introduction of differential gel electrophoresis (DIGE) using fluorescence reagents such as CyDye DIGE Fluor minimal dye[6−8] and saturation dye[9,10] has somewhat improved the reproducibility, sensitivity, and dynamic range.

LC-based methods offer flexibility of choice over a wide range of stationary and mobile phases to resolve complex biological samples at the protein or peptide level. In these methods, proteins are usually digested into peptides prior to separation by separation columns. The advantage of this approach is that the resolved peptides from the column can be directly introduced into an MS system. To obtain high sensitivity and quantification, the stable-isotope labeling reagents, that is, the isotope-coded affinity tag (ICAT),[11] the cleavable ICAT (cICAT),[3,12,13] and isobaric tags for relative and absolute quantitation (iTRAQ),[3,14] were developed and have gained in popularity. However, a major disadvantage of these strategies is that the obtained peptides cannot be correctly identified as any given protein. Moreover, low-abundance peptides are masked by high-abundance peptides with similar *m/z* ratios. Thus, for highly complex samples, such as tissue homogenates, these methods are not suitable for the quantification of specific low-abundance proteins unless extensive purification is employed before analysis.[2−4,12,13]

We recently reported a method for proteomics studies called the FD-LC−MS/MS method.[15−18] This method involves fluorogenic derivatization (FD) of proteins using fluorogenic reagents such as 7-chloro-*N*-[2-(dimethylamino)ethyl]-2,1,3-benzoxadiazole-4-sulfonamide (DAABD-Cl), followed by HPLC separation of the derivatized proteins, isolation of the subject proteins, enzymatic digestion of the isolated proteins, and identification of the proteins utilizing HPLC and tandem MS with a database-searching algorithm. The FD-LC−MS/MS method has unique features, differing from other proteome approaches in using a fluorogenic reagent to derivatize proteins

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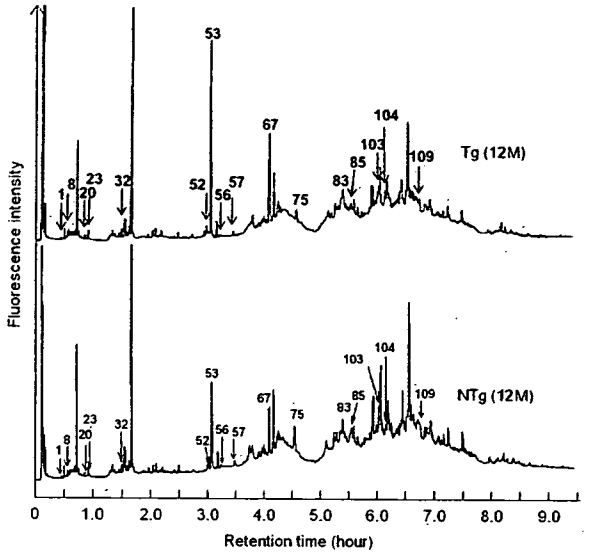

Figure 1. Chromatograms of proteins (8.0 μg protein) in mouse liver derivatized with DAABD-Cl. The chromatograms above and below were obtained from transgenic (12 months) and non-transgenic (12 months) mice, respectively. The altered peaks (106 proteins) between transgenic and non-transgenic mice were numbered, and as representatives, significantly altered peaks (15 proteins) and a peak (no. 53 for accuracy measurement) are described.

and HPLC to separate the derivatized proteins. The fluorogenic reagent is highly reactive and selective to thiols, is nonfluorescent itself, and is water-soluble, so there are few limitations to the complete derivatization of cysteine residues of proteins. The proposed method enables highly sensitive detection of derivatized proteins at the femtomol level,[17,18] whereas other derivatized reagents, such as CyDye DIGE Fluoro minimal dye, would have difficulty forcing the labeling reaction into saturation.[7,8] Separation by HPLC led to highly reproducible quantification. In addition, a protein can be isolated and identified from the corresponding peak fraction without losing any amino acid sequence information, including protein isoforms and post-translational modifications, because the isolated protein itself is digested into peptides following isolation by HPLC. Although this method has already identified more than 100 proteins in a soluble extract of *Caenorhabditis elegans*[15−17] and has identified altered proteins in the islet of Langerhans in dexamethazone-treated rats,[18] there have been no studies involving clinical proteomics analysis utilizing DAABD-Cl as a fluorogenic reagent. Therefore, we attempted to apply it to the quantification and differential profiling analysis of liver proteins taken from hepatitis C virus (HCV) core gene transgenic (Tg) and non-transgenic (NTg) as a model. HCV is the main cause of chronic hepatitis. Chronic hepatitis ultimately results in the progression of hepatocellular carcinoma (HCC). However, the mechanism of hepatocarcinogenesis associated with HCV infection is still unclear. K. Moriya et al. have suggested that the HCV core protein plays a critical role in the progression of HCC and that transgenic mice provide a good animal model for determining the molecular and pathological events in hepatocarcinogenesis with HCV infection.[19−22] Such mice have been investigated previously in terms of morphological and biochemical changes in HCV infection, so far. Therefore, this study investigated the long-term consequences of HCV core gene expression from the viewpoint of proteomics and evaluated the proposed method as the quantification and differential profiling analysis.

Materials and Methods

Transgenic Mice. The production of HCV core gene transgenic mice has been described.[19] Because HCC develops preferentially in male transgenic mice, male mice were used for analysis. Male non-transgenic littermates were utilized as controls. At least three mice were used in each experiment, with the data then subjected to statistical analysis. In the previous studies,[19,20] these transgenic mice developed hepatic steatosis, one of the characteristic histological features of chronic hepatic C, as early as 3 months of age. As the mice grew to 12 months of age, steatosis slowly progressed without neoplastic change. At the age of 16 months, one-fourth of the male mice had experienced hepatic tumors. Moreover, older transgenic mice (>12 months of age) morphologically exhibited an age-dependent increase in oxidative stress. Therefore, in this study, the transgenic and non-transgenic mice used were aged 6, 12, and 16 months, representing the early, medium, and late stages of hepatocarcinogenesis. Also, to exclude the influence of protein variations with advancing age, the amount of protein change due to HCV infection was calculated based on the Tg-to-NTg (Tg/NTg) ratio. All studies were performed according to the Helsinki Declaration and have passed our institutional review board.

Preparation of Sample and Determination of Total Proteins. Liver samples (100 mg) were homogenized in 500 μL of 10 mM CHAPS aq with a pestle on ice. The homogenate was centrifuged at 20 400*g* for 15 min at 4 °C. The supernatant was then collected and stored as a soluble fraction at −20 °C until use. The liver total proteins were determined with the Quick Start Bradford Protein assay kit (Bio-Rad Laboratories, Inc.) by following the written instructions. Bovine serum albumin was used as a protein standard.

FD and HPLC Conditions. The previous method[16] was used for the FD procedure for liver proteins with DAABD-Cl, except that the borate buffer was replaced with a pH 8.7 buffer solution (6.0 M guanidine hydrochloride, Tokyo Chemical Industry). Briefly, homogenized liver tissue was diluted with the CHAPS aq to 4.0 mg/mL, and 10 μL of the sample was mixed with 60 μL of a mixture of 0.83 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), ethylenediamine-*N,N,N',N'*-tetraacetic acid sodium salt (Na_2EDTA), and 16.6 mM CHAPS in the pH 8.7 buffer solution; 25 μL of the buffer solution; and 5.0 μL of 140 mM DAABD-Cl in acetonitrile. After the reaction mixture was placed in a 40 °C water bath for 10 min, 3.0 μL of 20% trifluoroacetic acid (TFA) was added to stop the derivatization reaction. Twenty microliters of the reaction mixture (8.0 μg proteins) was injected into the HPLC system at a flow rate of 0.55 mL/min. The overall system consisted of a Hitachi L-7000 series HPLC system and a fluorescence detector (Jasco FP-2025 plus; λex. 395 nm; λem, 505 nm). Since the derivatives offer adaptable selectivity for the stationary phase, a protein column (Intrada WP-RP, 250 × 4.6 mm i.d., Imtakt Co.) with a column temperature of 60 °C was adopted to further improve the column separation. The mobile phases consisted of 0.15% TFA in acetonitrile/isopropanol/water (A) 9.0/1.0/90 and (B) 69/1.0/30. Mobile phase (C) was the same as (A), except with 0.20% TFA. The gradient condition was established with the following elution: 5.0% B and 1.0% C held for 5.0 min; to 30% B and 35% C in 30 min, and then held for 35 min; to 35% B and 35%

