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

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

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

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

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Appendix A. Supplementary data

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

References

[1] Iso H, Sato S, Kitamura A, et al. Metabolic syndrome and the risk of ischemic heart disease and stroke among Japanese men and women. Stroke 2007;38:1744–51.
[2] Kwon HM, Kim BJ, Lee SH, Choi SH, Oh BH, Yoon BW. Metabolic syndrome as an independent risk factor of silent brain infarction in healthy people. Stroke 2006;37:466–70.
[3] Wang J, Ruotsalainen S, Moilanen L, Lepisto P, Laakso M, Kuusisto J. The metabolic syndrome predicts incident stroke: a 14-year follow-up study in elderly people in Finland. Stroke 2008;39:1078–83.
[4] Fadini GP, Coracina A, Inchiostro S, Tiengo A, Avogaro A, de Kreutzenberg SV. A stepwise approach to assess the impact of clustering cardiometabolic risk factors on carotid intima-media thickness: the metabolic syndrome no-more-than-additive. Eur J Cardiovasc Prev Rehabil 2008;15:190–6.
[5] Kahn R, Buse J, Ferrannini E, Stern M. The metabolic syndrome: time for a critical appraisal: joint statement from the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care 2005;28:2289–304.
[6] Murakami S, Otsuka K, Hotta N, et al. Common carotid intima-media thickness is predictive of all-cause and cardiovascular mortality in elderly community-dwelling people: Longitudinal Investigation for the Longevity and Aging in Hokkaido County (LILAC) study. Biomed Pharmacother 2005;59(Suppl 1):S49–53.
[7] Mannami T, Baba S, Ogata J. Strong and significant relationships between aggregation of major coronary risk factors and the acceleration of carotid atherosclerosis in the general population of a Japanese city: the Suita Study. Arch Intern Med 2000;160:2297–303.
[8] Ishizaka N, Ishizaka Y, Hashimoto H, et al. Metabolic syndrome may not associate with carotid plaque in subjects with optimal, normal, or high-normal blood pressure. Hypertension 2006;48:411–7.
[9] Hsu PF, Chuang SY, Cheng HM, et al. Clinical significance of the metabolic syndrome in the absence of established hypertension and diabetes: a community-based study. Diabetes Res Clin Pract 2008;79:461–7.
[10] Stern MP, Williams K, Hunt KJ. Impact of diabetes/metabolic syndrome in patients with established cardiovascular disease. Atheroscler Suppl 2005;6:3–6.
[11] Ishizaka N, Ishizaka Y, Toda E, Hashimoto H, Nagai R, Yamakado M. Association between cigarette smoking, metabolic syndrome, and carotid arteriosclerosis in Japanese individuals. Atherosclerosis 2005;181:381–8.
[12] Ishizaka N, Ishizaka Y, Takahashi E, et al. Association between insulin resistance and carotid arteriosclerosis in subjects with normal fasting glucose and normal glucose tolerance. Arterioscler Thromb Vasc Biol 2003;23:295–301.
[13] Fowkes FG, Murray GD, Butcher I, et al. Ankle brachial index combined with Framingham Risk Score to predict cardiovascular events and mortality: a meta-analysis. JAMA 2008;300:197–208.
[14] Paras E, Mancini GB, Lear SA. The relationship of three common definitions of the metabolic syndrome with sub-clinical carotid atherosclerosis. Atherosclerosis 2008;198:228–36.
[15] Vaidya D, Szklo M, Liu K, et al. Defining the metabolic syndrome construct: Multi-Ethnic Study of Atherosclerosis (MESA) cross-sectional analysis. Diabetes Care 2007;30:2086–90.
[16] Lakka HM, Laaksonen DE, Lakka TA, et al. The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. JAMA 2002;288:2709–16.
[17] Wilson PW, D'Agostino RB, Parise H, Sullivan L, Meigs JB. Metabolic syndrome as a precursor of cardiovascular disease and type 2 diabetes mellitus. Circulation 2005;112:3066–72.
[18] Leoncini G, Ratto E, Viazzi F, et al. Metabolic syndrome is associated with early signs of organ damage in nondiabetic, hypertensive patients. J Intern Med 2005;257:454–60.
[19] Kawamoto R, Tomita H, Ohtsuka N, Inoue A, Kamitani A. Metabolic syndrome, diabetes and subclinical atherosclerosis as assessed by carotid intima-media thickness. J Atheroscler Thromb 2007;14:78–85.
[20] Tzou WS, Douglas PS, Srinivasan SR, et al. Increased subclinical atherosclerosis in young adults with metabolic syndrome: the Bogalusa Heart Study. J Am Coll Cardiol 2005;46:457–63.
[21] Pannier B, Thomas F, Bean K, et al. The metabolic syndrome: similar deleterious impact on all-cause mortality in hypertensive and normotensive subjects. J Hypertens 2008;26:1223–8.

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Administration of angiotensin II, but not catecholamines, induces accumulation of lipids in the rat heart

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ABSTRACT

Accumulation of lipids in the heart may cause cardiac dysfunction in various disorders, such as obesity and diabetes. In the current study, we have investigated whether administration of angiotensin II or norepinephrine induces accumulation of lipids and/or changes in the expression of genes related to lipid metabolism in the rat heart. Lipid deposition was found in myocardial, vascular wall, and perivascular cells of the angiotensin II-infused rat heart, and superoxide generation was increased in these lipid-positive cells. By contrast, intracardiac lipid deposition was not found in the heart of norepinephrine-induced hypertensive rats. Triglyceride content in the heart tissue of angiotensin II-infused rats increased more than 3-fold as compared with untreated controls. Losartan completely, but hydralazine only partially, suppressed the angiotensin II-induced intracardiac lipid deposition and increase in tissue triglyceride content. Administration of angiotensin II upregulated the mRNA expression of sterol regulatory element-binding protein-1c and fatty acid synthase, but downregulated that of uncoupling protein 2 and 3, in a manner dependent on the angiotensin AT_1 receptor. Collectively, these results suggest that angiotensin II may be involved in modulating both intracardiac lipid content and lipid metabolism-related gene expression, in part via an angiotensin AT_1 receptor-dependent and pressor-independent mechanism.

1. Introduction

Accumulation of lipids in non-adipose tissues can occur in certain disease conditions, including aging, over-nutrition, obesity, and diabetes, and may play a crucial role in the pathogenesis of tissue damage (Schaffer, 2003), a phenomenon referred to as lipotoxicity (Unger, 2002). Inappropriate accumulation of free fatty acids and neutral lipids can also be observed in the myocardium; this accumulation may result in both functional and morphological damage, such as systolic and/or diastolic dysfunction of the left ventricle (Chiu et al., 2005, Zhou et al., 2000), ventricular wall hypertrophy (Finck et al., 2003, Horiuchi et al., 1993), and interstitial fibrosis (Lee et al., 2004). In previous studies, we found that administration of angiotensin II to rats causes deposition of lipids in tubular epithelial and vascular wall cells in the kidney (Ishizaka et al., 2006, Saito et al., 2005), where cellular proliferation may be promoted. In the current study, we have investigated whether administration of two different pressor agents, angiotensin II and a catecholamine, causes intracardiac accumulation of lipids, and modulates the expression of genes related to lipid metabolism.

2. Materials and methods

2.1. Animal models

The experiments were performed in accordance with the guidelines for animal experimentation approved by the Animal Center for Biomedical Research, Faculty of Medicine, University of Tokyo. Angiotensin II-induced hypertension was induced in male Sprague-Dawley rats (250 to 300 g) by subcutaneous implantation of an osmotic minipump (Alza Pharmaceutical) as described previously (Ishizaka et al., 1997). Briefly, Val^5-angiotensin II (Sigma Chemical) was infused at doses of 0.7 mg/kg/day. Norepinephrine (Sigma Chemical) was infused at a dose of 2.8 mg/kg/day for 7 days using the same system. In some angiotensin II-infused rats, angiotensin AT_1 receptor antagonist, losartan (25 mg/kg/day), or the nonspecific vasodilator, hydralazine (15 mg/kg/day) (Sigma Chemical), both of which normalized the blood pressure of angiotensin II-infused rats, was given in the drinking water (Ishizaka et al., 2002).

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Table 1
Oligonucleotide primers used in this study

Gene	GenBank no.	Forward primer	Reverse primer
PPAR-α	NM_013196	GTGGCTGCTATAATTTGCTG	TGAAGGAGTTTTGGGAAGAG
PPAR-γ	NM_013124	ATCAGCTCTGTGGACCTCTC	AGGCTCTACTTTGATCGCAC
SREBP-1c	XM_213329	CTGATGGAGACAGGGAGTTC	ATCACCACGGCTGTCAGT
FAS	M76767	CTGGAACGTGAACATGATCT	TTCACCAGCAGGATACTCAG
HMG-CoA reductase	NM_013134	GACACTTACAATCTGTATGATG	CTTGGAGAGGTAAAACTGCCA
CPT-1	NM_031559	ATCGACCGCCATCTCTTC	CTCAAAGTCAAAGAGCTCCAC
CPT-2	NM_012930	TGACCAAAGAAGCAGCGAT	TTGTGGTTCATCTGCTGGTA
DGAT-1	NM_053437	TCTTCCTACCGGGATGTCAATC	TCCCTGCAGACACAGCTTTG
PGC-1α	AY237127	TCATTACCTACCGTTACACCT	CATACTTGCTCTTGGTGGAA
UCP2	BC062230	TGGTCGGAGATACCAGAG	GTCTGTCATGAGGTTGGCT
UCP3	AF035973	GTCCGATTTCAAGCCATGAT	CTTGTGATGTTGGGCCAAGT
Nox1	MN_053683	TGGACGAATTAGGCAAACCG	TTGGGGTGGGCAGTAGCTAT
Nox4	AY027527	AACACTGGTGAAGATTTGC	CTGAGGGATGATTGATTACTG
GAPDH	NM_017008	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA

PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element-binding protein; FAS, fatty acid synthase; HMG-CoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; CPT, carnitine palmitoyltransferase; DGAT, diacylglycerol acyltransferase; PGC, PPAR-γ coactivator; UCP, uncoupling protein; and GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2.2. Measurement of lipid contents in the serum and the heart

Serum levels of total cholesterol, triglycerides, and nonesterified fatty acid were measured by enzymatic methods (SRL). Contents of triglycerides, total cholesterol, and free cholesterol in the heart tissue were measured from homogenate extracts by enzymatic colorimetric determination using Triglyceride-E Test, Cholesterol-E Test, and Free cholesterol-E Test Wako, respectively (Wako Pure Chemicals).

2.3. Histological analysis

Oil red O staining was performed on sections of unfixed, freshly frozen heart samples (3 μm in thickness). The areas of lipid deposition were calculated by using the image analysis software, Photoshop (Adobe), and semiquantification of the lipid deposition was performed as described elsewhere (Ishizaka et al., 2006). Staining with the oxidative fluorescent dye dihydroethidium (DHE) was performed as described previously (Saito et al., 2004). Images were obtained with a fluorescent microscope BX51 (Olympus), and the fluorescence intensity, obtained from at least five fields for each section, was presented as the percentage of that of untreated control.

2.4. Western blot analysis

Western blot analysis was performed as described previously (Aizawa et al., 2000). Antibodies against total and phosphorylated forms AMP-activated protein kinase (Cell Signaling), sterol regulatory element-binding protein (SREBP)-1 (Santa Cruz Biotechnology), SREBP-2 (Santa Cruz Biotechnology), ATP-binding cassette transporter subfamily A1 (ABCA1) (Novus Biologicals), scavenger receptor class B type 1 (SR-B1) (Novus Biologicals), and mitochondrial superoxide dismutase (mt SOD) (Upstate) were used at a dilution of 1/1000.

2.5. Real time reverse transcription-polymerase chain reaction (RT-PCR)

Expression of lipid metabolism-related gene mRNA was analyzed by real time quantitative PCR performed by LightCycler together with hybriprobe technology (Roche Diagnostics). Expression of target genes was normalized to the mRNA expression of endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The target genes were as follows: peroxisome proliferator-activated receptor (PPAR)-α (Nihon Gene Research Lab's Inc., Sendai, Japan), PPAR-γ, SREBP-1c, fatty acid synthase (FAS), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR), carnitine palmitoyltransferase (CPT)-1, CPT-2, diacylglycerol acyltransferase (DGAT)-1, PPAR-γ coactivator (PGC)-1α, uncoupling protein (UCP)2, UCP3, Nox1, and Nox4. The forward and backward primers used are described in Table 1.

