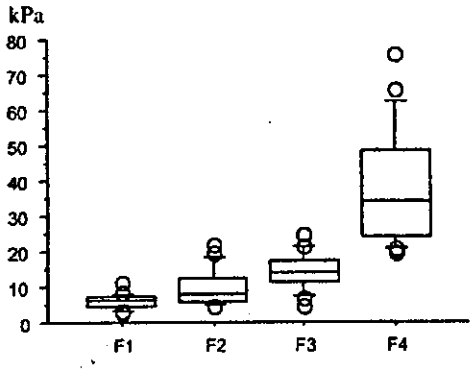

	Median level	P value
F1	6.25	P=0.009 (F1 vs. F2)
F2	7.80	P=0.018 (F2 vs. F3)
F3	13.7	P<0.0001 (F3 vs. F4)
F4	34.0	

Fig. 5. The median and 50% of the levels of elastomtric measurement in each fibrotic stage of the New Inuyama Classification of chronic hepatitis C. The *P*-value in the table means the difference of levels between F1 vs. F2, F2 vs. F3, and F3 vs. F4, respectively, from the top. The significant difference was found between all the groups.

suffered from uniformed etiology, chronic hepatitis C virus infection. The statistical difference between each group was; F1 versus F2 ($P = 0.068$); F2 versus F3 ($P = 0.048$); F3 versus F4 ($P = 0.020$). Thus, this marker also distinguishes between F3 and F4, but differences among from F1 to F3 were not statistically significant.

On the contrary, the elastometry correlated well to the stage of fibrosis ($P < 0.0001$) and the deviation was not so broad (Fig. 5). The actual measurement levels in each stage (median and 50%) were F1, 6.25 kPa (4.60–7.80); F2, 7.80 kPa (5.70–12.48); F3, 13.85 kPa (11.20–17.40); F4, 34.00 kPa (24.00–48.20). The data of F4 were rather significantly large than other stages, but the data of F1–F3 were differentiated well. This condition was quite different from that in serum fibrosis markers. The other series of examination revealed that the elastometry of the livers from 20 healthy subjects all showed less than 5 kPa (median 4.31 kPa; 25–75%, 4.03–4.90 kPa). The statistic difference between the groups was; F1 versus F2 ($P = 0.009$); F2 versus F3 ($P = 0.018$); F3 versus F4 ($P < 0.0001$). These differences were much superior than those in platelet counts. There was no correlation between platelet counts and elasticity measurement. Examples of pathologic findings and the elastometric measurements were shown in Fig. 6. Fibrosis extends from the portal area in the upper photograph of Fig. 6 but bridging fibrosis is not clearly found. The elastometric measurement showed that the elasticity of this liver was 7.9 kPa. On the other hand, the lower photograph shows histology of a case with liver cirrhosis and the measurement result showed that the elasticity of this liver was 20.7 kPa. These

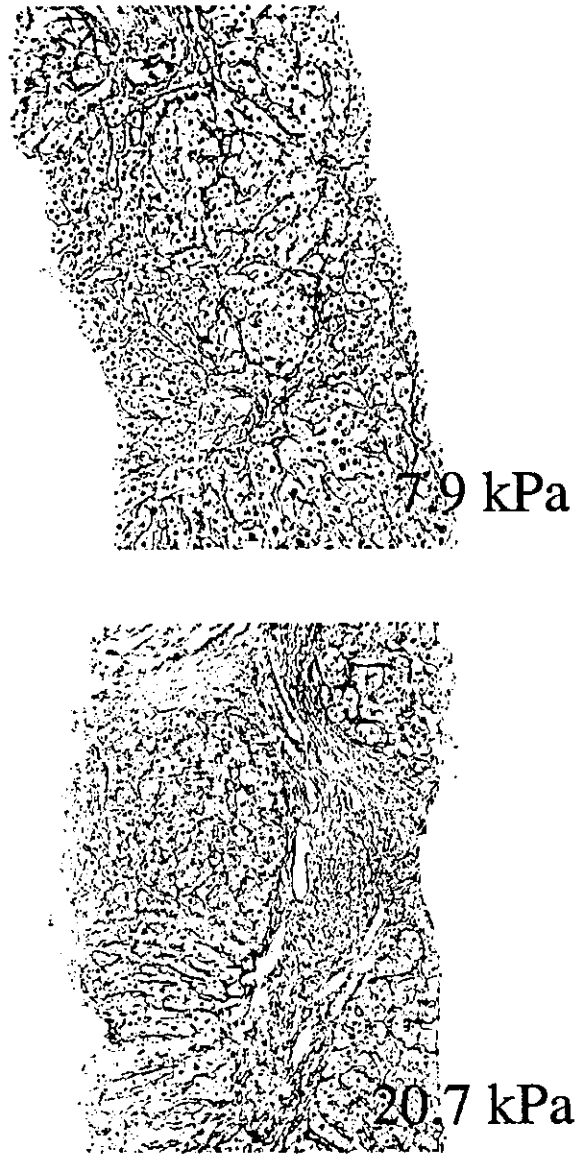

Fig. 6. Typical histology of the livers and correspondent elastometric values. Number shown in photographs indicates the elastometry (kPa). Liver biopsy was performed with 16-gauge needle biopsy apparatus (TopNotch, Boston Scientific Japan Co., Tokyo, Japan).

data were quite compatible for the aspects of the liver histology.