Table 1. Altered Proteins between Tg and NTg Mouse Livers for 6 Months[a]

peak number	Tg/NTg ratio	protein name	molecular mass (Da)	GI number[b]
		Down-Regulated		
		marker		
54	0.55	Major urinary protein (MUP)	20680	gi|295910
56	0.64*	MUP	17549	gi|53271
58	0.58	MUP	17549	gi|53271
55	0.63	Glial fibrillary acidic protein	46498	gi|14193690
		respiration		
52	0.52*	α-globin	15076	gi|49901
		electron-transfer system		
57	0.64	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	18752	gi|16741459
		apoptosis		
1	0.54*	Eukaryotic translation elongation factor 1 α 1 (EF-1 α 1)	50140	gi|13278382
		glycolytic system		
77	0.74	PREDICTED: similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	35789	gi|51768209
		other		
34	0.62	ND***		
		Up-Regulated		
		respiration		
27	1.28	α-globin	15076	gi|49900
29	1.28	α-globin	15076	gi|49900
37	1.35	α-globin	15076	gi|49900
		defense		
44	1.14	Cu/Zn-superoxide dismutase (SOD)	15955	gi|201006
75	1.69	Glycine *N*-methyltransferase	32712	gi|15679953
78	1.22	Aldo-keto reductase family 1, member C6	37024	gi|13487925
79	1.32	Glutathione S-transferase, mu 1	25953	gi|61402231
95	1.29	Glutathione S-transferase, α 3	25344	gi|31981724
		fatty acid metabolism (containing β-oxidation)		
35	1.43	Fatty acid-binding protein, hepatic (fragment)	10173	gi|90485
36	1.24	Fatty acid-binding protein, hepatic (fragment)	10173	gi|90485
42	1.37	Fatty acid-binding protein, hepatic (fragment)	10173	gi|90485
82	1.31	Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	41831	gi|20810027
		apoptosis		
3	2.21	EF-1α1	50140	gi|13278382
		glycolytic system		
61	1.40	Fructose-bisphosphate aldolase B	39548	gi|15723269
99	1.36	Enolase 1, α non-neuron	47095	gi|12963491
		metabolism		
68	1.37	Carbonic anhydrase 3	29348	gi|31982861
		other		
26	1.41	Unnamed protein product	58587	gi|12852157
33	1.40	Unnamed protein product	57807, 58587, 57007, 52653	gi|12852157, gi|26345440, gi|2634914, gi|26349459
112	1.42	put. β-Actin (aa 27–375)	39161	gi|49868

[a] Peak numbers correspond to those in Figure 1. Asterisks indicate significant differences (two-tailed Student's *t* test, $*P \leq 0.05$, $** P \leq 0.01$). ***ND, not detected. [b] GI number is simply a series of digits that are assigned consecutively to each sequence record processed by NCBI. The GI system of sequence identifiers runs parallel to the accession.version system, which was implemented by GenBank, EMBL, and DDBJ in February 1999. Therefore, if the protein sequence changes in any way, it will receive a new GI number. (http://www.ncbi.nlm.nih.gov/Sitemap/samplerecord.html#ProteinIDB).

C in 70 min, then to 38% B and 35% C in 130 min; to 44% B and 55% C in 250 min, and then held for 50 min; to 47% B and 53% C in 330 min; to 60% B and 40% C in 480 min; to 70% B and 30% C in 520 min; and then to 90% B and 10% C in 570 min.

Because of the differential profiling of proteins in transgenic and non-transgenic mice, the corresponding peak heights in the different elution profiles were compared for each month age. The correspondence of the peak was judged not only from the specific retention time of the derivatives, but also confirmation of the protein following isolation and identification of the derivatives. The Tg/NTg ratio was also compared between three developmental stages to investigate the consequences of HCV core gene expression during the progression of hepatocarcinogenesis.

Identification of Derivatized Proteins. Each eluate of the subject proteins was concentrated to 5.0 μL under reduced pressure. The residue was diluted with 50 μL of 50 mM ammonium bicarbonate solution (pH 7.8) containing 0.50 U trypsin and 10 mM calcium chloride, and the resultant mixture was incubated for 2.0 h at 37 °C. The peptide mixture (20 μL) was directly subjected to a nanoLC–ESI-tandem MS spectrometer (HCT plus, Bruker Daltonics). Chromatography was performed using an Ultimate/Famos/Switchos suite of instruments (LC Packings, Dionex). The sample was loaded onto a nanoprecolumn (300 μm i.d. × 1.0 mm, C18 PepMap) in the

Table 2. Altered Proteins between Tg and NTg Mouse Livers for 12 Months[a]

peak number	Tg/NTg ratio	protein name	molecular mass (Da)	GI number[b]
		Down-Regulated		
		respiration		
29	0.64	α-globin	15076	gi|49900
		defense		
75	0.56	Glycine *N*-methyltransferase	32712	gi|15679953
76	0.72	Glutathione *S*-transferase, mu 1	25953	gi|61402231
91	0.79	Methionine adenosyltransferase I, α	43481	gi|19526790
		fatty acid metabolism (containing β-oxidation)		
36	0.74	Fatty acid-binding protein, hepatic	10173	gi|90485
40	0.80	Fatty acid-binding protein, hepatic	10173	gi|90485
102	0.77	Peroxisomal acyl-CoA oxidase	74608	gi|2253380
		metabolism		
68	0.75	Carbonic anhydrase 3	29348	gi|31982861
105	0.80	Aldehyde dehydrogenase family 1, subfamily A1	54447	gi|7304881
		amino acid synthesis		
80	0.61	4-Hydroxyphenylpyruvate dioxygenase	45054	gi|849053
		other		
106	0.68	Heat-responsive protein	18462	gi|1255116
		Up-Regulated		
		marker		
55	1.52	Glial fibrillary acidic protein	46498	gi|14193690
56	1.23	MUP	17549	gi|53271
58	1.68	MUP	17549	gi|53271
70	1.51	α-Fetoprotein	47195	gi|191765
		respiration		
4	2.50	Hemoglobin, β adult major chain	15738	gi|31982300
66	1.45	Hemoglobin, β adult major chain	15738	gi|31982300
67	1.98*	Hemoglobin β	15653	gi|229301
27	2.27	α-globin	15076	gi|49900
28	2.43	α-globin	15076	gi|49900
30	1.70	α-globin	15076	gi|49901
31	1.64	α-globin	15076	gi|49900
51	2.12	α-globin	15076	gi|49900
53	2.05	α-globin	15076	gi|49902
		electron-transfer system		
20	1.85*	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	5834	gi|13385484
57	1.56*	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	18752	gi|16741459
		protein synthesis		
10	2.43	Ribosomal protein L28	15700	gi|56541228
46	2.33	Ribosomal protein S16	16319	gi|70920
		defense		
9	1.80	SOD	15955	gi|201006
11	2.28	SOD	15752	gi|226471
15	1.24	SOD	15955	gi|201006
18	1.96	SOD	15955	gi|201006
44	1.85	SOD	15955	gi|201006
12	1.42	60S Ribosomal protein	24692	gi|899445
43	1.31	Thioredoxin 1	11668	gi|6755911
50	1.99	Chain C, Crystal Structure Of Macrophage Migration Inhibitory Factor	12365	gi|5542287
69	1.60	D-Dopachrome tautomerase	13069	gi|6753618
71	2.24	Albumin 1	68648	gi|19353306
73	2.11	Albumin 1	68648	gi|19353306
89	1.63	Albumin 1	68648	gi|19353306
83	1.47**	Betaine-homocysteine methyltransferase (BHMT)	44992	gi|62533211
90	2.17	Methionine adenosyltransferase I, α	43481	gi|19526790
100	1.25	Glycine *N*-methyltransferase	32712	gi|15679953
		fatty acid metabolism (containing β-oxidation)		
7	1.95	3-Ketoacyl-CoA thiolase B	43968	gi|18043769
35	1.27	Fatty acid-binding protein, hepatic - (fragment)	10173	gi|90485
41	1.28	Fatty acid-binding protein, hepatic - (fragment)	10173	gi|90485
82	1.32	Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	41831	gi|20810027
		apoptosis		
1	2.86	EF-1α1	50139	gi|13278381
2	2.79	EF-1α1	50140	gi|13278382
3	1.64	EF-1α1	50140	gi|13278382
8	2.02	Ribosomal protein S29, isoform 1	6672	gi|22267962
24	2.06	Ribosomal protein L14	23549	gi|13385472

Table 2. (Continued)

peak number	Tg/NTg ratio	protein name	molecular mass (Da)	GI number[b]
		glycolytic system		
59	2.96	Fructose-bisphosphate aldolase B	39548	gi|15723268
62	1.21	Fructose-bisphosphate aldolase B	39548	gi|15723268
77	1.39	PREDICTED: similar to GAPDH	35789	gi|51768209
112	1.64	Lactate dehydrogenase 1, A chain	36475	gi|6754524
		metabolism		
21	1.79	TI-225	14167	gi|1167510
22	2.02	TI-225	14167	gi|1167510
32	2.02	Cystatin B	11039	gi|6681071
72	3.23	Carbonic anhydrase 3	29348	gi|31982861
104	1.83*	Acetaldehyde dehydrogenase (ALDH)	54410	gi|9755362
110	1.82	Aldh2 protein	56502	gi|13529509
115	1.90	Malate dehydrogenase (EC 1.1.1.37)	31692	gi|164543
116	2.10	Argininosuccinate lyase	51707	gi|19526986
		signal transduction		
47	1.70	Phosphatidylethanolamine binding protein	20847	gi|9256572
		amino acid synthesis		
74	2.21	Glycine-*N*-acyltransferase	34076	gi|22122359
		other		
5	2.02	ND***		
6	1.99	ND***		
118	1.97	ND***		
19	3.56	γ-actin	40992	gi|809561
23	1.40*	Diazepam binding inhibitor, splice form 1b	15219	gi|67511482
39	2.80	Saposin	61353	gi|249387
45	2.13	Unnamed protein product	58587, 57007, 52653, 49471	gi|12852157, gi|26345440, gi|26349141, gi|26349459,
49	1.42	Peptidylprolyl isomerase A	17960	gi|71051228
103	1.53*	Sorbitol dehydrogenase precursor	40066	gi|1009706
117	3.03	Unnamed protein product	57614	gi|52787