2.6. Statistical analysis

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

3. Results

3.1. Characteristics of experimental animals

The hemodynamic parameters in each group have been reported elsewhere (Aizawa et al., 2000). Angiotensin II and norepinephrine elevated the blood pressure to a similar extent, and both hydralazine and losartan completely suppressed the blood pressure elevation induced by angiotensin II. Angiotensin II, but not norepinephrine, significantly increased the serum levels of triglycerides and non-esterified fatty acids, and these increases were inhibited by losartan, but not by hydralazine (Fig. 1A–C).

3.2. Tissue contents of lipids

The tissue content of triglycerides, total cholesterol, and free cholesterol was found to be increased in the heart of angiotensin II-

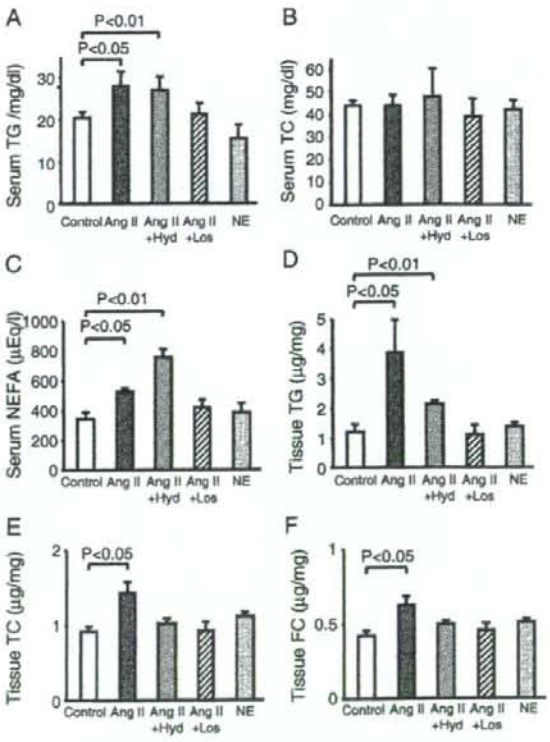

Fig. 1. Serum levels and tissue content of lipids. A–C. Serum levels of triglycerides (TG) (A), total cholesterol (TC) (B), and non-esterified fatty acids (NEFA) (C). D–E. Content of triglycerides (D), total cholesterol (E), free cholesterol (FC) (F) in the heart tissue. Shown in a summary of data from 4–6 rats in each group. Ang II, angiotensin II; Hyd, hydralazine; Los, losartan; and NE, norepinephrine.

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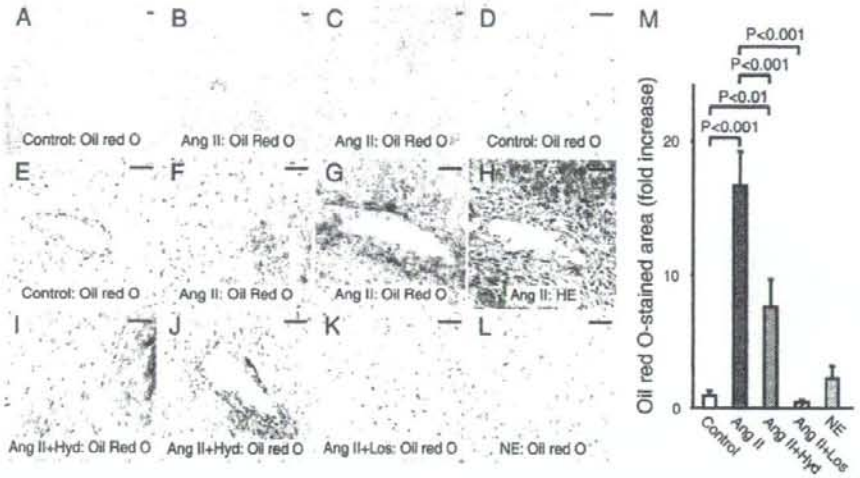

Fig. 2. Accumulation of lipids in the heart. A, D, E. Heart section from a control rat. B, C, F–H. Heart sections from angiotensin II (Ang II)-infused rats. I, J. Heart section from a rat given both angiotensin II and hydralazine (Hyd). K. Heart section from a rat given both angiotensin II and losartan (Los). L. Heart section from a rat given norepinephrine (NE). F and G are serial sections. A–G, I–L. Oil red O staining. H. Hematoxylin eosin (HE) staining. Lipid droplets were not observed in the myocardium or vascular regions (A, D, E) of control rats. Lipid droplets were present in both the myocardium (B, C, F) and perivascular regions (G) in the heart of angiotensin II-infused rats. Lipid droplets in the myocardium (I) and perivascular regions (J) were observed in the heart of rats given both angiotensin II and hydralazine, but not in the heart of rats given angiotensin II plus losartan (K) or those given norepinephrine (L). Original magnification, ×100 (A–C), and ×200 (D–L). Scale bars indicate 50 μm. M. Semiquantification of the oil red O-stained area. Shown is a summary of data from 5–7 experiments in each group.

infused rats, but not norepinephrine-infused rats (Fig. 1). Hydralazine only partially suppressed the angiotensin II-induced increase in intracardiac triglyceride content, but it completely suppressed the increase in intracardiac total cholesterol and free cholesterol content (Fig. 1D–F). Losartan suppressed the angiotensin II-induced increase in all three lipid fractions tested. Administration of losartan alone or hydralazine alone did not significantly alter the lipid content of the heart (losartan: triglycerides, 1.53±0.12 μg/mg, $n=4$; total cholesterol, 1.16±0.07 μg/mg, $n=3$; free cholesterol, 0.53±0.04 μg/mg, $n=4$; hydralazine: triglycerides, 1.40±0.14 μg/mg, $n=4$; total cholesterol, 1.09±0.14 μg/mg, $n=4$; free cholesterol, 0.43±0.04 μg/mg, $n=5$).

3.3. Staining for lipids

Oil red O staining of heart sections showed no apparent lipid deposition in the heart of untreated rats (Fig. 2A, D, E). By contrast, accumulation of oil red O-stainable lipid was observed in the myocardium as well as the arterial wall of angiotensin II-infused rats (Fig. 2B, C, F, G). In the angiotensin II-infused rat heart, lipid accumulation was also observed in perivascular regions, especially where remodeling of perivascular regions was apparent (Fig. 2G, H), and in granulation regions (data not shown). Lipid deposition remained present in the heart when angiotensin II-infused rats were concomitantly treated with hydralazine (Fig. 2I, J). On the other hand, lipid deposition was not apparent, or was very minor when present, in heart sections from rats treated with both angiotensin II and losartan or from rats treated with norepinephrine infusion (Fig. 2K, L). Semiquantitative measurements of the oil red O-stained areas are summarized in Fig. 2M.

3.4. Co-localization of lipid deposition and superoxide

As compared with untreated controls, DHE staining-positive signals were increased in the heart of angiotensin II-infused rats, and

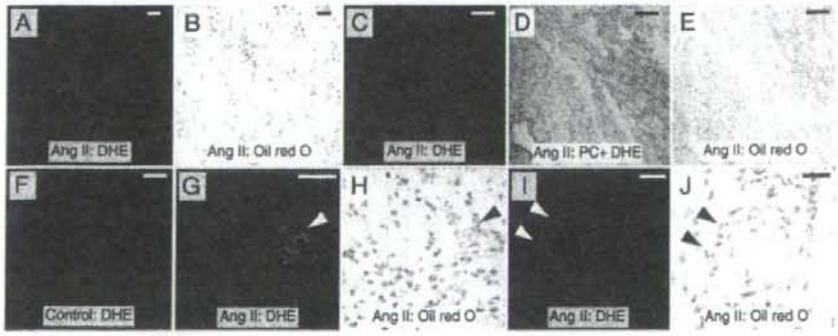

Fig. 3. Lipid and superoxide staining of the heart section. A–E, F–J. Heart sections from angiotensin (Ang) II-infused rats. F. Heart section from a control rat. A, C, F, G, I Dihydroethidium (DHE) staining. D. Phase contrast (PC) microscopic image overlaid with DHE staining image. B, E, H, J. Oil red O staining. C and D are the same section. C (=D)–E, G–H, and I–J are serial sections. Some cells with intense DHE staining (arrowheads in G and I) contained lipid deposits (arrowheads in H and J). Original magnification, ×100 (A–C), ×200 (C–H), and ×400 (I, J). Scale bars indicate 50 μm.

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Table 2
mRNA levels of genes related to lipid metabolism

Gene	Control (n=6)	Ang II (n=6)	P	Ang II+Hyd (n=5)	P	Ang II+Los (n=6)	P	NE (n=7)	P
PPAR-α	1±0.17	1.88±0.34	0.030	2.99±0.64	0.005	1.41±0.20	0.080	2.00±0.18	0.001
PPAR-γ	1±0.17	3.30±0.98	0.019	4.25±1.13	0.032	0.83±0.09	0.19	3.56±0.44	<0.001
SREBP-1c	1±0.24	3.66±1.02	0.008	2.67±0.96	0.039	0.71±0.12	0.14	0.77±0.17	0.21
FAS	1±0.17	2.97±0.32	<0.001	3.46±1.00	<0.001	1.30±0.17	0.18	1.28±0.13	0.18
HMG-CoA reductase	1±0.20	2.29±0.30	<0.001	2.50±0.66	0.009	0.99±0.23	0.49	0.98±0.33	0.48
CPT-1	1±0.06	0.55±0.09	<0.001	1.08±0.29	0.395	0.82±0.15	0.154	0.16±0.03	<0.001
CPT-2	1±0.04	0.63±0.06	<0.001	0.67±0.10	<0.001	0.66±0.06	<0.001	0.67±0.05	<0.001
DGAT-1	1±0.04	1.20±0.12	0.071	0.58±0.18	0.003	0.60±0.04	<0.001	0.87±0.12	0.14
PGC-1α	1±0.09	0.52±0.06	<0.001	0.59±0.18	<0.005	0.94±0.18	0.395	1.34±0.31	0.17
UCP2	1±0.08	0.51±0.08	<0.001	0.39±0.08	<0.001	0.80±0.09	0.055	1.53±0.79	0.276
UCP3	1±0.06	0.75±0.09	0.020	0.50±0.11	<0.001	0.74±0.14	0.037	2.10±0.49	0.038
Nox1	1±0.21	3.31±0.61	0.006	4.87±1.82	0.026	0.90±0.19	0.378	1.17±0.42	0.367
Nox4	1±0.21	5.25±2.22	0.047	0.72±0.10	0.093	1.17±0.12	0.199	1.17±0.14	0.206

P values are versus untreated control. Ang II, angiotensin II; Hyd, hydralazine; Los, losartan; and NE, norepinephrine. Other abbreviations were same as Table 1.

semiquantitative measurements showed that the DHE-stained area was significantly greater after angiotensin II infusion (control 100±37%, $n=5$, versus angiotensin II 342±125%, $n=5$; $P<0.05$). In the heart of angiotensin II-infused rats, some myocardial cells that had increased superoxide staining were found to be positive for lipid deposition (lower magnification in Fig. 3A, B, and higher magnification in Fig. 3C–D). Similarly, some vascular wall and perivascular cells with increased superoxide staining were found to contain lipid deposits (Fig. 3G–J).

3.5. Regulation of genes related to lipid metabolism

Next, we examined the expression of lipid metabolism-related genes after infusion of the pressor agents (Table 2). mRNA expression of PPAR-α, PPAR-γ, SREBP-1c, FAS, and HMG-CoAR was found to be increased in the heart of rats that received angiotensin II infusion. Of the genes tested, mRNA expression of PPAR-α and PPAR-γ was also increased in the heart of the norepinephrine-infused rat. The expression of PGC-1α, UCP2 and UCP3 was decreased after angiotensin II infusion, but not after norepinephrine infusion. The angiotensin II-induced regulation of these genes (PPAR-α, PPAR-γ, SREBP-1c, FAS, HMG-CoAR, PGC-1α, UCP2, and UCP3) was suppressed by losartan, but not by hydralazine. On the other hand, mRNA expression of CPT-1 and CPT-2 was downregulated by angiotensin II. We found that the angiotensin II-induced CPT-1 downregulation was suppressed by depressor agents, and that norepinephrine also downregulated CPT-1 mRNA expression; therefore, the angiotensin II-induced CPT-1 mRNA downregulation might be induced by hypertension per se. Angiotensin II increased the mRNA expression of two components of NAD(P)H oxidase, Nox1 and Nox4.