To evaluate the reliability of the result, elastometry was twicely performed in the same 15 patients 3 months after the initial examination. Table 1 shows the result and the two data obtained from the same patient were around the same suggesting that this elastometry is reliable.

5. Discussion

Liver biopsy may be necessary for confirming the diagnosis, evaluating disease severity, and ruling out other

Table 1
Reproducibility of the elastometry (kPa). Two data of the same case were measured 3 months after the initial measurement

Cases	Before (kPa)	Three months later (kPa)
A	10.4	12.6
B	12.5	12.5
C	14.3	19.0
D	17.4	13.1
E	46.7	48.2
F	10.0	9.7
G	12.9	12.9
H	10.2	13.9
I	10.4	10.3
J	11.9	11.9
K	14.3	10.1
L	16.7	17.4
M	6.9	8.8
N	9.0	9.2

unexpected cause of liver diseases, such as metabolic diseases [16,23–26]. It is also useful for identifying early fibrosis that may allow earlier intervention well before progression to cirrhosis, and especially in the natural history of chronic hepatitis C, the establishment of fibrotic stage may be helpful for early detection of hepatocellular carcinoma [26–28]. In case of the patient with chronic hepatitis C in older age such as more than 70, and the liver is slight fibrotic, he or she has no need for anti-viral therapy. Another benefit of liver biopsy is providing motivation of the patient for having treatment early or lifestyle change. On the other hand, although risks can be possibly reduced by operator experience and using an ultrasound guidance, several known risks of obtaining the tissue, such as pain, bleeding, pneumothorax, hemothorax, bile peritonitis, hemobilia, puncture of kidney and intestine, infection, anxiety and even death, do not seem to be entirely avoidable [16–18,29]. The present study showed the non-invasiveness, no risk and reliability of the elastometry in the evaluating hepatic fibrosis, and this procedure can be substituted for liver biopsy in the risky patient. The most useful indication of this measurement may be the sequential changes of hepatic fibrosis in follow-up of the same patient with or without treatment.

A reliable way to determine the presence of advanced fibrosis with non-invasive procedures is needed in chronic hepatitis C. The combination of biochemical markers and clinical and imaging findings can identify much advanced fibrosis [27]. A high aspartate/alanine aminotransferase ratio or low platelet count in patients with chronic hepatitis C has a good predictive value of advanced fibrosis [30]. This study also suggested that platelet counts correlated with the stage of hepatic fibrosis, and the result suggests that we can estimate a patient who becomes cirrhosis when his or her platelet counts decreases under $100 \times 10^3\ \mu l^{-1}$. But the deviation is so large that the cross-sectional approach with platelet counts cannot directly predict hepatic fibrosis. In several European countries, a more complexed formula of tests with γ-globulin, γ-glutamyltranspeptidase, total bilirubin, α2-macroglobulin, haptoglobin, and apolipoprotein A1 achieved to identify patients with and without advanced fibrosis [31]. But this test could categolize patients into two groups with accuracy of 46%. Compared to the value of platelet counts, and also to the value of serum fibrosis markers in the present study, the result of elastometry seemed to be cross-sectionally more reliable, because a deviation was not so large. From this study, we may assess the fibrotic stage of the patient with chronic hepatitis C by elastometry. We can assess as F1 if the result of elastometry is around 5 kPa, as F2 if the result is around 10 kPa, and as F3 if the result is around 15 kPa. If the data were more than 20 kPa, the liver may be in F4 stage, according to the new Inuyama classification [22].

It is thought that the elasticity measured by this device may be influenced by the fibrosis patterns, such as a difference between thick fibrosis and fine fibrosis, that may vary even if the total extracellular matrix content is same. Therefore, the most indicatable case of this device may be the follow-up of the same individual. The elasticity of the liver might be different in patients with chronic viral infection and alcoholic liver diseases, because the intrahepatic fibrotic patterns are different. This is because we investigated only the group of patients with chronic hepatitis C. Liver fibrosis is a complex process, involving injury to liver cells and inflammatory response and scaring [1,6]. In response to liver injury, hepatic stellate cells are activated and proliferate [32,33], resulting in secretion of cytokines [2,3], matrix proteins [34] and their counterparts [35]. The balance of their dynamisms may determine the pattern of fibrotic change in the liver. There are many potential therapies, including antioxidants [36–38], cytokine modulators [35,39–41], anti-inflammatory drugs [42–45], inhibitors of collagen deposition, and inhibitors of stellate cell activation [5,46]. The most primary therapy to reverse fibrosis is elimination or control of the injuring agent such as virus, alcohol and iron, and this therapy successfully reverses cirrhosis [12,47–49]. Although it is not sure that genetic treatment that can stop or reverse fibrosis when the primary pathogenetic agent cannot be eliminated, the elastometry seems to be a good marker for assessing changes achieved by the treatment of fibrosis.