[a] Peak numbers correspond to those in Figure 1. Asterisks indicate significant differences (two-tailed Student's t test, *$P \leq 0.05$, ** $P \leq 0.01$). ***ND, not detected. [b] GI number is simply a series of digits that are assigned consecutively to each sequence record processed by NCBI. The GI system of sequence identifiers runs parallel to the accession.version system, which was implemented by GenBank, EMBL, and DDBJ in February 1999. Therefore, if the protein sequence changes in any way, it will receive a new GI number. (http://www.ncbi.nlm.nih.gov/Sitemap/samplerecord.html#ProteinIDB).

injection loop, and washed using 0.10% TFA in 2.0% acetonitrile at 30 μL/min using the Switchos pump. Peptides were then separated on a nanoflow column (75 μm i.d. × 15 cm, C18 PepMap) at a flow rate of 170 μL/min, employing a gradient from 5.0% to 60% buffer B (0.10% formic acid in 80% acetonitrile) over a period of 35 min (A buffer: 0.10% formic acid in 2.0% acetonitrile). One-second MS/MS scans were performed on each precursor ion. Ions observed with m/z between 350 and 1250 were fragmented with capillary energies from 1300 to 1800 V. The proteins were identified in accord with the previous method.[15,17] There were several candidates with the same score for the unnamed protein products (peak numbers 33 and 45).

Statistical Analysis. Results are expressed as the mean ± SD. The significance of the difference in means was determined by a two-tailed Student's t test.

Results and Discussion

Validation of The FD-LC–MS/MS Method. With the FD-LC–MS/MS method, more than 500 peaks were obtained from an extract of mouse liver tissue derivatized with DAABD-Cl. Typical chromatograms derived from transgenic and non-transgenic mice are depicted in Figure 1. Only the proteins which expression was estimated to fluctuate between transgenic and non-transgenic mice on the same months were identified after isolation, tryptic digestion, and LC–MS/MS identification of arbitrarily selected peak fractions (113 proteins). As a result, 106 proteins differed between transgenic and non-transgenic mice from 6 to 16 months of age, as summarized in Tables 1–3. The total protein amount required for quantification and identification was only 8.0 μg per injection, and identification of even low-abundance proteins was possible with 40 μg of total protein per injection into an HPLC column. In general, proteome analysis of biological samples labeled with CyDye, ICAT, cICAT, or iTRAQ requires from dozens to hundreds of micrograms of protein samples.[3,7–10,11,13,14,28] The accuracy of the method was acquired based on the reproducibility of the peak heights using peaks 53, 83, and 32 as representatives of the high, medium, and low peaks obtained from each individual mouse. The relative standard deviation (RSD, %) for each between-day peak was less than 16 (high peak), 17 (medium peak), and 23% (low peak) ($n = 3$). The reproducibility of the retention time was also calculated using peak 32. The between-day RSD was 0.41% ($n = 3$). As an additional benefit, the simple apparatus, consisting of a pump, a column, and a fluorescence detector, does not require a complex facility for operation. In this study, we attempted a comprehensive profiling analysis of an 11-h operation to evaluate the utility of the method. After the elution time of a subject protein has been determined, it will be possible to reduce the analysis time for an arbitrary analysis of the subject protein by re-optimizing the separation conditions. It would also be possible to reduce the overall analysis time if we could develop a higher-performance column.

Table 3. Altered Proteins between Tg and NTg Mouse Livers for 16 Months[a]

peak number	Tg/NTg ratio	protein name	molecular mass (Da)	GI number[b]
		Down-Regulated		
		respiration		
4	0.41	Hemoglobin, β adult major chain	15738	gi|31982300
67	0.69	Hemoglobin β	15653	gi|229301
53	0.70	α-globin	15076	gi|49902
108	0.70	Quininoid dihydropteridine reductase	25554	gi|21312520
		protein synthesis		
101	0.54	Regucalcin	33385	gi|6677739
		defense		
16	0.69	SOD	15955	gi|201006
65	0.64	Manganese superoxide dismutase	24662	gi|53450
40	0.53	Thioredoxin 1	11668	gi|6755911
63	0.72	Glutathione peroxidase (GSHPx-1) (Cellular glutathione peroxidase)	22268	gi|121666
75	0.49*	Glycine *N*-methyltransferase	32712	gi|15679953
79	0.61	Glutathione S-transferase, mu 1	25953	gi|61402231
83	0.69	BHMT	44992	gi|62533211
95	0.61	Glutathione *S*-transferase, α 3	25344	gi|31981724
97	0.51	Chain B, Glutathione *S*-Transferase Yfyf Cys 47-Carboxymethylated Class Pi, Free Enzyme	23350	gi|2624496
		fatty acid metabolism (containing β-oxidation)		
36	0.69	Fatty acid-binding protein, hepatic (fragment)	10173	gi|90485
82	0.58	Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	41831	gi|20810027
107	0.70	Acetyl-Coenzyme A acyltransferase 1	43926	gi|18700004
85	0.32*	Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), β subunit (HADHB)	51353	gi|13542763
102	0.58	Peroxisomal acyl-CoA oxidase	74608	gi|2253380
		apoptosis		
3	0.69	Eukaryotic translation elongation factor 1 α 1	50140	gi|13278382
8	0.58*	Ribosomal protein S29, isoform 1	6672	gi|22267962
		glycolytic system		
61	0.79	Fructose-bisphosphate aldolase B	39548	gi|15723269
98	0.42	Enolase 1, α non-neuron	47095	gi|12963491
99	0.58	Enolase 1, α non-neuron	47095	gi|12963491
		metabolism		
32	0.46**	Cystatin B	11039	gi|6681071
68	0.80	Carbonic anhydrase 3	29348	gi|31982861
72	0.53	Carbonic anhydrase 3	29348	gi|31982861
109	0.30**	PREDICTED: Carbamoyl-phosphate synthetase 1 (CPS1)	165705	gi|51705066
84	0.50	Argininosuccinate synthetase	46555	gi|6996911
		signal transduction		
87	0.65	Electron transferring flavoprotein, α polypeptide	35018	gi|13097375
		amino acid synthesis		
80	0.39	4-Hydroxyphenylpyruvate dioxygenase	45054	gi|849053
		other		
38	0.54	Histidine triad nucleotide binding protein 1	13768	gi|33468857
49	0.71	Peptidylprolyl isomerase A	17960	gi|71051228
64	0.69	Nit protein 2	30483	gi|12963555
86	0.64	γ-actin	40992	gi|809561
96	0.50	Unknown (protein for IMAGE:6414729)	50209	gi|53734652
103	0.70	Sorbitol dehydrogenase precursor	40066	gi|1009706
106	0.68	Heat-responsive protein	18462	gi|1255116
48	0.59	Unnamed protein product	65586	gi|12859782
		Up-Regulated		
		respiration		
37	1.33	α-globin	15076	gi|49900
		fatty acid metabolism (containing β-oxidation)		
35	1.60	Fatty acid-binding protein, hepatic (fragment)	10173	gi|90485
		other		
17	1.30	ND***		
33	1.34	Unnamed protein product	57807, 58587, 57007, 52653	gi|12852157, gi|26345440, gi|2634914, gi|26349459

[a] Peak numbers correspond to those in Figure 1. Asterisks indicate significant differences (two-tailed Student's *t* test, *$P \leq 0.05$, **$P \leq 0.01$). ***ND, not detected. [b] GI number is simply a series of digits that are assigned consecutively to each sequence record processed by NCBI. The GI system of sequence identifiers runs parallel to the accession.version system, which was implemented by GenBank, EMBL, and DDBJ in February 1999. Therefore, if the protein sequence changes in any way, it will receive a new GI number. (http://www.ncbi.nlm.nih.gov/Sitemap/samplerecord.html#ProteinIDB).

Differential Profiling. Differential profiling analysis was performed using liver tissue from HCV core gene transgenic and non-transgenic mice as model samples to evaluate the feasibility of the FD-LC–MS/MS method for clinical proteomics. To investigate the differential expression of proteins in transgenic and non-transgenic mice, the heights of the peaks corresponding to specific retention times were compared for each month of age, with 106 altered proteins observed. The differentially expressed proteins were classified by age, regulation and function (see Tables 1–3). Tg/NTg ratios over 1.2 were defined as up-regulated, and those below 0.8 were defined as down-regulated. Many proteins were up- or down-regulated during the progression of HCV-associated liver disease. Fifteen proteins were significantly altered in their levels of protein contents (Figure 2). At the age of 6 months, there were fewer down-regulated proteins than up-regulated (9 vs 19 proteins). In contrast, many kinds of proteins were different between transgenic- and non-transgenic mice at 12 months, with 11 proteins being down-regulated and 65 being up-regulated. At 16 months, there were more down-regulated proteins than in any other months (39 proteins), but only a small minority (four) of proteins were up-regulated.