Angiotensin II did not alter the protein expression of AMPKα; however, it increased the levels of phosphorylated AMPKα, and this increase was inhibited by either depressor agent (Fig. 4). Protein expression of matured SREBP-1 was increased by angiotensin II, and this increase was suppressed by losartan, but not by hydralazine.

We also examined the expression of several other lipid metabolism-related proteins. In the heart of control ($n=4$) and angiotensin II-

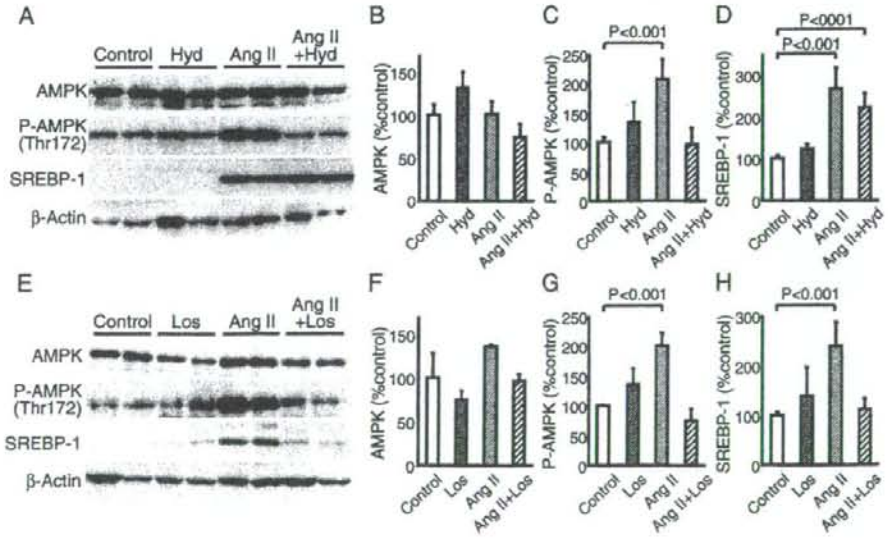

Fig. 4. Western blot analysis of AMP-activated protein kinase (AMPK), phosphorylated (activated) form of AMPKα (P-AMPK), and SREPB-1. A, E. Representative blots. B–D, F–H. Summary of data from 4–6 experiments in each group. Abbreviations are same as Table 1.

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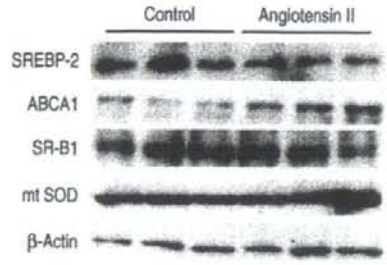

Fig. 5. Western blot analysis of proteins related to lipid metabolism. Shown are the results of the expression in the heart of control and angiotensin II-infused rats of the following proteins: Sterol regulatory element-binding protein (SREBP)-2, ATP-binding cassette transporter subfamily A-1 (ABCA1), scavenger receptor class B type 1 (SR-B1), mitochondrial superoxide dismutase (mt SOD).

infused ($n=4$) rats, the expression of these proteins was, respectively (% control): SREBP-2: 100±17 versus 78±14 (P=NS); ABCA1: 100±10 versus 172±7 ($P<0.001$); SR-B1: 100±17 versus 127±18 (P=NS); mit SOD: 100±5 versus 110±18 (P=NS) (Fig. 5).

4. Discussion

In the present study, we showed that administration of angiotensin II, but not catecholamines, caused accumulation of lipids in myocardial, vascular wall, and perivascular cells in the rat heart. Such angiotensin II-induced lipid deposition, as well as the increases in tissue triglyceride content in the heart, was suppressed completely by losartan, but only partially by hydralazine. These findings collectively indicate that the accumulation of intracardiac lipids induced by angiotensin II was, at least in part, independent of the pressor properties of angiotensin II.

Intracardiac lipid accumulation, which is sometimes designated 'cardiac steatosis' (McGavock et al., 2007), is known to occur in humans in certain diseased conditions, such as diabetes and heart failure (McGavock et al., 2007, Sharma et al., 2004). By means of genetic engineering, several animal models showing an amount of intracardiac lipids have been generated; these models include mice with cardiac-specific overexpression of acyl CoA synthase (Lee et al., 2004), fatty acid transport protein 1(Chiu et al., 2005), and PPAR-α (Finck et al., 2003), and mice with cardiac-restricted deletion of PPAR-delta (Cheng et al., 2004). The observation that accumulation of excessive fatty acids aggravates, whereas reduction of cardiac lipid content ameliorates, the structural and functional damage in these models supports the notion that accumulation of excessive lipid may indeed be cardiotoxic. In our previous studies, we found that administration of angiotensin II, but not catecholamines, caused marked accumulation of neutral lipids in the kidney (Ishizaka et al., 2006, Saito et al., 2005), leading us to investigate whether these two pressor agents affect cardiac lipid content differently in the current study.

What would be the mechanism underlying angiotensin II-induced intracardiac lipid deposition? We found that angiotensin II upregulated the expression of SREBP-1c, FAS, and HMG-CoAR, and downregulated that of UCP2, and UCP3; in addition, the pattern of regulation paralleled intracardiac lipid accumulation. It has been reported that angiotensin II upregulates the expression of SREBP-1c and FAS, resulting in increased lipogenesis in adipocytes in vitro (Jones et al., 1997, Kim et al., 2001). In addition, although the physiological functions of UCP2 and UCP3 are not well-established, downregulation of these new UCPs may augment the production of reactive oxygen species and decrease the catalysis of transported fatty acids (Affourtit et al., 2007). We also found that angiotensin II upregulated PPAR-α mRNA expression. Overexpression of PPAR-α in the heart may also cause lipotoxic cardiomyopathy (Finck et al., 2003, Vikramadithyan et al., 2005), suggesting that PPAR-α upregulation might be an underlying mechanism linking angiotensin II administration and cardiac lipid deposition.

Several previous studies have shown that PPAR-α activator may ameliorate myocardial damage induced by angiotensin II (Fujita et al., 2008, Ichihara et al., 2006). In the current study, we also found that PPAR-α expression was increased by norepinephrine infusion, which did not cause apparent cardiac lipid accumulation, indicating that upregulation of cardiac PPAR-α may not solely account for lipid accumulation in the heart. Whether or not PPAR-α activator acts to enhance or to suppress angiotensin II-induced lipid accumulation in the heart should be examined in future studies.

Activation of AMPK may result in the phosphorylation of acetyl CoA carboxylase, followed by the reduction of malonyl CoA and the subsequent activation and upregulation of CPT-1, leading to the stimulation of fatty acid oxidation (Affourtit et al., 2007). In the current study, we found that angiotensin II activated cardiac AMPK; however, it downregulated CPT-1 mRNA expression. Tian et al. (2001) have recently reported that pressure overload-induced cardiac hypertrophy causes a significant increase in AMPK activity in the heart that is, unexpectedly, accompanied by a downregulation of CPT-1 expression. They presumed that, unlike short-term activation, prolonged activation of AMPK might result in a downregulation of the enzymes that would be critical to fatty acid oxidation. With regard to this, it may be of note that, in the current study, both AMPK activation and CPT-1 downregulation by angiotensin II were suppressed not only by losartan, but also by hydralazine, and that CPT-1 mRNA downregulation was also induced by norepinephrine-induced hypertension, suggesting that these events were induced not in an angiotensin II-specific manner, but rather by hypertension itself.

It has been reported that UCP2 may reduce the generation of ROS, and conversely, downregulation of uncoupling proteins may increase the generation of ROS (Arsenijevic et al., 2000). On the other hand, enhanced oxidative stress or increased amounts of ROS may activate or upregulate SREBP-1 and FAS (Furuta et al., 2008, Gharavi et al., 2006). In addition, CuZn-SOD deficiency has been reported to increase lipid accumulation in the liver (Uchiyama et al., 2006). We found in our previous study (Saito et al., 2005) and the current one that superoxide is histologically co-localized with lipid deposition in the heart and kidney of angiotensin II-infused rats. Taken together, these findings may collectively suggest that angiotensin II-induced deposition of lipid in the heart may be evoked, at least in part, by enhanced oxidative stress (Fig. 5). This hypothesis should be examined in future studies (Fig. 6).

In conclusion, administration of angiotensin II to rats induced intracardiac lipid accumulation in regions where superoxide

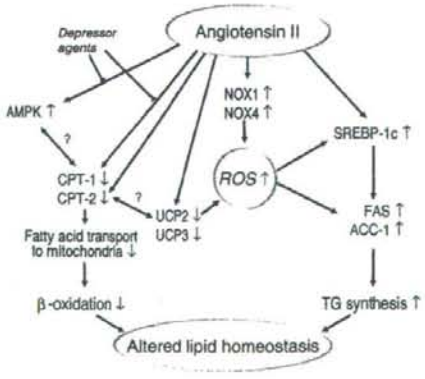

Fig. 6. Working hypothesis on angiotensin II-induced altered lipid homeostasis. Abbreviations are same as in Table 1. ROS indicates reactive oxygen species.

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production was found to be increased. The angiotensin II-induced accumulation of intracardiac lipids, in addition to regulation of the expression of several lipid metabolism-related genes (SREBP-1c, FAS, HMG-CoAR, PGC-1α, UCP2, and UCP3), events that were not mimicked by catecholamine infusion, were found to be dependent on the angiotensin AT_1 receptor. The physiological significance of angiotensin II-induced cardiac lipid accumulation and the role of enhanced oxidative stress on this phenomenon await further investigation.

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References

Affourtit, C., Crichton, P.G., Parker, N., Brand, M.D., 2007. Novel uncoupling proteins. Novartis Found. Symp. 287, 70–80 Discussion 80–91.

Aizawa, T., Ishizaka, N., Taguchi, J., Nagai, R., Mori, I., Tang, S.S., Ingelfinger, J.R., Ohno, M., 2000. Heme oxygenase-1 is upregulated in the kidney of angiotensin II-induced hypertensive rats: possible role in renoprotection. Hypertension 35, 800–806.

Arsenijevic, D., Onuma, H., Pecqueur, C., Raimbault, S., Manning, B.S., Miroux, B., Couplan, E., Alves-Guerra, M.C., Goubern, M., Surwit, R., Bouillaud, F., Richard, D., Collins, S., Ricquier, D., 2000. Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. Nat. Genet. 26, 435–439.

Cheng, L., Ding, G., Qin, Q., Huang, Y., Lewis, W., He, N., Evans, R.M., Schneider, M.D., Brako, F.A., Xiao, Y., Chen, Y.E., Yang, Q., 2004. Cardiomyocyte-restricted peroxisome proliferator-activated receptor-delta deletion perturbs myocardial fatty acid oxidation and leads to cardiomyopathy. Nat. Med. 10, 1245–1250.

Chiu, H.C., Kovacs, A., Blanton, R.M., Han, X., Courtois, M., Weinheimer, C.J., Yamada, K.A., Brunet, S., Xu, H., Nerbonne, J.M., Welch, M.J., Fettig, N.M., Sharp, T.L., Sambandam, N., Olson, K.M., Ory, D.S., Schaffer, J.E., 2005. Transgenic expression of fatty acid transport protein 1 in the heart causes lipotoxic cardiomyopathy. Circ. Res. 96, 225–233.

Finck, B.N., Han, X., Courtois, M., Aimond, F., Nerbonne, J.M., Kovacs, A., Gross, R.W., Kelly, D.P., 2003. A critical role for PPARalpha-mediated lipotoxicity in the pathogenesis of diabetic cardiomyopathy: modulation by dietary fat content. Proc. Natl. Acad. Sci. U. S. A. 100, 1226–1231.

Fujita, K., Maeda, N., Sonoda, M., Ohashi, K., Hibuse, T., Nishizawa, H., Nishida, M., Hiuge, A., Kurata, A., Kihara, S., Shimomura, I., Funahashi, T., 2008. Adiponectin protects against angiotensin II-induced cardiac fibrosis through activation of PPAR-alpha. Arterioscler. Thromb. Vasc. Biol. 28, 863–870.

Furuta, E., Pai, S.K., Zhan, R., Bandyopadhyay, S., Watabe, M., Mo, Y.Y., Hirota, S., Hosobe, S., Tsukada, T., Miura, K., Kamada, S., Saito, K., Iiizumi, M., Liu, W., Ericsson, J., Watabe, K., 2008. Fatty acid synthase gene is up-regulated by hypoxia via activation of Akt and sterol regulatory element binding protein-1. Cancer Res. 68, 1003–1011.