The remarkable decrease in major urinary protein (MUP) and eukaryotic translation elongation factor 1 α 1 (EF-1α1) seen in Figure 2a represents an early event in the progression of HCV-associated liver disease (at 6 months). MUP has been known as a negative tumor marker.[23] Suppression of EF-1α1 expression prevents the induction of apoptosis, with the regulation reflected in an antiapoptotic mode.[24] Although one of the α-globin peaks (peak no. 52) decreased significantly, the other three peaks of α-globin (peak nos. 27, 29, and 37) tended to increase (see Table 1). The expression of α-globin has been shown to be up-regulated in apoptotic stimuli.[25] Therefore, the phenomenon might be considered a trend in apoptosis at this stage. Another observation made at the age of 6 months was the up-regulation of enzymes related to β-oxidation.

At 12 months of age, proteins related to respiration, the electron-transfer system, and defense against reactive oxygen species (ROS) were significantly up-regulated (Figure 2b). Moreover, a majority of proteins involved in respiration, protein synthesis, defense, apoptosis, the glycolytic system, and metabolism were more up-regulated than the changes observed at 6 months (Table 2).

Finally, at 16 months, proteins related to defense, β-oxidation, and apoptosis significantly decreased. Cystatin B[26] and carbamoyl-phosphate synthetase 1 (CPS1)[27] are known to be down-regulated in tumor and/or carcinoma and exhibited a significant decrease with the proposed method (Figure 2c). It was also established that various biological functions such as respiration, protein synthesis, defense, and metabolism tended to decline (Table 3).

As a whole, the investigation of the differential expression of proteins in transgenic and non-transgenic mice revealed that many proteins related to biological functions such as respiration, protein synthesis, defense, β-oxidation, and apoptosis fluctuate during the progression of chronic hepatitis C. These changes may reflect a gross effect derived from the loss of liver function in the various stages of chronic hepatitis in HCV infection.

Additionally, these data support, from the viewpoint of proteomics, the former results obtained from morphological and biochemical observation.[19–21] For example, previous reports suggested that HCV core protein might affect a specific pathway in the lipid metabolism.[19,21] In fact, the core protein has a specific effect on lipid metabolism; fat droplets are formed and accumulate in the liver, leading to steatosis. An analysis of the composition of these lipid droplets determined that the concentration of carbon 18 monosaturated (C18:1) fatty acids, such as oleic and vaccenic acid, significantly increased in the livers of transgenic mice as well as in chronic hepatitis C patients.[21] In the present study, hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein) β subunit (HADHB), which catalyzes fatty-acid metabolism, significantly decreased after 16 months (Figure 2). However, at 12 months, other enzymes associated with β-oxidation tended to increase (Table 2: peak nos. 7 and 82). In addition, up-regulation of ATP synthase led to an increase in the synthesis and metabolism of fatty acid at 12 months (Figure 2). Furthermore, acetaldehyde dehydrogenase (ALDH), which catalyzes the acetaldehyde metabolism, tended to be up-regulated in the same month (Figure 2). The metabolic reactions of fatty acid and acetaldehyde generate $NADH_2^+$, and the overexpression then causes suppression of both metabolisms. Hence, these results suggest that the fatty-acid metabolism may become milder and resulted from the multiple protein changes related to β-oxidation, ATP synthase, and acetaldehyde metabolism with the progression of HCV-associated liver disease.

Previous reports also suggested that HCV core protein might alter the oxidant/antioxidant state in the liver.[20] The reports demonstrated that there is no significant difference in the levels of lipid peroxidation at 3 and 12 months of age, resulting in cellular and tissue damage by ROS. In contrast, after 16 months, the peroxidation and hydrogen peroxide levels increased remarkably and the levels of total and reduced glutathione, which plays an important role as an antioxidant, decreased. While, our results demonstrate that enzymes related to the antioxidant effect, such as betaine-homocysteine methyltransferase (BHMT) and Cu/Zn-superoxide dismutase (SOD), were up-regulated in transgenic mice at 12 months (Table 2: defense). Subsequently, up to 16 months, a decrease in BHMT and glycine *N*-methyltransferase related to the methylation cycle was observed (Figure 2). The decrease in these enzymes led to a deficiency of adenosylmethionine, impairing mitochondrial function and generating oxidative stress in the liver.[29,30] It has recently been shown that a chronic deficiency of adenosylmethionine in the liver results in the spontaneous progression of steatohepatitis and HCC.[31] In addition, the down-regulation of glycine *N*-methyltransferase would inhibit the synthesis of glutathione resulting in a shift to the oxidizing state, thereby reducing cell proliferation and increasing apoptosis.[32] Therefore, the observed expression of antioxidants might reflect direct oxidative stress status; although at 12 months the up-regulated antioxidants protected against oxidative stress, the oxidative stress might become dominant by the deficiency of antioxidants among the progression of liver disease. Also, these results, derived from both studies, strongly suggest that HCV core protein induces ROS in an age-dependent manner. After 16 months, a biochemical[20] and proteomic analysis revealed a lack of glutathione, suggesting that supplying glutathione might be more effective than SOD in the progression of HCC in the late stage. Although a further animal experiment should be required for reliable clarification of the hepatocarcinogenesis mechanism, the proposed method was demonstrated to be extremely

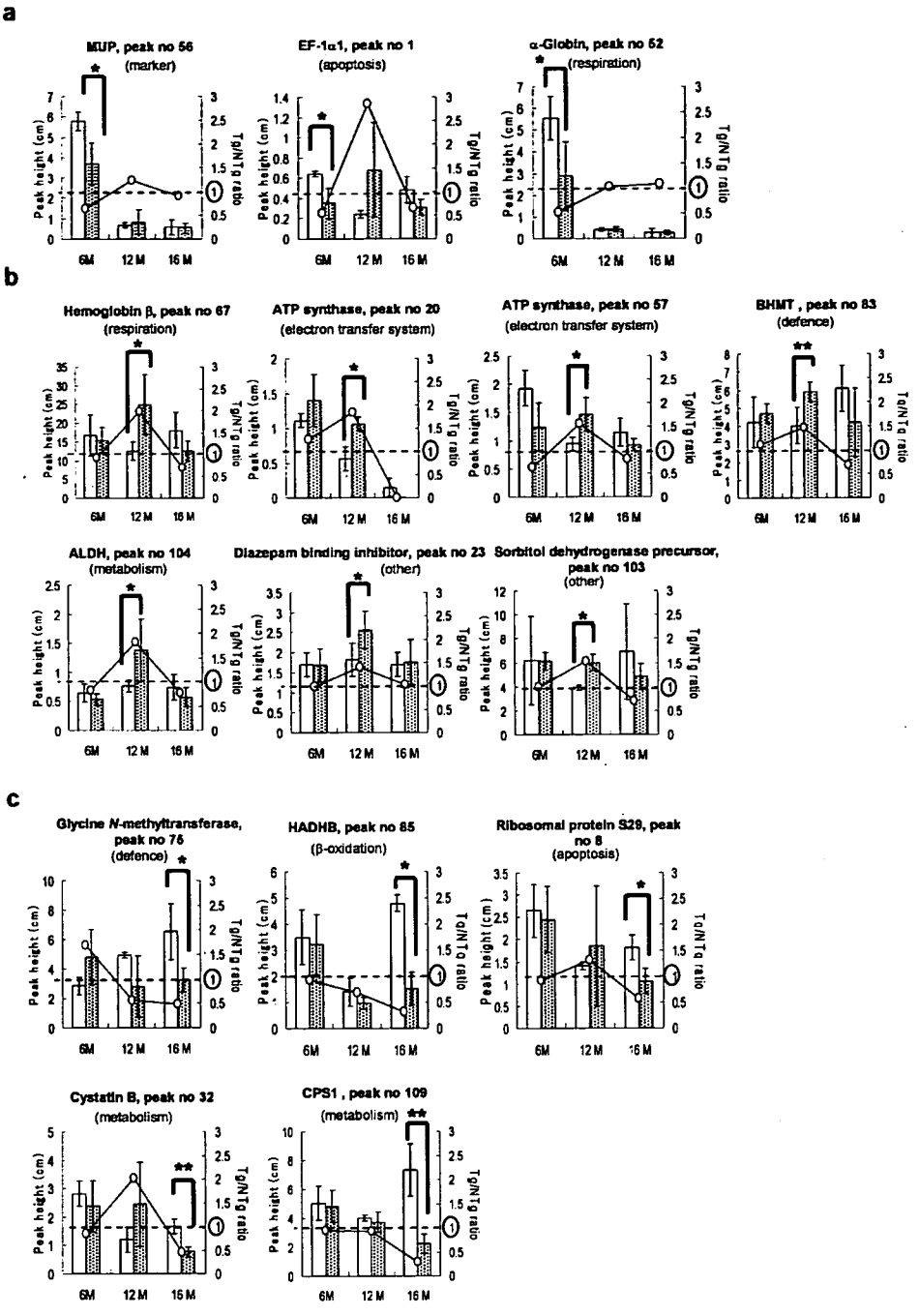

Figure 2. Comparison of peak heights between transgenic (Tg; gray bar) and non-transgenic (NTg; white bar) mice, and the Tg-to-NTg ratio (open circle) from 6 months (6M) to 16 months (16M). Significantly altered proteins are seen at 6M (a), 12M (b), and 16M (c). Peak numbers correspond to those in Figure 1. Mean values ± SD are plotted. Asterisks indicate significant differences (two-tailed Student's *t* test of all data points, $*P \leq 0.05$, $**P \leq 0.01$).

useful for understanding biotransformation from the viewpoint of proteomics. Also, the data obtained in this experiment could support the understanding of hepatocarcinogenesis with HCV infection in terms of proteomics in addition to the morphological and biochemical observations mentioned above.