Gharavi, N.M., Baker, N.A., Mouillesseaux, K.P., Yeung, W., Honda, H.M., Hsieh, X., Yeh, M., Smart, E.J., Berliner, J.A., 2006. Role of endothelial nitric oxide synthase in the regulation of SREBP activation by oxidized phospholipids. Circ. Res. 98, 768–776.

Horiuchi, M., Yoshida, H., Kobayashi, K., Kuriwaki, K., Yoshimine, K., Tomomura, M., Koizumi, T., Nikaido, H., Hayakawa, J., Kuwajima, M., et al., 1993. Cardiac hypertrophy in juvenile visceral steatosis (jvs) mice with systemic carnitine deficiency. FEBS Lett. 326, 267–271.

Ichihara, S., Obata, K., Yamada, Y., Nagata, K., Noda, A., Ichihara, G., Yamada, A., Kato, T., Izawa, H., Murohara, T., Yokota, M., 2006. Attenuation of cardiac dysfunction by a PPAR-alpha agonist is associated with down-regulation of redox-regulated transcription factors. J. Mol. Cell. Cardiol. 41, 318–329.

Ishizaka, N., de Leon, H., Laursen, J.B., Fukui, T., Wilcox, J.N., De Keulenaer, G., Griendling, K.K., Alexander, R.W., 1997. Angiotensin II-induced hypertension increases heme oxygenase-1 expression in rat aorta. Circulation 96, 1923–1929.

Ishizaka, N., Aizawa, T., Yamazaki, I., Usui, S., Mori, I., Kurokawa, K., Tang, S.S., Ingelfinger, J.R., Ohno, M., Nagai, R., 2002. Abnormal iron deposition in renal cells in the rat with chronic angiotensin II administration. Lab. Invest. 82, 87–96.

Ishizaka, N., Matsuzaki, G., Saito, K., Noiri, E., Mori, I., Nagai, R., 2006. Expression and localization of PDGF-B, PDGF-D, and PDGF receptor in the kidney of angiotensin II-infused rat. Lab. Invest. 86, 1285–1292.

Jones, B.H., Standridge, M.K., Moustaid, N., 1997. Angiotensin II increases lipogenesis in 3T3-L1 and human adipose cells. Endocrinology 138, 1512–1519.

Kim, S., Dugail, I., Standridge, M., Claycombe, K., Chun, J., Moustaid-Moussa, N., 2001. Angiotensin II-responsive element is the insulin-responsive element in the adipocyte fatty acid synthase gene: role of adipocyte determination and differentiation factor 1/sterol-regulatory-element-binding protein 1c. Biochem. J. 357, 899–904.

Lee, Y., Naseem, R.H., Duplomb, L., Park, B.H., Garry, D.J., Richardson, J.A., Schaffer, J.E., Unger, R.H., 2004. Hyperleptinemia prevents lipotoxic cardiomyopathy in acyl CoA synthase transgenic mice. Proc. Natl. Acad. Sci. U. S. A. 101, 13624–13629.

McGavock, J.M., Lingvay, I., Zib, I., Tillery, T., Salas, N., Unger, R., Levine, B.D., Raskin, P., Victor, R.G., Szczepaniak, L.S., 2007. Cardiac steatosis in diabetes mellitus: a 1H-magnetic resonance spectroscopy study. Circulation 116, 1170–1175.

Saito, K., Ishizaka, N., Aizawa, T., Sata, M., Iso, O.N., Noiri, E., Ohno, M., Nagai, R., 2004. Role of aberrant iron homeostasis in the upregulation of transforming growth factor-beta1 in the kidney of angiotensin II-induced hypertensive rats. Hypertens. Res. 27, 599–607.

Saito, K., Ishizaka, N., Hara, M., Matsuzaki, G., Sata, M., Mori, I., Ohno, M., Nagai, R., 2005. Lipid accumulation and transforming growth factor-beta upregulation in the kidneys of rats administered angiotensin II. Hypertension 46, 1180–1185.

Schaffer, J.E., 2003. Lipotoxicity: when tissues overeat. Curr. Opin. Lipidol. 14, 281–287.

Sharma, S., Adrogue, J.V., Golfman, L., Uray, I., Lemm, J., Youker, K., Noon, G.P., Frazier, O.H., Taegtmeyer, H., 2004. Intramyocardial lipid accumulation in the failing human heart resembles the lipotoxic rat heart. Faseb. J. 18, 1692–1700.

Tian, R., Musi, N., D'Agostino, J., Hirshman, M.F., Goodyear, L.J., 2001. Increased adenosine monophosphate-activated protein kinase activity in rat hearts with pressure-overload hypertrophy. Circulation 104, 1664–1669.

Uchiyama, S., Shimizu, T., Shirasawa, T., 2006. CuZn-SOD deficiency causes ApoB degradation and induces hepatic lipid accumulation by impaired lipoprotein secretion in mice. J. Biol. Chem. 281, 31713–31719.

Unger, R.H., 2002. Lipotoxic diseases. Annu. Rev. Med. 53, 319–336.

Vikramadithyan, R.K., Hirata, K., Yagyu, H., Hu, Y., Augustus, A., Homma, S., Goldberg, I.J., 2005. Peroxisome proliferator-activated receptor agonists modulate heart function in transgenic mice with lipotoxic cardiomyopathy. J. Pharmacol. Exp. Ther. 313, 586–593.

Zhou, Y.T., Grayburn, P., Karim, A., Shimabukuro, M., Higa, M., Baetens, D., Orci, L., Unger, R.H., 2000. Lipotoxic heart disease in obese rats: implications for human obesity. Proc. Natl. Acad. Sci. U. S. A. 97, 1784–1789.

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Original Paper

Influence of Risk Factors for Metabolic Syndrome and Non-Alcoholic Fatty Liver Disease on the Progression and Prognosis of Hepatocellular Carcinoma

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ABSTRACT

Background/Aims: We investigated a relationship between the risk factors for metabolic syndrome, such as obesity, diabetes mellitus, hypertension, and hyperlipidemia, and the pathogenesis and outcome of hepatocellular carcinoma (HCC).

Methodology: One hundred twenty four patients who underwent curative resections for HCC were classified into 3 groups: those patients who were positive for hepatitis B surface antigen (group B), those positive for antibody to hepatitis C virus (group C), and those negative for both of them (non-B non-C) (group NBNC). The preoperative laboratory data, risk factors for metabolic syndrome, history of alcohol abuse, and outcome after surgery were investigated. The presence of non-alcoholic steatohepatitis (NASH) was also evaluated.

Results: The incidence of diabetes mellitus, hyperlipidemia, and alcohol abuse, and the serum level of triglyceride were significantly higher in group NBNC than in groups B or C. The risk factors for metabolic syndrome tended to lower the survival rates in group B and C, but not in group NBNC. Three of the 37 non-B non-C patients were associated with NASH.

Conclusions: It is suggested that the pathogenesis of non-B non-C HCC may be more closely associated with the risk factors for metabolic syndrome than that of hepatitis virus related HCC.

KEY WORDS: Hepatocellular carcinoma; Non-B non-C; Metabolic syndrome; Nonalcoholic steatohepatitis

ABBREVIATIONS: Hepatocellular Carcinoma (HCC); Hepatitis B Virus (HBV); Hepatitis C Virus (HCV); Hepatitis B Surface Antigen (HBsAg); Antibody Against HCV (HCVAb); Metabolic Syndrome (MS); Nonalcoholic Fatty-liver Disease (NAFLD); Nonalcoholic Steatohepatitis (NASH); Anibody Against Hepatitis B Surface Antigen (HBsAb); Antibody against Hepatitis B Core Antigen (HBcAb); Platelet Count (PLT); Asparate Aminotransferase (AST); Alanine Aminotransferase (ALT); Total Cholesterol (TC); Triglyceride (TG); Body Mass Index (BMI)

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers, and its incidence has increased worldwide (1). Epidemiological studies and recent molecular biological investigations have revealed that hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are major causes of HCC, and hence numerous basic and clinical studies have been conducted from the view point of hepatitis virus infection (1-6). On the other hand, little attention has been paid to HCC in patients without both hepatitis B surface antigen (HBsAg) and antibody against HCV (HCVAb), possibly because of the small population of these HCC patients (7-12). However, the number of HCC patients without apparent hepatitis virus infection has been increasing, and the so-called non-B non-C HCC has been the subject of investigation in recent years (10,11).

Recently, other factors such as obesity and type 2 diabetes mellitus, which were regarded as risk factors of metabolic syndrome (MS) (13), have been implicated in steatosis and fibrosis of the liver, and HCC (14-18). In addition, although the natural histories of non-alcoholic fatty-liver disease (NAFLD) or nonalcoholic steatohepatitis (NASH) have remained controversial, they have been considered to be one of the possible causes of cryptogenic cirrhosis of the liver and HCC (19-25). However, the relationship between HCC and NASH, as well as the incidence of HCC developing in the liver in response to NASH, has not been well understood. Moreover, the relationship between risk factors for MS, such as hyperlipidemia and hypertension, and HCC still remain unclear.

Based on this point of view, the present study investigated the participation of risk factors for MS in the pathogenesis and progression of HCC, and the outcome after surgical treatment. Evidence will be presented indicating that non-B non-C HCC is more closely related to those diseases with risk factors for MS than HCC associated hepatitis virus infection.

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METHODOLOGY

A total of 124 patients who underwent curative resections for HCC from April 2000 to March 2005 were enrolled in this study, and their medical records were reviewed retrospectively.

Twenty-six of the 124 patients had already undergone prior treatments including surgery, transcatheter arterial (chemo) embolization, and/or ablation therapy for HCC before being referred to our department.

The patients were divided into 3 groups: groups B, C, and NBNC. Those patients who were positive for HBsAg but negative for HCVAb were classified into the group B, and those who were negative for HBsAg but positive for HCVAb were classified as group C. The NBNC group was composed of the non-B non-C patients. In the non-B non-C patients, the antibody against hepatitis B surface antigen (HBsAb) and the antibody against hepatitis B core antigen (HBcAb) were also measured.

We examined the platelet count (PLT) and serum levels of asparate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), and triglyceride (TG) preoperatively. In addition, we investigated the body mass index (BMI), any history of alcohol abuse and risk factors for MS, which consisted of diabetes mellitus, hypertension, and hyperlipidemia (fasting plasma level of TC≥220mg/dL and/or TG≥150mg/dL), in each patient. All of the patients with diabetes mellitus and/or hypertension had already been diagnosed and treated before consulting us. Being overweight was defined as a BMI of 25kg/m^2 or more. A daily ethanol intake of 86g or more for 10 years was regarded as alcohol abuse.

The liver histology as well as the presence of NAFLD and NASH was evaluated in the resected specimens microscopically, especially in those patients without a history of alcohol consumption (daily alcohol intake within 20g) in the NBNC group. If there was moderate to severe macrovesicular steatosis, hepatocellular ballooning, lobular inflammation with necrosis of the hepatocytes and perisinusoidal fibrosis, we considered that NASH was present. The stage of the HCC was defined according to the Japanese general rules of primary liver cancer (26).

After surgical treatment, all of the patients were followed as out patients, and none of the patients underwent adjuvant therapies. Tumor markers such as alpha-fetoprotein and protein induced by vitamin K antagonist-II were measured every month, and ultrasonography, enhanced computed tomography, and/or magnetic resonance imaging were performed every 3 months. If an intrahepatic recurrence was suspected, computed tomography with angiography was performed to confirm.

We calculated the cumulative overall and disease free survival rates in each group, particularly for those patients with and without risk factors for MS.

The data are expressed as means ± standard deviation. Statistical analyses were performed by a one-way analysis of variance for parametric data, and by a Kruskal-Wallis test for categorical data among the three groups. If there was a significant difference on the Kruskal-Wallis test, a further analysis was performed by the Mann-Whitney test with Bonferroni's correction. Both the cumulative overall survival and disease free survival rates were calculated using the Kaplan-Meier method, and comparisons were made by using the log-rank test. A p-value of less than 0.05 was considered statistically significant.

TABLE 1 The Backgrounds of the 124 Patients

	B	C	NBNC	p
N	19	68	37	
Age	55.9±11.3	67.8±6.6	67.7±8.4	<0.0001[a]
Gender (Male/Female)	15 / 4	49 / 19	30 / 7	
Child-Pugh				N.S.
A	18	58	35	
B	1	10	1	
C	0	0	1	
Stage*				N.S.
I	1	13	2	
II	5	16	12	
III	7	27	17	
IVA	6	12	6	
Liver histology				0.03[b]
Normal liver	2	0	7	
Chronic hepatitis	8	31	17	
Liver cirrhosis	9	37	13	
Surgical procedure*				N.S.
Hr0	4	31	12	
HrS	8	14	8	
Hr1	2	12	9	
Hr2	5	10	8	
Hr3	0	1	0	
History of previous treatment	3	18	5	

*Stage and surgical procedure were defined according to the general rules for the clinical and pathological study of primary liver cancer, 4th edition. Hr 0: partial resection; Hr S: subsegmentectomy; Hr1: segmentectomy; Hr2: liver resection of 2 segments; Hr3: liver resection of 3 segments. N.S.: Not significant; [a]: B *vs.* NBNC and C; [b]: NBNC *vs.* C

TABLE 2 Preoperative Laboratory Data

	B	C	NBNC	p
AST (IU/L)	58.4±58.0	67.9±37.6	36.7±18.5	<0.001[a]
ALT (IU/L)	47.8±25.3	65.5±46.4	34.1±19.5	<0.001[b]
PLT (×104/mm^3)	15.5±5.5	13.7±7.7	19.6±12.2	<0.01[b]
Total Cholesterol (mg/dL)	174.0±46.3	152.1±35.9	180.1±39.1	<0.01[c]
Triglyceride (mg/dL)	76.0 ± 33.3	95.4 ± 43.9	138.3 ± 81.6	<0.001[a]

[a]: NBNC *vs.* B and C; [b]: NBNC *vs.* C; [c]: NBNC and B *vs.* C

TABLE 3 Associated Conditions

		B	C	NBNC	p
N		19	68	37	
Body Mass Index (kg/m^2)		22.0±3.1	22.8±3.2	24.1±4.1	N.S.
BMI ≥ 25	+/-	4 / 15	12 / 56	11 / 26	N.S.
Diabetes mellitus	+/-	1 / 18	15 / 53	17 / 20	<0.01[a]
Hypertension	+/-	3 / 16	32 / 36	19 / 18	0.03[b]
Hyperlipidemia	+/-	3 / 16	11 / 57	14 / 23	0.03[c]
Alcohol abuse	+/-	6 / 13	13 / 55	16 / 21	0.03[c]

BMI: Body mass index; N.S.: Not significant; a: NBNC *vs.* B and C; [b]: B *vs.* NBNC and C; [c]: NBNC *vs.* C

RESULTS

Of the 124 patients, there were 94 men and 30 women, and the mean age was 65.9±9.0 years (range 30-82 years). The median postoperative follow-up period was 18.5 months (range 1-58 months).

The number of patients in groups B, C, and NBNC were 19 (15.3%), 68 (54.8%), and 37 (29.8%), respectively. In the NBNC patients, 17 (45.9%) of patients were positive for HBcAb and 16 patients were negative for HBcAb. Four patients were not examined for their HBsAb and/or HBcAb status. The background of these patients is summarized in Table 1. The mean age was significantly younger in group B than in groups C or NBNC ($p<0.0001$). The preoperative Child-Pugh scores were not significantly different among the 3 groups, but there was significant difference in liver histology ($p=0.03$); the incidence of chronic hepatitis and liver cirrhosis was significantly higher in group C than in groups B or NBNC. Both the stage of the HCC and the surgical procedure performed were decided based on the Japanese rules for HCC (26), and were not significantly different among the 3 groups.

Table 2 shows the preoperative laboratory data. Serum level of ALT was lower and the PLT was significantly higher in the NBNC group than in group C. The serum TG level was significantly higher in the NBNC group than in groups B or C.

The BMI and number of overweight patients were not significantly different among the three groups, as shown in **Table 3**. The frequency of diabetes mellitus was significantly higher in the NBNC group than in either group B or C. The incidences of both hyperlipidemia and alcohol abuse were significantly higher in the NBNC group as compared with group C.

Moreover, we made the same comparisons using those patients without alcohol abuse to exclude the influence of alcohol intake (**Table 4**). Consequently, we found that the laboratory data were not affected by alcohol abuse, whereas ratios of having associated conditions, such as diabetes mellitus, hyperlipidemia, and hypertension, became statistically insignificant among the 3 groups.

The cumulative overall survival rates at 1 and 3 years in groups B, C, and NBNC were 88.1% / 66.1%, 77.8% / 58.9%, and 85.5% / 69.8%, respectively (**Figure 1**). There were no significant differences among the three groups ($p=0.46$). The disease free survival rates at 1 and 3 years in groups B, C, and NBNC were 48.5% / 29.1%, 56.5% / 22.5%, and 73.8% / 27.6%, respectively (**Figure 2**). Although the NBNC group tended to be higher, the differences in the disease free survival rates among these three groups were not significant ($p=0.12$). In addition, to estimate the influence of risk factors for MS on survival after surgery, we compared the cumulative overall (**Figure 3**) and disease free (**Figure 4**) survival rates between those patients with *vs.* those without risk factors for MS in each group. In groups B and C, there was a trend that the survival rate was better in those patients without risk factors for MS. On the other hand, the survival rate was slightly better with risk factors for MS in the NBNC group. However, there were no significant differences between the groups. In groups B and C, the disease free survival rate was almost same with *vs.* without the risk factors for MS, and they were not significantly different in each group. In the NBNC group, the disease free survival seemed to be better in those patients with the risk factors for MS, but again there was no statistically significant difference ($p=0.24$).

Moreover, in the NBNC group, the overall ($p=0.50$) and disease free ($p=0.15$) survival rates were not significantly different between the two subgroups

TABLE 4 Laboratory Data, Liver Histology, and Associated Conditions in Patients without Alcohol Abuse

		B	C	NBNC	p
N		13	55	21	
AST (IU/L)		69.0±67.3	68.6±38.0	38.3±19.4	0.01[a]
ALT (IU/L)		52.6±27.1	63.9±45.3	39.0±21.5	< 0.05[b]
PLT (×10⁴/mm³)		16.3±6.5	13.1±7.9	21.6±14.0	< 0.01[b]
Total Cholesterol (mg/dL)		173.1±54.2	151.7±30.0	184.6±38.5	< 0.01[b]
Triglyceride (mg/dL)		76.4±34.6	95.3±39.4	124.1±69.7	< 0.05[a]
Liver histology					0.001[b]
Normal liver	1	0	5		
Chronic hepatitis	5	20	11		
Liver cirrhosis	7	35	5		
Diabetes mellitus	+/-	0 / 13	11 / 44	7 / 14	N.S.
Hypertension	+/-	3 / 10	27 / 28	9 / 12	N.S.
Hyperlipidemia	+/-	4 / 9	8 / 47	6 / 15	N.S.

N.S.: Not significant; [a]: NBNC *vs.* B and C; [b]: NBNC *vs.* C

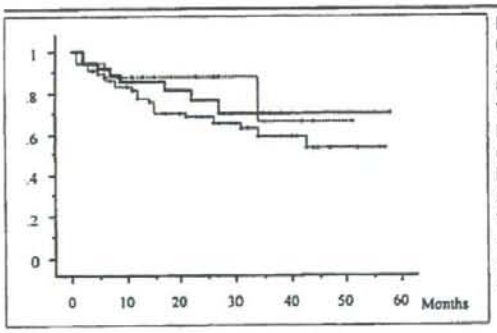

FIGURE 1
Cumulative overall survival curves for HCC after surgery. The bold line, dotted line, and thin line denote groups NBNC, B, and C, respectively. There were no significant differences in the overall survival rates among the 3 groups.

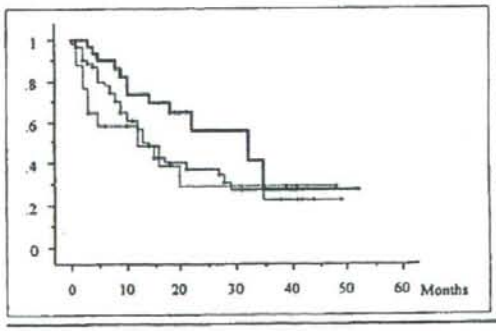

FIGURE 2
Disease-free survival curves for HCC after surgery. The bold line, dotted line, and thin line denote groups NBNC, B, and C, respectively. There were no significant differences in the disease-free survival rates among the 3 groups.

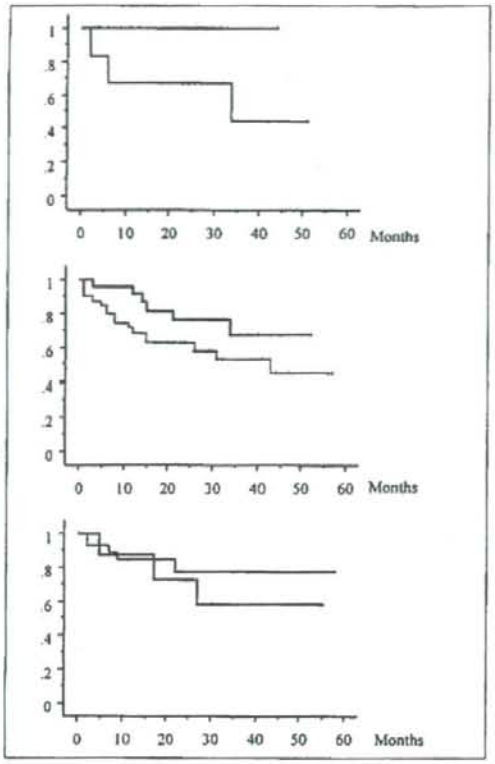

FIGURE 3
Cumulative overall survival curves with or without risk factors of metabolic syndrome.Upper panel: group B; middle panel: group C; and lower panel: group NBNC. The thin and bold lines denote patients with and without risk factors of metabolic syndrome, respectively. There were no significant differences among all groups, although groups B and C with risk factors for metabolic syndrome had a tendency toward lower survival rates than those without.

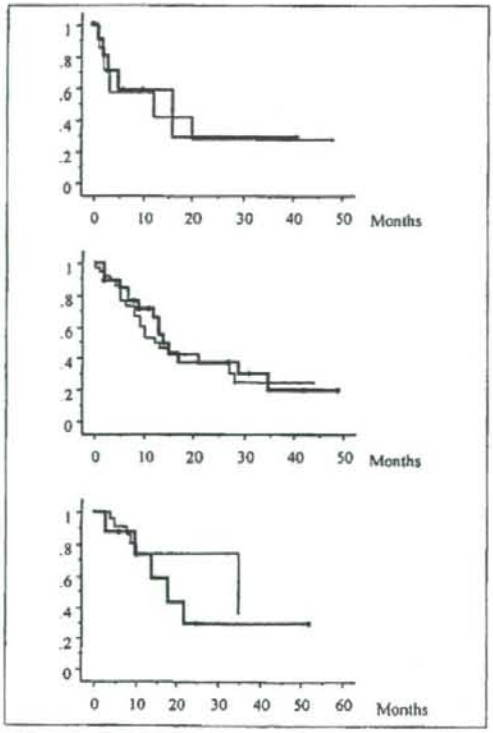

FIGURE 4
Disease-free survival curves with or without risk factors of metabolic syndrome.Upper panel: group B; middle panel: group C; and lower panel: group NBNC. The thin and bold lines denote patients with and without risk factors of metabolic syndrome, respectively. There were no significant differences among all groups.

with or without HBcAb (data not shown).

Seven out of the 37 patients from the NBNC group showed various degrees of fatty changes in their liver histology. Among them, 3 patients who did not have a history of alcohol abuse were strongly suspected to have NASH (8.1%). The clinical features of these 3 patients with HCC associated with NASH are shown in **Table 5**. We estimated both the necroinflammatory grade and the fibrosis score according to the definitions proposed by Brunt *et al.* (27). The BMIs of these three patients were 23.1, 24.8, and 24.4, respectively. These figures were within the normal range according to western standards but are considered to be overweight by Asian standards (28). Two of the 3 patients had associated diabetes mellitus, and 1 patient had no risk factors for MS.

DISCUSSION

In this study, the background and associated conditions of patients with HCC who underwent surgery in relation to viral markers was investigated, and then compared to determine participation of risk factors for MS in the development of HCC, in particular non-B non-C HCC. At first, the prevalence of chronic hepatitis and liver cirrhosis in non-B non-C HCC was confirmed to be almost the same as in group B, and was significantly lower than in group C, although there were no differences in the preoperative Child-Pugh classifications among the 3 groups.

One of the current topics in this field is the relationship between obesity and HCC (15,18). In this study, the mean BMI was less than 25kg/m^2 in all 3 groups, and there were no significant differences among them. However, the NBNC group showed the highest ratio of patients with a BMI greater than 25kg/m^2, suggesting the possible participation of obesity in non-B non-C HCC.