Conclusions

The proposed method demonstrated for the first time the existence of several event-marker proteins at the three progression stages of hepatocarcinogenesis in transgenic mice. It should be stressed that the FD-LC–MS/MS method would also

be worthwhile for clinical proteomics analysis, as a supplement to gel- and LC-based methods.

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Review

Hepatitis C as a systemic disease: virus and host immunologic responses underlie hepatic and extrahepatic manifestations

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Introduction

Hepatitis C virus (HCV) causes liver diseases. Approximately 2 million people in Japan and approximately 170 million people worldwide are infected with HCV, and they often suffer from chronic hepatitis, followed by hepatic cirrhosis, leading to hepatic cancer. It was determined relatively soon after the discovery of HCV that HCV infection does not involve the liver only. Other than hepatitis, many complicating diseases of the organs and tissues other than the liver, referred to as extrahepatic lesions, occur in association with HCV infection (Table 1). This review provides an overview of typical extrahepatic lesions associated with hepatitis C.

Cryoglobulinemia

Cryoglobulins are abnormal immunoglobulins that solidify into white deposits at 4°C and liquefy at 37°C.[1] The etiology of cryoglobulinemia in HCV infection has not yet been clarified. However, the involvement of apoptosis suppression by B lymphocytes, which produce monoclonal IgM, induced by the association of *bcl-2* and *IgJ(H)* as a result of the translocation of chromosome t(14:18), is suspected. Intrahepatic growth of CD5- and CD81-positive B lymphocytes has been observed, suggesting monoclonal IgM induction as a possible cause.[7]

Cryoglobulins are classified into three types, namely, monoclonal cryoglobulins (type I), polyclonal cryoglobulins (type III), and mixed cryoglobulins (type II). Cryoglobulinemia associated with HCV infection mainly involves the mixed type. More specifically, it involves monoclonal IgM and polyclonal IgG antibodies having rheumatoid factor activity.[8,9]

The clinical symptoms of essential mixed cryoglobulinemia (EMC) include purpura, arthralgia, and renal impairments.[10] Renal impairments are particularly known for showing membranoproliferative glomerulonephritis histologically and progressing to renal insufficiency.[11] Approximately 80% of EMC patients are infected with HCV.[12] When the high-sensitivity gel diffusion method is used, cryoglobulins are detected in 70% of patients chronically infected with HCV.[13] Many patients with HCV-associated cryoglobulinemia show subclinical symptoms, but the incidence of EMC is highest as an extrahepatic complication of hepatitis C.

Interferon (IFN) therapy has been used for HCV-associated cryoglobulinemia.[14] Misiani et al.[15] reported that, following the administration of IFN to 25 patients with HCV-associated cryoglobulinemia, cryoglobulinemia symptoms improved in 15 patients after the start of treatment but that the symptoms recurred after treatment ended. The combination of IFN and ribavirin has become standard therapy for chronic hepatitis C. It has also been used to treat HCV-associated cryoglobulinemia, with particular efficacy expected in patients for whom IFN monotherapy is ineffective. Zuckerman et al.[16] reported that the administration of both IFN and ribavirin to nine EMC patients who had not responded to IFN monotherapy alleviated cryoglobulinemia in all and improved clinical symptoms in seven of the nine patients.

In addition, for patients with severe cryoglobulinemia, antiviral therapy based on IFN and combination therapy with a steroid or an immunosuppressant are considered effective.[17] Other treatment strategies, including plasma exchange therapy[17] and splenectomy,[18]

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Table 1. Extrahepatic manifestations of chronic hepatitis C

Complication	Pathogenesis	Prevalence of HCV antibody (%)	Treatment with antiviral drug	References
Cryoglobulinemia	Apoptosis suppression of B lymphocytes. monoclonal IgM production caused by translocation of chromosome t(14:18)	50–90	Interferon Pegylated Interferon plus ribavirin	1–17
Renal impairment	Accumulation of an immune complex formed by monoclonal or polyclonal IgM-κ with rheumatoid factor activity produced by HCV-infected B lymphocytes in the glomerular vascular endothelium and mesangium	10–60	Interferon Pegylated Interferon plus ribavirin	18–26
Myocardial impairment	Involvement of host immunologic responses to HCV, particularly human MHC class II antigen	6–10	Not reported	27–31
Porphyria cutanea tarda	Reduced activity of uroporphybrinogen decarboxylase associated with an excessive deposition of iron in the liver induced by HCV infection	60–100	Interferon	32–37
Sjögren's syndrome	Involvement of host immunologic responses to HCV	0–45	Not reported	38–43
Lichen planus	Involvement of HCV-specific T cells	0–65	Interferon	44–63
Oral cancer	Unknown	70–100 (HCV-RNA)	Not reported	64–65
Diabetes mellitus	Involvement of insulin resistance and insulin secretory deficiency. Disruption of tyrosine phosphorylation of IRS-1. Involvement of TNF-α	50	Not reported	66–77
Malignant lymphoma	Involvement of *myc* gene mutation in some cryoglobulinemia patients	0–33	Interferon Pegylated Interferon plus ribavirin	78–94
Autoimmune thyroid disease	Involvement of LKM1	10	Not reported	95–102
Idiopathic interstitial pneumonitis	Involvement of activated T lymphocytes and eosinophils	28	Not reported	103–107
Mooren's ulcer	Unknown	Unknown	Not reported	108–114

HCV, hepatitis C virus; MHC, major histocompatibility; IRS, insulin receptor substrate; TNF, tumor necrosis factor; LKM1, liver/kidney microsomal antibody 1

have also been attempted, and future development of these strategies is promising.

Renal impairments

Reported renal impairments associated with HCV infection include membranoproliferative glomerulonephritis, membranous nephropathy, mesangial proliferative glomerulonephritis, Henoch-Schönlein purpura nephritis, and tubulointerstitial nephritis.[19]

Membranoproliferative glomerulonephritis, in particular, is considered a typical example of hepatic disease involving renal impairment associated with HCV and is referred to as HCV-associated nephritis. In 1993, Johnson et al.[11] first reported on eight patients with HCV infection complicated by membranoproliferative glomerulonephritis.[11] The incidence of HCV-associated nephritis developing as a complication of hepatitis C has not been confirmed. In a study of 188 autopsied cases of chronic hepatitis C, Arase et al.[20] reported that 11.2% of patients exhibited membranoproliferative glomerulonephritis, 2.7% membranous nephropathy, and 17.6% mesangial proliferative glomerulonephritis. The pathogenic mechanism underlying HCV-associated nephritis is considered to be the accumulation of an immunocomplex formed by monoclonal or polyclonal IgM-κ with rheumatoid factor activity produced by HCV-infected

peripheral blood B lymphocytes in the glomerular vascular endothelium and mesangium.[21]

Histopathological features of HCV-associated nephritis are similar to those of typical membranoproliferative glomerulonephritis type I, but the former sometimes show cryoglobulin deposition.[22] In essential cryoglobulinemia and nephrotic syndrome with a rheumatoid factor, HCV-associated nephritis is suspected; therefore, the presence or absence of HCV infection should be determined.

IFN therapy has been reported to be efficacious for HCV-associated nephritis.[23,24] Johnson et al.[23] reported that the administration of IFN to 14 patients with HCV-associated nephritis improved proteinuria, but they observed a relapse of nephritis in association with HCV reexpression after the end of IFN therapy in many patients.[23] Recently, IFN and ribavirin combination therapy, which shows a low relapse rate, has been tested.[25,26] Sabry et al.[26] reported on the effectiveness of IFN and ribavirin combination therapy administered to 16 patients with HCV-associated nephritis for whom IFN monotherapy had proved ineffective; a follow-up study is awaited. Steroid and cyclophosphamide have been used for immunosuppression therapy, but satisfactory results using an immunosuppressant alone have not yet been obtained.[27] Because patients with HCV-associated nephritis have been reported to have a poor prognosis,[11] early establishment of a therapeutic procedure based mainly on IFN and ribavirin combination therapy is desirable.

Myocardial impairments

Myocardial impairments for which a causal relationship with HCV infection has been suspected to date include dilated cardiomyopathy, hypertrophic cardiomyopathy, arrhythmogenic right ventricular dysplasia cardiomyopathy, and chronic myocarditis.[28–30]

A study by Matsumori[28] observed positivity for serum anti-HCV antibody in 6.3% (42/663) of patients with hypertrophic cardiomyopathy and in 10.6% (74/697) of patients with dilated cardiomyopathy. These positivity rates were higher than the rate (2.4%) observed among age-matched Japanese blood donors.[28] Positive- and negative-strand HCV RNAs were detected in cardiac muscle samples of these patients, indicating potential intramyocardial HCV multiplication.[29,30] HCV RNA has also been detected in cardiac muscle samples of patients with arrhythmogenic right ventricular dysplasia cardiomyopathy and chronic myocarditis, indicating that HCV potentially plays an important role in the onset of myocardial impairments.[32]

With regard to the cause of myocardial impairments associated with HCV, the involvement of host immunologic responses to HCV, particularly that of the human major histocompatibility (MHC) class II antigen, has been suggested.[30] There are many patients with normal liver enzyme levels among hepatitis C patients with a concomitant myocardial impairment.[28] No established therapy is currently available, but the use of IFN-based antiviral therapy should be considered.