This study also demonstrated that patients in NBNC group had a significantly higher prevalence of both diabetes mellitus and hyperlipidemia, and their mean serum TG was significantly higher than groups B and C. This result showed that non-B non-C HCC may be strongly associated with metabolic or insulin resistance syndrome, as suggested in other reports (16,20). Diabetes mellitus has been recognized as a risk factor of not only chronic liver disease, but also HCC (14-17). Bugianesi *et al.* (20) reported that the prevalence of diabetes and the plasma levels of both TC and TG were significantly higher in those patients with HCC associated with cryptogenic cirrhosis than in patients with alcohol and hepatitis virus infection related HCC. However, in this study, the prevalence of alcohol abuse was significantly higher in the NBNC group than in group C. Therefore, we performed an additional analysis after excluding those patients with alcohol abuse. Based on that analysis, no significant difference in the prevalence of diabetes mellitus and hyperlipidemia among the 3 groups was found, although the serum TG and TC levels were still higher. In contrast to these results, some studies have indicated that alco-

Table 5 Three cases of HCC associated with NASH

	Case 1	Case 2	Case 3
Age	69	72	79
Gender	Male	Female	Male
BMI (kg/m²)	23.1	24.8	24.4
Diabetes mellitus	-	+	+
Hypertension	-	+	-
Hyperlipidemia	-	+	+
AST (IU/L)	23	59	59
ALT (IU/L)	23	46	76
PLT (×10⁴/mm³)	15.6	16	11.8
Total Cholesterol (mg/dL)	212	217	207
Triglyceride (mg/dL)	70	95	205
Steatosis*	mild	moderate	moderate
Degeneration of hepatocyte*	mild	ballooning and acidophilic bodies	Mallory bodies and acidophilic bodies
Intraacinar inflammatory cells*	scattered microabscess	many microabscess	many microabscess
Portal inflammation*	mild	moderate to severe	moderate to severe
Necroinflammatory grade*	1	2	3
Fibrosis stage*	3	4	4

*Definitions according to Brunt *et al.* (27)

hol intake was associated with a lower prevalence of MS (29,30). In the patients studied here, however, chronic liver disease, risk factors for MS, and alcohol abuse might all participate in the carcinogenesis of HCC in a very complex fashion.

More recently, NAFLD including NASH has been considered to be one of the causes of chronic liver disease (15,31) and HCC (19-25). Although the prevalence of NASH in HCC patients remains unknown, in the literature, HCC has been observed in 2.4-13% of NASH patients (21,23,24). Bugianesi *et al.* (20) reported that among 641 cirrhosis-associated HCCs, 44 patients (6.9%) had cryptogenic cirrhosis which was associated with NASH. On the other hand, Yano *et al.* (12) stated that there were no non-B non-C patients with HCC in whom NASH was thought to be the cause. However, it is difficult to clearly determine the participation of NASH in the pathogenesis of HCC associated with cryptogenic cirrhosis, because the steatosis might decrease or disappear when the NASH progressed to cirrhosis (17,18,20,23,31). In the present study, 3 patients were judged to be associated with NASH based on microscopic findings of moderate to severe macrovesicular steatosis, hepatocellular ballooning, lobular inflammation with necrosis of the hepatocytes, and perisinusoidal fibrosis. Although there was some discussion about the histopathological findings in NAFLD (19), macro-vesicular steatosis, Mallory's hyaline, ballooning, lobular inflammation, and perisinusoidal fibrosis were considered to be histological features in general (19,31,32).

In this study, the cumulative overall and disease free 3-year survival rates for HCC in the non-B non-C patients after surgery were 69.8% and 27.6%, respectively, and there were no significant differences in both the cumulative overall and disease free survival rates among the B, C, and NBNC patients. Although this study population contained 26 patients who had already underwent some treatment for HCC, these results were considered to be almost the same as with previous studies (8,10,11,33). HCC in the non-B non-C patients has been reported to have a worse prognosis than HCC due to other causes. With respect to the influence of the risk factors for MS on survival, the interesting result was obtained that these risk factors might exhibit an adverse effect on the survival of hepatitis virus related HCC patients, but not on non-B non-C HCC patients, although their survival rates did not show any statistically significant differences because of the small number of cases studied. One could speculate that the risk factors for MS had a positive impact on the occurrence of non-B non-C HCC, but may exert a negative impact on the prognosis of hepatitis virus related HCC patients.

In conclusion, the prevalence of diabetes mellitus, hyperlipidemia, and the plasma triglyceride level were all significantly higher in non-B non-C HCC patients as compared with those with HCC related to both HBV and HCV infection. This suggests that the pathogenesis of non-B non-C HCC was more closely associated with the risk factors for MS than viral related HCC. However, alcohol abuse affected the pathological mechanisms at least in part. With respect to the participation of NASH, 3 out of 37 non-B non-C HCC patients were clearly associated with NASH from the viewpoint of their clinical and histological analyses. The prognosis of patients with positive viral markers tended to be worse in those patients who also had risk factors for MS than those without. On the other hand, there was no such tendency in the non-B non-C patients. To our knowledge, this is the first report of its kind on HCC patients with and without hepatitis virus markers, and the findings presented here may be informative in a clinical setting.

REFERENCES

1 **Llovet JM, Burroughs A, Bruix J:** Hepatocellular carcinoma. Lancet 2003; 362:1907-1917.

2 **Ercolani G, Grazi GL, Ravaioli M, Del Gaudio M, Gardini A, Cescon M, Varotti G, Cetta F, Cavallari A:** Liver resection for hepatocellular carcinoma on cirrhosis: univariate and multivariate analysis of risk factors for intrahepatic recurrence. Ann Surg 2003; 237:536-543.

3 **Fong Y, Sun RL, Jarnagin W, Blumgart LH:** An analysis of 412 cases of hepatocellular carcinoma at a Western center. Ann Surg 1999; 229:790-800.

4 **Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, Furuta S, Akahane Y, Nishioka K, Purcell RH, Alter HJ:** Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 1990; 12:671-675.

5 **Nishioka K, Watanabe J, Furuta S, Tanaka E, Iino S, Suzuki H, Tsuji T, Yano M, Kuo G, Choo QL, Houghton M, Oda T:** A high prevalence of antibody to the hepatitis C virus in patients with hepatocellular carcinoma in Japan. Cancer 1991; 67:429-433.

6 **Takenaka K, Yamamoto K, Taketomi A, Itasaka H, Adachi E, Shirabe K, Nishizaki T, Yanaga K, Sugimachi K:** A comparison of the surgical results in patients with hepatitis B *versus* hepatitis C-related hepatocellular carcinoma. Hepatology 1995; 22:20-24.

7 **Kato Y, Nakata K, Omagari K, Furukawa R, Kusumoto Y, Mori I, Tajima H, Tanioka H, Yano M, Nagataki S:** Risk of hepatocellular carcinoma in patients with cirrhosis in Japan. Analysis of infectious hepatitis viruses. Cancer 1994; 74:2234-2238.

8 **Koike Y, Shiratori Y, Sato S, Obi S, Teratani T, Imamura M, Hamamura K, Imai Y, Yoshida H, Shiina S, Omata M:** Risk factors for recurring hepatocellular carcinoma differ according to infected hepatitis virus-an analysis of 236 consecutive patients with a single lesion. Hepatology 2000; 32:1216-1223.

9 **Shim J, Kim BH, Kim NH, Dong SH, Kim HJ, Chang YW, Lee JI, Chang R:** Clinical features of HBsAg-negative but anti-HBc-positive hepatocellular carcinoma in a hepatitis B virus endemic area. J Gastroenterol Hepatol 2005; 20:746-751.

10 **Uchiyama K, Ueno M, Hama T, Kawai M, Tani M, Terasawa H, Ozawa S, Uemura R, Nakase T, Yamaue H:** Recurrence of primary hepatocellular carcinoma after hepatectomy--differences related to underlying hepatitis virus species. Hepatogastroenterology 2005; 52:591-595.

11 **Yamanaka N, Tanaka T, Tanaka W, Yamanaka J, Yasui C, Kuroda N, Takada M, Okamoto E:** Correlation of hepatitis virus serologic status with clinicopathologic features in patients undergoing hepatectomy for hepatocellular carcinoma. Cancer 1997; 79:1509-1515.

12 **Yano Y, Yamashita F, Sumie S, Ando E, Fukumori K, Kiyama M, Oyama T, Kuroki S, Kato O, Yamamoto H, Tanaka M, Sata M:** Clinical features of hepatocellular carcinoma seronegative for both HBsAg and anti-HCV antibody but positive for anti-HBc antibody in Japan. Am J Gastroenterol 2002; 97:156-161.

13 **Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults:** Executive summary of the third report of the national cholesterol education program (ncep) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel iii). Jama 2001; 285:2486-2497.

14 **Adami HO, Chow WH, Nyren O, Berne C, Linet MS, Ekbom A, Wolk A, McLaughlin JK, Fraumeni JF Jr:** Excess risk of primary liver cancer in patients with diabetes mellitus. J Natl Cancer Inst 1996; 88:1472-1477.

15 **Caldwell SH, Crespo DM, Kang HS, Al-Osaimi AM:** Obesity and hepatocellular carcinoma. Gastroenterology 2004; 127:S97-103.

16 **Davila JA, Morgan RO, Shaib Y, McGlynn KA, El-Serag HB:** Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. Gut 2005; 54:533-539.

17 **El-Serag HB, Tran T, Everhart JE:** Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology 2004; 126:460-468.

18 **Powell EE, Jonsson JR, Clouston AD:** Steatosis: co-factor in other liver diseases. Hepatology 2005; 42:5-13.

19 **Brunt EM:** Nonalcoholic steatohepatitis. Semin Liver Dis 2004; 24:3-20.

20 **Bugianesi E, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, Musso A, De Paolis P, Capussotti L, Salizzoni M, Rizzetto M:** Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterology 2002; 123:134-140.

21 **Marrero JA, Fontana RJ, Su GL, Conjeevaram HS, Emick DM, Lok AS:** NAFLD may be a common underlying liver disease in patients with hepatocellular carcinoma in the United States. Hepatology 2002; 36:1349-1354.

22 **Mori S, Yamasaki T, Sakaida I, Takami T, Sakaguchi E, Kimura T, Kurokawa F, Maeyama S, Okita K:** Hepatocellular carcinoma with nonalcoholic steatohepatitis. J Gastroenterol 2004; 39:391-396.

23 **Powell EE, Cookaley WG, Hanson R, Searle J, Halliday JW, Powell LW:** The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. Hepatology 1990; 11:74-80.

24 **Shimada M, Hashimoto E, Taniai M, Hasegawa K, Okuda H, Hayashi N, Takasaki K, Ludwig J:** Hepatocellular carcinoma in patients with non-alcoholic steatohepatitis. J Hepatol 2002; 37:154-160.

25 **Zen Y, Katayanagi K, Tsuneyama K, Harada K, Araki I, Nakanuma Y:** Hepatocellular carcinoma arising in nonalcoholic steatohepatitis. Pathol Int 2001; 51:127-131.

26 **Liver Cancer Study Group of Japan (Eds.):** The General Rules for the Clinical and Pathological Study of Primary Liver Cancer. 4th Edition. Tokyo: Kanehara. 2000.

27 **Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR:** Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol 1999; 94:2467-2474.

28 **Weisell RC:** Body mass index as an indicator of obesity. Asia Pac J Clin Nutr 2002; 11:S681-684.

29 **Freiberg MS, Cabral HJ, Heeren TC, Vasan RS, Curtis Ellison R:** Alcohol consumption and the prevalence of the Metabolic Syndrome in the US: a cross-sectional analysis of data from the Third National Health and Nutrition Examination Survey. Diabetes Care 2004; 27:2954-2959.

30 **Djousse L, Arnett DK, Eckfeldt JH, Province MA, Singer MR, Ellison RC:** Alcohol consumption and metabolic syndrome: does the type of beverage matter? Obes Res 2004; 12:1375-1385.

31 **Sanyal AJ:** AGA technical review on nonalcoholic fatty liver disease. Gastroenterology 2002; 123:1705-1725.

32 **Angulo P:** Nonalcoholic fatty liver disease. N Engl J Med 2002; 346:1221-1231.

33 **Lang H, Sotiropoulos GC, Domland M, Fruhauf NR, Paul A, Husing J, Malago M, Broelsch CE:** Liver resection for hepatocellular carcinoma in non-cirrhotic liver without underlying viral hepatitis. Br J Surg 2005; 92:198-202.