Porphyria cutanea tarda

Porphyria cutanea tarda is an acquired condition in which patients exhibit solar photosensitivity and hepatic damage owing to decreased activity of uroporphyrinogen decarboxylase in the liver.[33] The involvement of alcohol, excess iron, and medications for hepatic impairments in porphyria cutanea tarda was previously considered. However, because HCV infection has been observed in 60%–100% of cases of porphyria cutanea tarda, the involvement of HCV infection in the pathogenesis of porphyria cutanea tarda is suspected.[34]

The mechanism underlying the pathogenesis of porphyria cutanea tarda associated with HCV infection has not yet been clarified. It is assumed, however, that porphyria cutanea tarda results from reduced uroporphyrinogen decarboxylase activity associated with excessive deposition of iron in the liver as a result of HCV infection.[34]

The efficacy of IFN therapy for the treatment of porphyria cutanea tarda has been demonstrated, in addition to avoidance of sun exposure, abstention from alcoholic beverages, and blood letting. Okano et al.[38] reported that IFN therapy given to porphyria cutanea tarda patients with HCV infection led to transaminase normalization, HCV RNA disappearance, and normalization of porphyrin and ferritin levels with improvement of clinical symptoms, including vesicle formation and hypertrichosis. These results demonstrate the efficacy of IFN therapy for porphyria cutanea tarda.

Sjögren's syndrome

Sjögren's syndrome is an aggregate of symptoms characterized by insufficient tear production by the lacrimal glands and insufficient saliva production by the salivary glands because of exocrine lymphocyte infiltration, causing dryness of the eyes and mouth. Patients with Sjögren's syndrome are classified roughly into two groups, those exhibiting only dryness and those exhibiting both dryness and connective tissue disease symptoms such as arthralgia.[39]

An association of Sjögren's syndrome with viral infection has been reported for some time, and 0%–45% of Sjögren's syndrome patients test positive for

anti-HCV antibody.[40] Differences in the anti-HCV antibody positivity rate are attributed to regional differences in the HCV infection rate. Koike et al.[41] verified that transgenic mice with the 1b HCV envelope genotype developed sialadenitis resembling Sjögren's syndrome. Takamatsu et al.[42] detected HCV RNA in salivary gland tissue from anti-HCV antibody-positive patients with Sjögren's syndrome by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Arreita et al.[43] performed in situ hybridization of 19 salivary gland tissue samples obtained from eight anti-HCV antibody-positive patients and 11 anti-HCV antibody-negative patients with chronic sialoadenitis or Sjögren's syndrome, and detected HCV RNA in all salivary gland tissue samples from the anti-HCV antibody-positive patients. Moreover, the HCV-infected salivary gland epithelium showed viral multiplication.[43] These reports indicate that HCV plays some role in the development of sialoadenitis in Sjögren's syndrome, but it has not yet been determined whether HCV itself or immunologic responses to HCV infection induce sialoadenitis.

Current therapies for Sjögren's syndrome mainly aim to alleviate the symptoms. Artificial lacrimal fluid and artificial saliva are used to alleviate dryness, and a nonsteroidal anti-inflammatory drug or a steroid is administered for treatment of fever and articular symptoms.[39] There are no reports regarding the efficacy of IFN therapy for HCV-associated sialadenitis,[44] and it is necessary to establish a treatment protocol in the future on the basis of accumulated case reports.

Lichen planus

Lichen planus is an inflammatory disease associated with abnormal chronic dermal and intraoral keratinization of unknown etiology. The assumed causes of lichen planus include viral or bacterial infection, immunologic responses, circulatory disorder, allergy, mental stress, abnormal autonomic function, medication, and glucose metabolism disorder.[45,46]

There are many reports of a relationship between lichen planus and HCV infection, but the anti-HCV antibody positivity rate in lichen planus shows marked regional differences, ranging from 0% to 65%.[47–53] HCV reproduction in the skin and oral mucosal epithelium has been examined by in situ hybridization and RT-PCR analysis.[54–56] HCV-specific T cells are reported to be associated with the pathogenesis of lichen planus,[57] but its pathogenesis is not associated with HCV level, genotype, or pathologic severity.[58,59]

The intravenous administration of a glycyrrhizinate preparation has been demonstrated to have efficacy for treatment of HCV-associated lichen planus.[60] Antiviral therapy based on IFN has also been attempted recently and has been reported to be effective,[61] but other investigators have reported that IFN is a lichen-planus-inducing factor[62] or that it can be aggravating factor.[63] No definite conclusion on the effectiveness of IFN against lichen planus is possible. Nagao et al.[64] reported that when intraoral lichen planus lesions in chronic hepatitis C patients administered IFN were observed over time, no macroscopic changes were observed in the lesions 1 year after the end of IFN administration, but that macroscopic and histological improvements were observed 3 or more years after the end of IFN administration. They also assumed that, since positive-strand HCV RNA was detected in the oral mucous membrane of some patients despite the demonstration of histological recovery from lichen planus following IFN therapy, host immunologic responses to HCV infection were related to the development of oral lichen planus.[64] The early establishment of a treatment procedure for lichen planus is desired, because lichen planus is also considered to be a precancerous condition.[45,46]

Oral cancer

A relationship between HCV infection and oral cancer was first reported by Nagao et al.[65] They showed that the HCV infection rate was higher in oral cancer patients than in esophageal, gastric, or colorectal cancer patients.[65] The HCV infection rate has also been found to be higher in patients with cervical squamous cell carcinoma than in controls.[66] When HCV-RNA was examined in cancer tissues from 17 oral cancer patients by RT-PCR analysis, positive-strand HCV RNA was detected in all anti-HCV antibody-positive patients and negative-strand HCV RNA was detected in 71.4% of the anti-HCV antibody-positive patients.[55] These findings interestingly indicate the possibility of HCV multiplication in cancer tissue. No definite conclusion has been arrived at regarding the relationship between oral lichen planus and oral cancer. However, because lichen planus is considered precancerous, as mentioned above, oral examination is also important for patients with chronic hepatitis C.

Diabetes mellitus

In 1994, Allison et al.[67] reported a relationship between HCV-associated cirrhosis and diabetes mellitus, because the rate of diabetes mellitus complication in patients with both cirrhosis and HCV infection was 50%, which is much higher than that (9%) in patients with cirrhosis but without HCV infection. A large-scale epidemiologic survey showed that the rate of non-insulin-dependent

diabetes mellitus occurring as a complication of chronic hepatic diseases associated with HCV infection was higher than that of other chronic hepatic diseases, and that anti-HCV antibody-positive patients aged 40 years or more had a 3.77-fold higher risk of becoming diabetic than anti-HCV antibody-negative patients.[68] In addition, it has been demonstrated that complication by diabetes mellitus is both a risk factor for hepatocellular carcinoma[69] and a prognostic factor in cirrhosis patients.[70] These reports suggest a correlation between HCV infection and type 2 diabetes. Increased insulin resistance and insulin secretory deficiency are considered to be highly involved in the pathogenesis of type 2 diabetes.[71] Petit et al.[72] reported that insulin resistance increases even in chronic hepatitis C patients with slight hepatic impairment and that the index of impairment (HOMA-IR) correlates with the severity of the liver tissue disorder. Tumor necrosis factor (TNF)-α, which closely correlates with hepatic inflammation and fibrillation in chronic hepatitis C,[73] is considered to enhance glucose uptake in peripheral tissue and to promote gluconeogenesis in the liver, leading to the induction of insulin resistance.[74] Shintani et al.[75] confirmed that in transgenic mice with the 1b HCV core genotype, tyrosyl phosphorylation of the insulin receptor substrate 1 in the insulin signal transduction pathway is disrupted and that this disruption causes gluconeogenesis inhibition by insulin in the liver, leading to the induction of marked insulin resistance. These transgenic mice exhibited a high anti-TNF-α antibody level, and insulin resistance was improved by the administration of an anti-TNF-α antibody. These results indicate a close relationship between HCV infection and the pathogenesis of diabetes mellitus. The relationship between HCV infection and hepatocyte fat modification has also attracted attention.[76] Moriya et al.[77] suggested the possible direct involvement of HCV core protein in hepatocyte fat modification, because they observed hepatocyte fat deposits in transgenic mice expressing the HCV core gene. In summary, the above-described findings strongly indicate that hepatitis C has the characteristics of a metabolic disease, and nutritional management is also considered important in the treatment of chronic hepatitis C.

Malignant lymphoma

HCV reproduces in lymphocytes, and studies of a short-term HCV culture system using lymphocytes have been reported.[78,79] Infected lymphocytes may undergo malignant transformation, leading to the development of malignant lymphoma. HCV infection is considered to be associated with the development of malignant lymphoma, particularly in association with the pathogenesis of non-Hodgkin B-cell lymphoma, and many reports suggest a relationship between HCV infection and malignant lymphoma.[80–88] It has been assumed that some cryoglobulinemia patients develop non-Hodgkin B-cell lymphoma in association with *myc* gene mutation.[89] The anti-HCV antibody positivity rates in patients with non-Hodgkin B-cell lymphoma range from 0% to 33%.[80–88] These differences in HCV antibody positivity rates are considered to relate to regional differences in the HCV infection rate. The HCV antibody prevalence tends to be higher in Japan and Italy but lower in Britain and Canada. Studies indicating a relationship between HCV infection and malignant lymphoma have been reported by Ferri et al.[90] and De Vita et al.[91] Ferri et al.[90] reported that 14 of 500 patients with chronic hepatitis C were complicated with non-Hodgkin B-cell lymphoma, and they detected HCV RNA in peripheral blood lymphocytes in all of these patients. De Vita et al.[91] detected positive-strand and negative-strand HCV RNAs in the parotid glands of patients with parotid non-Hodgkin B-cell lymphoma associated with HCV infection, and confirmed the presence of HCV in the parotid gland by in-situ hybridization.[91] As shown by these findings, many patients with HCV-associated non-Hodgkin B-cell lymphoma show involvement of extranodal sites such as the liver and salivary glands.[92]

Treatment of HCV-associated malignant lymphoma is similar to that of HCV-associated non-Hodgkin B-cell lymphoma; however, recently, IFN monotherapy or IFN and ribavirin combination therapy have been reported to be effective.[93–96] Vallisa et al.[96] reported that administration of both pegylated IFN and ribavirin to 13 patients with HCV-associated non-Hodgkin B-cell lymphoma achieved a complete response in seven of these patients. It is interesting that IFN-based antiviral therapy has been demonstrated to be useful for malignant lymphoma associated with HCV-associated non-Hodgkin B-cell lymphoma in addition to conventional chemotherapy.

Autoimmune thyroid disease

The relationship between HCV infection and thyroid disease has been analyzed in many studies,[97,100] and a causal relationship between HCV infection and autoimmune thyroid disease has been particularly suggested.[98–100] Antonelli et al.[98] assessed the incidence of thyroid dysfunction in 630 chronic hepatitis C patients without cirrhosis or hepatocellular carcinoma who had not been treated with IFN by recruiting 389 patients from an iodine-deficient area, 268 patients from an iodine-sufficient area, and 86 patients with chronic hepatitis B aged 40 years or older as study subjects. The chronic hepatitis C patients exhibited a higher thyroid-

stimulating hormone level and lower free thyroxine and triiodothyronine levels than the controls. In addition, the chronic hepatitis C patients exhibited hypothyroidism and tended to have antithyroglobulin antibodies and anti-thyroid peroxidase antibodies. These findings suggest a relationship between HCV infection and thyroid disorder.[98] A possible relationship between HCV infection and thyroid cancer has also attracted attention recently.[99] The mechanism underlying the pathogenesis of thyroid disease associated with HCV infection has not yet been elucidated, but a relationship with liver/kidney microsomal antibody type 1 has been suggested.[99] Many patients with thyroid disorder caused by HCV infection are asymptomatic, requiring no special treatment. Thyroid disorder is also known to be an adverse reaction to IFN-α therapy for chronic hepatitis C.[99,101–104] Thyroid hypofunction caused by the administration of IFN-α is usually transient, and the patient recovers spontaneously after the end of the therapy. Hence, discontinuation of IFN-α is not required in many cases.[103]

Idiopathic interstitial pneumonitis

Recently, viral infection has been suggested to be a cause of idiopathic interstitial pneumonitis.[104] With regard to the relationship between HCV infection and idiopathic interstitial pneumonitis, Ueda et al.[106] reported in 1992 that the anti-HCV antibody positivity rate in 66 patients with idiopathic interstitial pneumonitis determined by enzyme-linked immunosorbent assay was 28.8%, which was significantly higher than that in 9464 normal subjects serving as controls.[106] It has not yet been clarified how HCV infection is associated with the pathogenesis of idiopathic interstitial pneumonitis. Kubo et al.[107] suggested that activated T lymphocytes and eosinophils are related to the pathogenesis of idiopathic interstitial pneumonitis associated with HCV infection, because they observed increased activated T-lymphocyte and eosinophil counts in the bronchoalveolar fluid of 13 chronic hepatitis C patients, despite their having the same total cell counts as normal subjects. On the other hand, studies disagree regarding the relationship between HCV infection and idiopathic interstitial pneumonitis,[108] and in-depth studies of this issue are expected. Idiopathic interstitial pneumonitis is also reported to be an adverse reaction to IFN therapy in chronic hepatitis C patients.[109] Such patients often have a high pretreatment KL-6 level, and the potential of their developing idiopathic interstitial pneumonitis is suggested. Recovery from IFN therapy-induced idiopathic interstitial pneumonitis is achieved by the discontinuation of the therapy,[109] but steroid administration is required in some cases.

Rheumatoid arthritis

HCV-associated rheumatoid arthritis complicated by cryoglobulinemia or Sjögren's syndrome has been reported.[10,39] For further information, please refer to the cited references.

Mooren's ulcer

Mooren's ulcer is a progressive ulcer associated with congestion and pain around the cornea.[110] HCV infection has been suggested to contribute to the development of this disease.[111–113] The effectiveness of IFN therapy for HCV-associated Mooren's ulcer has been reported,[110,111] but the exacerbation of ocular pain following the discontinuation of IFN therapy has also been observed; hence, caution is required.[111] Systemic corticoid administration has also been reported to be effective.[112] However, other investigators reported a negative correlation between HCV infection and Mooren's ulcer.[114–116] It is hoped that further detailed studies will clarify this issue.

Conclusion

It is necessary to consider possible complications associated with extrahepatic diseases in the treatment of HCV-infected patients.

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Hepatitis C virus core protein induces spontaneous and persistent activation of peroxisome proliferator-activated receptor α in transgenic mice: Implications for HCV-associated hepatocarcinogenesis

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Persistent infection of hepatitis C virus (HCV) can lead to a high risk for hepatocellular carcinoma (HCC). HCV core protein plays important roles in HCV-related hepatocarcinogenesis, because mice carrying the core protein exhibit multicentric HCCs without hepatic inflammation and fibrosis. However, the precise mechanism of hepatocarcinogenesis in these transgenic mice remains unclear. To evaluate whether the core protein modulates hepatocyte proliferation and apoptosis *in vivo*, we examined these parameters in 9- and 22-month-old transgenic mice. Although the numbers of apoptotic hepatocytes and hepatic caspase 3 activities were similar between transgenic and nontransgenic mice, the numbers of proliferating hepatocytes and the levels of numerous proteins such as cyclin D1, cyclin-dependent kinase 4 and c-Myc, were markedly increased in an age-dependent manner in the transgenic mice. This increase was correlated with the activation of peroxisome proliferator-activated receptor α (PPARα). In these transgenic mice, spontaneous and persistent PPARα activation occurred heterogeneously, which was different from that observed in mice treated with clofibrate, a potent peroxisome proliferator. We further demonstrated that stabilization of PPARα through a possible interaction with HCV core protein and an increase in nonesterified fatty acids, which may serve as endogenous PPARα ligands, in hepatocyte nuclei contributed to the core protein-specific PPARα activation. In conclusion, these results offer the first suggestion that HCV core protein induces spontaneous, persistent, age-dependent and heterogeneous activation of PPARα in transgenic mice, which may contribute to the age-dependent and multicentric hepatocarcinogenesis mediated by the core protein.

Key words: cell-cycle regulator; peroxisome; nuclear stabilization; heterogeneous PPARα activation

Hepatitis C virus (HCV) is one of the major causes of chronic hepatitis, and persistent infection with this virus can lead to a high incidence of hepatocellular carcinoma (HCC).[1,2] The prevalence of HCC because of chronic HCV infection has increased over the past two decades,[3,4] and chronic HCV infection has therefore been recognized as a serious disease. However, the precise mechanism of hepatocarcinogenesis during chronic HCV infection remains unclear.

Many experiments using cell culture systems have suggested the possibility that HCV core protein itself can modulate various cellular functions and can be directly linked to the development of HCV-related HCC.[5] For example, HCV core protein transforms rat embryo fibroblasts to a tumorigenic phenotype in cooperation with the H-*ras* oncogene,[6] suppresses *c-myc*-related apoptosis[7] and transcription of the *p53* gene,[8] interacts with a variety of proteins, including helicase, lymphotoxin-β receptor, or dead box protein, and modulates their functions.[9] We further established transgenic mouse lines carrying the HCV core gene, in which the core protein is constitutively expressed in the liver at levels similar to that found in chronic hepatitis C patients.[10] These mice exhibited multicentric hepatic adenomas, and developed HCCs in an age-dependent manner.[11] The livers of these mice were almost free of inflammation, necrosis and fibrosis,[10,11] suggesting that the core protein itself has a hepatocarcinogenic potential *in vivo*. However, the molecular mechanism of the development of HCC in the transgenic mice has not been fully understood.