シグナル伝達を理解するために必要な知識

Eカドヘリンによるシグナル伝達

高島基樹*　池嶋健一*　渡辺純夫*

はじめに

カドヘリンはCa^{2+}依存性のI型膜蛋白質で，細胞表面の同種のカドヘリンと結合し細胞間接着に中心的役割を果たしている[1]．近年，カドヘリンスーパーファミリーとよばれるカドヘリンリピートをもつ多数の膜蛋白質が同定されているが，なかでも上皮細胞間の接着に不可欠なEカドヘリン機構は多くの発生過程にかかわっている．また，Eカドヘリンの不活化はその下流に存在するカテニンを介した細胞内シグナル伝達にも変化をもたらし，細胞間接着の消失のみならず癌の浸潤性にも寄与している．本稿では，代表的なカドヘリン分子であるEカドヘリンに関連したシグナル伝達に焦点を絞って概説する．

カドヘリン複合体の構造とその生理的役割

カドヘリンはCa^{2+}依存性の細胞間接着分子であり，分子量120～130kDaの糖蛋白でカドヘリン同士が結合することにより細胞間を接着する．カドヘリンの細胞外ドメインは約110のアミノ酸残基からなる5つのくり返し構造からなり，アミノ末端に位置するEC1が同種のカドヘリンを発現する細胞を認識し，結合特異性を規定している[2]．カドヘリンの細胞質ドメインのカルボキシ末端にはβカテニンあるいはγカテニンが直接結合する．これらの分子を介しαカテニンが結合し，さらに細胞膜近傍領域にはp120が結合している（図❶）．これらの分子による複合体の形成は細胞間接着に不可欠であり，実際にカテニンのない細胞では細胞間接着は低下し[3]，カテニンと結合できない変異カドヘリンでも同様に接着能は低下する[4]．カドヘリンを介した細胞間接着のシグナルはカテニンおよび細胞骨格蛋白を通じて細胞内に伝達される．そのシグナル伝達において中心的な役割を果たすのがβカテニンである．βカテニンは781個のアミノ酸からなる分子量95kDaの蛋白質で，アミノ末端にGSK-3βによりリン酸化されるドメインをもち，中央にはアルマジロリピート（Armリピート）とよばれる42アミノ酸からなる13回のくり返し領域がある．Armリピートにはカドヘリン以外にもβカテニンのリン酸化に関与するTcf/Lef-1およびAPCがそれぞれ競合的に結合する（図❷）．通常，カドヘリン複合体は発生段階における器官の形成や創傷治癒に深く関与し，その生理的役割を果たしている．カドヘリンスーパーファミリーの一種であるデスモグレインの機能低下は落葉状天疱瘡を引き起こすことがわかっている．

KEY WORDS
◎ Eカドヘリン
◎ βカテニン
◎ Wnt

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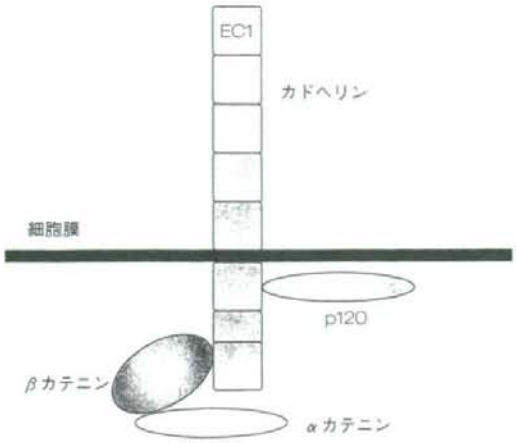
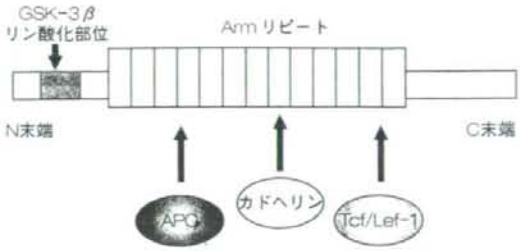


図❶ カドヘリン・カテニン複合体の構造
カドヘリンの細胞外ドメインは約 110 のアミノ酸残基からなるカドヘリンリピートとよばれるくり返し構造（EC1～5）をもつ．アミノ末端に位置する EC1 が同種のカドヘリンを発現する細胞を認識し，結合特異性を規定している．カドヘリンの細胞質ドメインのカルボキシ末端にはβカテニンが直接会合する．これらの分子を介しαカテニンが結合し，さらに細胞膜近傍領域には p120 が結合している．



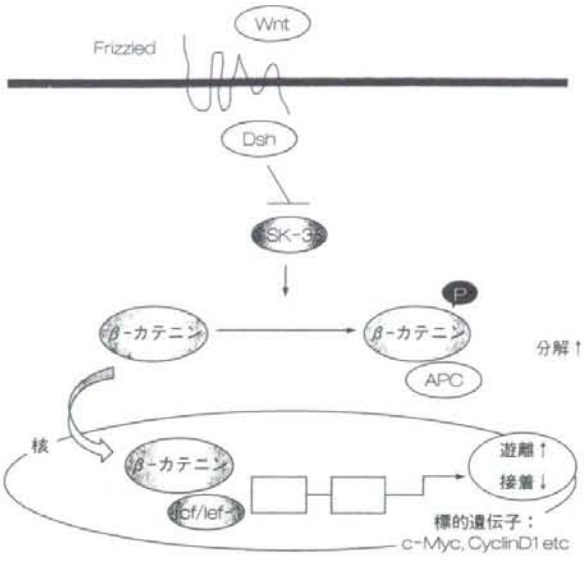
図❷ βカテニンの構造
βカテニンは分子量 95 kDa の蛋白質で 781 個のアミノ酸からなる．N 末端には GSK-3β によってリン酸化される Ser/Thr がある．中央に位置する 42 個のアミノ酸からなる 13 回のくり返し構造（アルマジロリピート：Arm リピート）にはカドヘリン，APC および Tcf/Lef-1 が結合する．αカテニンは Arm リピートの N 末端側に結合する．

Wnt/βカテニンによるシグナル伝達

E カドヘリン複合体と相互作用する代表的なシグナル伝達系に Wnt シグナル経路がある．βカテニンは Wnt シグナルを受けて Tcf/Lef-1 ファミリーと複合体を形成した後に核内へ移行し，標的遺伝子の転写活性を誘導する．Wnt は動物の発生初期段階において体軸の決定や形態形成などに重要な役割を果たす蛋白分子である．この経路の活性は細胞質に存在するβカテニンの量によって決定され，通常状態ではβカテニンの量は APC，GSK-3β を介したプロテアソーム依存性蛋白分解によって低いレベルに保たれている．Wnt 経路では Wnt の刺激がない時には下流に存在する GSK-3β は活性化されており，βカテニンのアミノ末端がリン酸化され分解が起こる．逆に Wnt がそのレセプターである Frizzled に結合し刺激が入力された時には Dishevelled（Dsh）が活性化され，βカテニンの分解を誘導する GSK-3β は不活化される．その結果，βカテニンのリン酸化が抑制され分解を免れることにより遊離βカテニンが細胞質内に増加する．遊離したβカテニンは Tcf/Lef-1 と結合し核内へ移動し標的遺伝子のエンハンサー，プロモータ領域に結合し転写を活性化させる．現在，Wnt シグナルの標的遺伝子として c-Myc や cyclinD1 などを含む約 100 種類もの遺伝子が同定されている（**図❸**）[4]．

癌と E カドヘリンおよびシグナル伝達の異常

癌浸潤および転移とは，癌細胞が原発巣から遊離し血流に乗り体内の別の部位に到達しそこで新たに増殖をはじめるという，悪性腫瘍に特有の現象である．この特性により悪性腫瘍の治癒率は良性腫瘍のそれにくらべかなり低いため，癌転移のメカニズムの研究は癌研究の重要なテーマの一つにあげられている．癌細胞においても細胞接着の中心的役割を果たす E カドヘリンの異常は必然的に細胞間接着の異常をきたすことは想像にかたくない．その代表的なものとして低分化胃癌があげられる．実際，多くの低分化胃癌では E カドヘリン蛋白の発現が消失あるいは減少している．胃癌，食道癌，大腸癌，乳癌において E カドヘリンの発現が正常上皮にくらべ低下あるいは消失している例では有意に浸潤傾向が強く，リンパ節への転移が多いことが示されている[5]．低分化胃癌細胞株での研究では E カドヘリン遺伝子においてスプライシング異常が起こっていることが明らかになった[6]．さらには，低分化胃癌において高頻度に E カドヘリン遺伝子の欠失，突然変異などの異常が見つかり[7]，



図❸ Wnt シグナル経路における β カテニンの制御機構と役割
液性因子 Wnt は細胞表面上の 7 回膜貫通型レセプターである Frizzled に結合し，Dsh を活性化する．その結果，GSK-3β は抑制され APC による β カテニンの分解が阻害される．細胞内には遊離した β カテニンが蓄積し Tcf/Lef-1 と結合し核内に移動し標的遺伝子の転写を活性化する．Wnt シグナルが存在しない時，β カテニンは APC を含む複合体に結合し GSK-3β によってリン酸化された後，ユビキチン化を受けプロテアソームによって分解される．

またニュージーランドの若年胃癌家系において生殖細胞系列に E カドヘリンのスプライシング異常が発見され，E カドヘリンは家族性胃癌における癌抑制遺伝子であることが明らかになった[8]．また，E カドヘリン遺伝子に異常は認められないが，E カドヘリンのプロモータのメチル化によって E カドヘリン遺伝子の発現低下が起こっている例も多く存在することも明らかになった[9]．その後，E カドヘリン遺伝子の発現調節において Snail という転写因子が E カドヘリン遺伝子の発現を抑制するはたらきをもつことが解明され[11][12]，さまざまな癌細胞において Snail の発現増加と E カドヘリン発現の低下の相関性が証明されている[12]．

Wnt シグナルの異常亢進は種々の腫瘍性疾患を引き起こすことが明らかにされている．なかでも APC 遺伝子変異はヒトにおいて最も高頻度に観察される遺伝子変化であり，大腸腺腫や大腸癌に密接に関連することがよく知られている．また，β カテニン遺伝子変異は恒常的な Wnt シグナルの亢進をもたらすことにより大腸癌やメラノーマの発生に関与することが報告されており，さらに乳癌，胃癌および食道癌においても Wnt の発現亢進が見出されている．したがって，癌細胞において Wnt シグナルの標的遺伝子の転写亢進は細胞増殖をもたらし，アポトーシス抑制にも関与しているものと考えられる．一方，Wnt シグナル低下ないし欠如は発生段階の異常を伴い，胸腺低形成，心奇形，Di George 症候群および知能障害などを引き起こすことがわかっている．

おわりに

以上のように，β カテニンを含む E カドヘリンシステムの破綻は発生段階の異常をきたすのみならず，さまざまな種類の癌の浸潤および転移に密接に関与している．Wnt シグナル伝達系と E カドヘリンの細胞間接着装置は β カテニンという同一の分子を利用して，複雑にクロストークをおこない細胞機能を制御しているものと考え

られる．細胞間接着と細胞増殖という現象はEカドヘリン・カテニン系だけでなくインテグリンファミリーをはじめとした他の接着分子群からのシグナルの影響を受けながら密接に制御されており，これらのクロストークの詳細はいまだ未解明な部分も多い．Eカドヘリンを介したシグナル伝達系は癌の浸潤・転移を抑制するための標的分子群としてもきわめて重要であり，今後の研究の展開が期待される．

文　献

1) Takeichi M：The cadherins cell-cell adhesion molecules controlling animal morphogenesis. *Development* **102**：639-655, 1988
2) Nose A, Nagafuchi A, Takeichi M：Expressed recombinant cadherins mediate cell sorting in model systems. *Cell* **54**：993-1001, 1988
3) Reynolds AB, Daniel J, McCrea PD *et al*：Identification of a new catenin：the tyrosine kinase substrate p120cas associates with E-cadherin complexes. *Mol Cell Biol* **14**：8333-8342, 1994
4) The Wnt gene homepage (http//www.stanford.edu/~rnusse/wntwindow.html)
5) Oka H, Shiozaki H, Kobayashi K *et al*：Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. *Cancer Res* **53**：1696-1701, 1993
6) Oda T, Kanai Y, Oyama T *et al*：E-cadherin gene mutations in human gastric carcinoma cell lines. *Proc Natl Acad Sci U S A* **91**：1858-1862, 1994
7) Muta H, Noguchi M, Kanai Y *et al*：E-cadherin gene mutations in signet ring cell carcinoma of the stomach. *Jpn J Cancer Res* **87**：843-848, 1996
8) Guilford P, Hopkins J, Harraway J *et al*：E-cadherin germline mutations in familial gastric cancer. *Nature* **392**：402-405, 1998
9) Yoshiura K, Kanai Y, Ochiai A *et al*：Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proc Natl Acad Sci U S A* **92**：7416-7419, 1995
10) Batlle E, Sancho E, Francí C *et al*：The transcription factor snail is a repressor of *E-cadherin* gene expression in epithelial tumour cells. *Nat Cell Biol* **2**：84-89, 2000
11) Cano A, Pérez-Moreno MA, Rodrigo I *et al*：The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* **2**：76-83, 2000
12) Blanco MJ, Moreno-Bueno G, Sarrio D *et al*：Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. *Oncogene* **21**：3241-3246, 2002