In the livers of HCV core gene transgenic mice, an age-dependent increase in oxidative stress and resultant DNA damage were found,[12] and these effects may contribute to or facilitate the development of HCC. Another possible mechanism of hepatocarcinogenesis is continuous enhancement of hepatocyte proliferation. Cell proliferation and apoptosis are highly regulated processes for maintaining homeostasis in many organs, and during the carcinogenic process, sustained imbalance generally precedes cancer.[13,14] For example, in patients with chronic HCV infection, high hepatocyte proliferative activity relative to apoptosis may reliably predict a new development of HCC.[15] However, there is no information about whether or not hepatocyte proliferation accelerates persistently in mice carrying the HCV core gene, and no information about how the core protein promotes hepatocyte proliferation *in vivo*. In the current study, we began to examine changes in the parameters of hepatocyte proliferation and apoptosis in the transgenic mice.

Material and methods

Animals and treatments

HCV core gene transgenic mice on a C57BL/6N genetic background were produced as described earlier.[10] Because HCC developed preferentially in male transgenic mice,[11] 9- and 22-month-old male mice ($n = 8$ for either age group) were adopted. Sex- and age-matched nontransgenic mice ($n = 8$ for either age group) were used as controls. These mice were fed an ordinary diet and were treated in a specific pathogen-free state according to the institutional guidelines. For additional experiment, male wild-type mice fed a control diet containing 0.5% clofibrate for 2 weeks ($n = 8$) were used. All mice were killed by cervical dislocation and the livers were excised. When a hepatic tumor was present, it was removed and the remaining liver tissue was used. All experiments were performed in accordance with animal study protocols approved by the Shinshu University School of Medicine.

Abbreviations: AOX, acyl-CoA oxidase; CDK, cyclin-dependent kinase; DAB, 3,3'-diaminobenzidine; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; L-FABP, liver-type fatty acid-binding protein; NEFA, nonesterified fatty acid; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PMSF, phenylmethylsulfonyl fluoride; PPAR, peroxisome proliferator-activated receptor; PT, peroxisomal thiolase; RXR, retinoid X receptor; SDS, sodium dodecyl sulfate; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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Preparation of hepatocyte nuclear fraction

Approximately 200 mg of liver tissues was transferred to a chilled Dounce homogenizer (Wheaton, Milliville, NJ) and homogenized on ice by 30 strokes in 1.2 mL of nuclei buffer [300 mM sucrose in 10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 5 mM $MgCl_2$ and 0.25 mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was filtered through gauze and centrifuged at 4,500*g* for 5 min at 4°C. The resulting pellet was resuspended, layered over 2 mL of nuclei buffer containing 2 M sucrose, and centrifuged at 23,000*g* for 1 hr at 4°C. The pellet obtained after ultracentrifugation was resuspended in 250 μL of nuclei buffer and used as the nuclear fraction. Preparation of nuclear fraction from isolated hepatocytes was performed as described elsewhere.[16]

Immunoblot analysis

Protein concentration was measured colorimetrically by a BCA™ Protein Assay kit (Pierce, Rockford, IL). For analysis of fatty acid-metabolizing enzymes and protein, whole liver lysate (10–20 μg protein) was subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.[17] For analysis of other proteins, hepatocyte nuclear fraction (100 μg protein) or whole liver lysate (200–300 μg protein) was subjected to electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membranes, which were incubated with the primary antibody, followed by alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG. The origin of the primary rabbit polyclonal antibodies against fatty acid-metabolizing enzymes and protein was described earlier.[17] For immunoblot analysis of peroxisome proliferator-activated receptor α (PPARα), a polyclonal anti-mouse antibody[18] or commercial antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used. The antibodies against cell-cycle regulators and oncogene products were purchased commercially (Santa Cruz Biotech.).[19] Equal loading of the protein obtained from whole liver lysate and nuclear fraction was confirmed by reprobing the membranes with an antibody against β-actin and histone H1, respectively. The band intensity of nuclear PPARα was quantified densitometrically, normalized to that of histone H1, and subsequently expressed as the fold changes relative to that of 9-month-old nontransgenic mice.

mRNA analysis

Total liver RNA was extracted with an RNeasy Mini Kit™ (Qiagen, Valencia, CA). Five microgram of RNA was electrophoresed on 1.1 M formaldehyde-containing 1% agarose gels and transferred to nylon membranes by capillary blotting in 20× SSC buffer (3 M NaCl and 300 mM sodium citrate, pH 7.0) overnight. The membranes were hybridized with ^{32}P-labeled cDNA probes. The blots were exposed to a phosphorimager screen cassette and were analyzed using a Molecular Dynamics Storm 860 Phosphorimager system (Sunnyvale, CA). The origin of the cDNA probes has been described elsewhere.[17–19] Northern blot of β-actin was used as the internal control. The blot intensity was quantified, normalized to that of β-actin and subsequently expressed as the fold changes relative to that of 9-month-old nontransgenic mice.

Pulse-label and pulse-chase experiment

Parenchymal hepatocytes were isolated from transgenic and control mice by the modified *in situ* perfusion method.[20] After perfusion with 0.05% collagenase solution (Wako, Osaka, Japan), the isolated hepatocytes were washed thrice by means of differential centrifugation and the dead cells removed by density gradient centrifugation on Percoll (Amersham Pharmacia Biotech, Buckinghamshire, UK). The live hepatocytes were washed and suspended in William's E medium containing 5% fetal bovine serum. When the viability of the isolated hepatocytes exceeded 85% as determined by the trypan blue exclusion test, the following experiments were conducted. The isolated hepatocytes were washed twice and incubated in methionine-free medium containing 5% dialyzed fetal bovine serum for 1 hr at 37°C. The medium was replaced with the same medium containing 300 μCi/mL of [^{35}S]methionine (Amersham Pharmacia Biotech.). After 3-hr of incubation, the labeled medium was changed to the standard medium and the preparation was chased for 4, 8 or 16 hr. The labeled cells were washed, homogenized and centrifuged for preparation of the nuclear fraction. The levels of radioactivity in the homogenates of the pulse-labeled preparations were similar between the transgenic and the nontransgenic mice, suggesting that the [^{35}S]methionine uptake capacity in the former hepatocytes is similar to that in the latter. The nuclear fraction was lysed in RIPA buffer [10 mM Tris-HCl, pH 7.4, 0.2% sodium deoxycholate, 0.2% Nonidet P-40, 0.1% SDS, 0.25 mM PMSF, 10 μg/mL aprotinin]. The lysate was incubated for 3 hr at 4°C with purified anti-PPARα antibody. The immune complexes were precipitated with *Staphylococcus aureus* protein A bound to agarose beads. After the precipitates had been washed in RIPA buffer, the labeled proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The nuclear fractions of the pulse-labeled preparations were also used for immunoblot analysis of PPARα.

Affinity chromatography for PPARα complex

All procedures were performed at 4°C. The nuclear fraction from the mouse liver was mixed with a 4-fold volume of a solution containing 12.5 mM potassium phosphate, pH 7.5, 25 mM NaCl, 0.25% Tween 20 and 0.1 mM PMSF. The mixture was briefly sonicated with a microsonicator, the Powersonic Model 50 (Yamato, Tokyo, Japan), and then centrifuged at 100,000*g* for 20 min. The supernatant was applied to an immobilized anti-PPARα IgG column (1.0 × 4.0 cm^2), prepared with the Affigel HZ Immunoaffinity kit^R (Bio-Rad, Hercules, CA) and equilibrated with 10 mM potassium phosphate, pH 7.5, 20 mM NaCl and 0.2% Tween 20. The solution was again passed through the column and this was repeated at least thrice. The column was washed and the elution performed with 150 mM sodium citrate, pH 3.0, and 200 mM NaCl, in a total volume of 2 mL. The eluate was resolved by 10 and 15% SDS-polyacrylamide gel electrophoresis for PPARα and the HCV core protein, respectively. The core protein expressed in COS cells was used as a positive marker.[21] The monoclonal antibody against the core protein was purchased commercially (ViroGen, Watertown, MA).

Cytochemical staining of peroxisomes

Liver peroxisome proliferation was evaluated by using 3,3′-diaminobenzidine (DAB) staining for catalase according to the method of Novikoff and Goldfischer with minor modifications.[22] Small pieces of liver were fixed with 2% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.2, for 3 hr at 4°C, rinsed with sodium cacodylate buffer and cut into 100-μm sections with a Lancer^R Vibratome 1000 (Lancer, Bridgeton, MO). These sections were then incubated for 1 hr at 37°C in the DAB reaction medium (0.2% DAB tetrahydrochloride in 50 mM propanediol, pH 9.7, 5 mM KCN, 0.05% H_2O_2) and postfixed with 1% OsO_4 in 100 mM sodium phosphate, pH 7.4 for 1 hr. The sections were dehydrated through a graded series of ethanol and acetone treatments and embedded in Epok 812 (Oken, Tokyo, Japan). One micrometer sections were prepared, counterstained with 0.1% toluidine blue solution and examined by light microscopy. For electron microscopic examination, 0.1-μm sections were cut with a diamond knife, collected on grid meshes, stained with lead citrate and uranyl acetate and visualized with a JEM 1200EX II electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 keV.

Morphometry of hepatic peroxisomes

Morphometric analysis of DAB-stained peroxisomes was carried out using electron photomicrographs. For each mouse, 10 independent fields in the pericentral area of liver lobuli were photomicrographed at an original magnification of 4,000×. At this magnification, peroxisomes smaller than 450 nm were clearly