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Review

Liver diseases and metabolic syndrome

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Emerging attention has been paid to metabolic syndrome, which comprises several metabolic disorders including visceral obesity, diabetes mellitus, dyslipidemia, and hypertension. Whether the severity of each disease is mild to moderate, the comorbidity of these metabolic disorders has a serious impact on the development of atherosclerosis. Nonalcoholic fatty liver disease (NAFLD) is the major hepatic disorder in patients with metabolic syndrome, and indeed it is the most common cause of abnormal liver function tests in the working population in industrialized countries. In recent years, it has become recognized that NAFLD is no longer just a trivial disease, and a rather considerable proportion of the patients develop liver cirrhosis. Furthermore, chronic infection of hepatitis C virus also develops a pathological feature of steatohepatitis, and extended hepatic steatosis has a serious impact not only on the progression of hepatic fibrosis but also on the antiviral efficacy of interferon therapy. Emerging lines of studies indicated that insulin resistance, abnormal lipid metabolism, and dysregulation of cytokines/adipokines (e.g., tumor necrosis factor-alpha, adiponectin, and leptin) are profoundly involved in the pathogenesis of NAFLD. This review aims to integrate the reported evidence and to provide the current point of view for comprehensive understanding of the pathophysiology of steatohepatitis.

Key words: metabolic syndrome, nonalcoholic fatty liver diseases, nonalcoholic steatohepatitis, chronic hepatitis C, pegylated interferon, ribavirin, sustained viral response

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Introduction

In Japan in recent years, the Western lifestyle has become common, and the morbidity rates of obesity, diabetes mellitus, dyslipidemia, and hypertension have gradually increased. Generally, the metabolic disorders, which are basically characterized by insulin resistance, have been recognized as risk factors for cardiovascular disease. Even if the severity of each disease is only moderate, it is most likely true that the comorbidity of these metabolic disorders has serious impacts on the development of atherosclerosis. From this point of view, metabolic syndrome has become a new hot topic, and suddenly many related papers have been published.[1,2] Moreover, it is a matter of interest that metabolic syndrome is closely associated with some gastrointestinal diseases as well.[3]

On the other hand, the liver is one of the most important organs associated with digestion, detoxification, production, and storage. Nonalcoholic fatty liver disease (NAFLD) has been noted as the most common cause of abnormal liver function,[4,5] and had, however, previously been recognized merely as a relatively benign disease mostly observed in some obese patients. In 1980, Ludwig et al. reported nonalcoholic steatohepatitis (NASH) as a novel liver disease entity, which is histologically characterized by zone 3-dominant hepatic steatosis with hepatocellular ballooning, lobular inflammation, zone 3 perisinusoidal fibrosis, and cirrhosis.[6] This notion indicated that NAFLD is no longer just a trivial disease, but rather potentially develops into liver cirrhosis.

At the same time, the number of obese patients steadily increased, and consequently obesity became one of the major causes of morbidity and mortality in the United States in the 1990s.[7,8] Also, obesity is also one of the most important risk factors for fatty liver disease. For this reason, the report of Ludwig et al. had a great impact on the management of patients with

NAFLD in the late 1990s. In Japan, the increase in the number of people with obesity caused the same health problems, following after the Western countries with a lag of a decade. Therefore, the metabolic abnormalities; so-called metabolic syndrome and fatty liver disease, have now become an important health issue in Japan as well.

Some recent studies have been trying to describe how liver diseases are closely associated with metabolic syndrome, because this is useful to elucidate the background and pathophysiology.[9-11] The aim of this review is to integrate the reported evidences of liver diseases and metabolic syndrome and find a way to establish a rational medical treatment of the metabolic disorders.

Nonalcoholic steatohepatitis (NASH) and metabolic syndrome

Pathological features of NASH/NAFLD

Nonalcoholic steatohepatitis (NASH) is currently recognized as an important disease that potentially progresses to cirrhosis and liver-related death. In 1980, Ludwig et al. first reported liver biopsy findings that resemble alcohol-induced liver disease pathologically but develop in patients without alcohol abuse.[6] Since the 1970s, the number of people with obesity gradually increased at a steady state, and in the 1990s, some obesity-related diseases became a serious problem in Western countries. On that occasion, Day et al. hypothesized the "two hit theory" as the pathophysiology of steatohepatitis in 1998. The review article noted that fat accumulation in hepatocytes, induced by insulin resistance and some other factors, is the "first hit"; and after that, cell injury induced by oxidant stress and free fatty acids is the "second hit."[12] Additionally, the title of the review was so catchy that the "two hit theory" spread quickly through the world. In 1999, Brunt et al. proposed the grading (1–3) and staging (0–4) system of the histological lesions in patients with nonalcoholic steatohepatitis.[13] Although the system is only for NASH, it is a useful and semiquantitative evaluation that is now popular among pathologists and clinical physicians. About that time, Matteoni et al. evaluated the entire spectrum of NAFLD by using comparative analysis, pathologically: type 1, fatty liver alone; type 2, fat accumulation and lobular inflammation; type 3, fat accumulation and ballooning degeneration; and type 4, fat accumulation, ballooning degeneration, and either Mallory hyaline or fibrosis. In fact, the poor outcomes, including cirrhosis and liver-related death, were more frequent in patients in whom biopsies show ballooning degeneration and Mallory hyaline or fibrosis.[14] This pathological evaluation system is valuable because it is most likely true that there was a positive association between the types of the pathological classification and the outcome in the patients with NAFLD.

After these reports, as the more extended term, nonalcoholic fatty liver disease (NAFLD) acquired a new meaning and became accepted to cover the wide spectrum of metabolic fatty liver disorders.[15] More recently, a NAFLD activity score (NAS) was proposed for NAFLD and NASH with reasonable interrater reproducibility. Actually, the scoring system seems to be somewhat complicated and difficult to approach for some clinicians; however, it is useful to evaluate the semiquantitative and reproducible activity score, i.e., steatosis (0–3), lobular inflammation (0–2), hepatocellular ballooning (0–2), and fibrosis (0–4). An NAS greater than 5 is correlated with a diagnosis of NASH, and biopsies with scores of less than 3 were diagnosed as "not NASH."[16]

Clinical features of NAFLD/NASH and metabolic syndrome

Focused on the prevalence of NAFLD, a recent population-based cohort study revealed that 34% of the adult population; included an age range of 18–65 years in the United States, has fatty liver mostly without excessive alcohol abuse.[17] Furthermore, another population-based cohort study described the natural history of nonalcoholic fatty liver disease (NAFLD). It was shown that mortality among NAFLD patients was higher than the general population. Liver-related death was the third most common cause and accounting for 13% of all deaths; however, the absolute risk was low.[18] From the other point of view, the result of the study has still been controversial because of the inevitably implicated selection bias in the study. Even though, it could be believed that the report has a great impact on regarding NAFLD as a critical disease.

On the other hand, metabolic disorders such as obesity, hyperglycemia, hypertriglyceridemia, and hypertension were reported as risk factors for NAFLD/NASH.[18-22] Recently, a clinical study revealed the close relationships between Japanese patients with metabolic syndrome and NAFLD detected by abdominal ultrasonography.[23] The limitations of the report were insufficient examination to detect NAFLD and inappropriate estimation of central obesity to defined metabolic syndrome so far. Although the concept has become believed that NAFLD is a manifestation of metabolic syndrome, it seems obvious that the background of each entity is necessarily overlapped.

Experimental facts related to obesity, metabolic disturbances, and NASH

The experimental data indicated that ingestion of a high-fat diet promotes obesity and the development of metabolic disturbances in rodents.[24-26] A homozygous defect of obese gene in the mouse (ob/ob mouse) presents an obese phenotype as well.[27] Further, homozygous mutations of the leptin receptor gene have been identified both in mice (i.e., db/db mouse) and in rats [Zucker (fa/fa) rat], which are also associated with obesity.[28-30]

Pathophysiology of NASH: insulin resistance, free fatty acids, and cytokines

Although it is not yet fully understood what is the pivotal cause of whether simple hepatic steatosis or steatohepatitis occurs, it is most likely true that insulin resistance and increased free fatty acids in the liver are highly associated with NASH.[31,32] Insulin resistance leads to fat accumulation in hepatocytes by lipolysis and hyperinsulinemia.

Recently, the cytokine–adipokine interaction related to NAFLD is increasingly drawing great attention to elucidate the underlying mechanism. Insulin resistance is thought to be regulated by proinflammatory cytokines, such as TNF-α (tumor necrosis factor-alpha), and some adipokines, e.g., adiponectin and leptin.[33,34]

TNF-α and NAFLD/NASH

TNF-α is an important inflammatory cytokine that is overexpressed in the adipose tissues of rodent models of obesity.[35] Clinically, enhanced TNF-α expression was shown in patients with NASH compared with patients with simple steatosis.[36] Experimental data described that free fatty acids induced production of TNF-α through promoting hepatic lipotoxicity.[37] Moreover, it was shown that antibody-mediated neutralization of TNF-α improves NAFLD in ob/ob mice.[38] Taken together, these findings indicated that TNF-α is one of the critical factors for occurrence and progression of NAFLD/NASH.

Adiponectin and NASH

There is a noteworthy adipokine, adiponectin, which is one of the important properties for antiinflammation, insulin sensitization, and antiatherosclerosis. Adipose tissue is the major site of endogenous adiponectin production. It is well known that hypoadiponectinemia is observed in patients with visceral obesity and insulin resistance, especially NASH and atherosclerosis.[39-41] Furthermore, Hui et al. showed that hypoadiponectinemia is a feature of the NASH independent of insulin resistance.[42]

Additionally, the experimental data suggested that adiponectin and TNF-α suppressed each other's synthesis locally in adipose tissue and suppressed each other's function remotely in muscle in adiponectin-deficient mice.[43] These data indicated that adiponectin has a key role in neutralization of TNF-α. Interestingly, adiponectin considerably alleviated hepatomegaly, steatosis, and abnormal liver function in nonalcoholic obese ob/ob mice as well.[44]

Leptin and hepatic fibrosis

Leptin, an obese gene product mainly produced from adipocytes, is also a cytokine-type hormone that regulates food intake and fat metabolism through actions on the central nervous system.[27] Leptin receptors (Ob-R) have originally been shown in hypothalamic neurons, through which leptin regulates food intake and body weight.[45] In the late 1990s, Potter et al. described that the activated stellate cells in culture during hepatic fibrosis can express leptin.[46] The findings lead to the hypothesis that leptin plays a pivotal role in profibrogenic responses in the liver caused by hepatotoxic chemicals.

Endogenous leptin and hepatic fibrosis

First, we demonstrated that administration of recombinant leptin augments profibrogenic responses in the liver caused by xenobiotics [i.e., carbon tetrachloride, thioacetamide (TAA)] in mice.[47] After that, to investigate whether endogenous leptin promotes hepatic fibrogenesis, we utilized ob/ob mice; which lack leptin because of naturally occurring disruption of the leptin gene. Interestingly, ob/ob mice demonstrated extremely poor profibrogenic responses against xenobiotic treatment. It was shown that leptin appears to promote profibrogenic responses in the liver, in part, by upregulation of transforming growth factor-beta (TGF-β), suggesting that leptin is one of the key regulators of hepatic fibrogenesis.[48,49]

Second, we evaluated that role of Ob-R in hepatic fibrogenesis using Zucker rats, which lack functional Ob-R as a result of a missense mutation in the common, extracellular domain.[50] Zucker rats presented extremely poor profibrogenic responses in the liver caused by chronic thioacetamide (TAA) treatment as compared to their lean littermates, indicating that Ob-R is involved in the profibrogenic responses in the liver.