There may be many factors influencing to the measurement of velocity of a transient vibration, but little is known about this apparatus. According to the report described by Sandrin et al. [19], who first developed this apparatus, elasticity measurements were related only to the fibrosis grade and not to the activity and steatosis grades. But he also described that fat tissue may absorb or diminish low-frequent vibration, resulting in a poor signal to noise ratio that affects the elasticity measurement algorithm in patients with obesity. The low-frequency elastic waves do not propagate through liquids, indicating that elastometry is not impossible in patients with ascites. The chest wall contributes to prevent the liver from being directly compressed by the probe itself, and to give a static and plane surface for the probe

positioning. Blood flow might be another consistent factor for the measurement. We wondered that fatty liver is softer than healthy liver parenchyma, suggesting that steatosis would be expected to induce a decrease of liver elasticity. Further evaluation for the elastometory of liver fibrosis in patients with steatosis is necessary. The growing awareness of increasing prevalence of non-alcoholic fatty liver diseases made increased frequency of biopsy, because this type of disease entity has not been established until its diagnostic criteria is determined. Diehl et al. [50] demonstrated the value of liver biopsy for identifying unsuspected but treatable forms of liver disease. This report suggested that the result of liver biopsy sometimes prevents patients from receiving inappropriate treatment. Although the elastometry may assess the changes of fibrotic stage in this type of liver diseases, it is conceivable that we can exactly assess the absolute elastic degree of the liver with fatty degeneration. This is why we selected the patients whose histology showed fatty deposit less than 10% of hepatocytes in whole biopsy samples. The correlation of elastometry and fibrotic stage of the liver in patients with other liver diseases is open for discussion in future, when the data of both histology and elastometry in individuals are collected in various liver diseases.

There are several semiquantitative scores used in clinical trials and in retrospective analysis such as the Knodell [51], the Scheuer [52], the Metavir [53], the Batts–Ludwig [54], and the Ishak [55] scores in the Western countries. In Japan, new Inuyama classification has been used since the older classification was largely modified in 1996 [22]. Criteria for staging of fibrosis was follows; F0, no fibrosis; F1, fibrous portal expansion; F2, bridging fibrosis (portal-portal or portal-central linkage); F3, bridging fibrosis with lobular distortion (disorganization); F4, cirrhosis. Compared with the Metavir score, this Japanese score may includes more severe fibrosis in F2–F4 than that of the Metavir system. Since morphologic features of chronic hepatitis C are sometimes interpreted with interobserver and intraobserver variations, and also the scoring system is different between countries, quantitative evaluation of hepatic fibrosis is necessary. The elastometry will be a good quantitative indicator for hepatic fibrosis if elastometry exactly reflects the whole extracellular matrix content of the liver, and influence of fatty change and other factors for measurement is elucidated.

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Review

Recent understanding of immunological aspects in alcoholic hepatitis

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Abstract

Alcoholic hepatitis is a rate-limiting step in the development of alcoholic liver disease into liver cirrhosis, and approximately half of the heavy drinkers with alcoholic hepatitis develop liver cirrhosis within 5 years. Immunologic mechanisms may be involved in the individual differences in the clinical course of this disease. Endotoxin from the intestine seems to play an important role in neutrophil infiltration of the liver, which induces, and at the same time is induced; by cytokines and chemokines. Kupffer cells and monocytes also have a key role in activating other cell types and producing several cytokines, chemokines, and free radicals. Both cytokines and chemokines up-regulate expression of various adhesion molecules, and adhesion molecules accelerate a cell-to-cell contact that stimulates cytotoxic lymphocytes to cause hepatocyte death. Self-antigens and adducts formed as a result of the degenerative effect of ethanol or aldehyde are targets of antibody-dependent cell-mediated cytotoxicity. Oxygen radicals, NF-kB, and AP-1 are key intracellular factors mediating hepatocyte death in alcoholic hepatitis. Viral infections and alcoholic hepatitis exacerbate each other. Integration of both human investigations and accumulated information from various animal models will gradually clarify the immunological mechanism of alcoholic hepatitis in future.

Keywords: Alcoholic hepatitis; Immunological mechanism; Kupffer cell; Neutrophil; Lymphocyte; Oxygen radicals

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1. Introduction

Alcoholic liver disease (ALD) includes fatty liver, liver fibrosis, alcoholic (steato)hepatitis, and liver cirrhosis. In the United States, about half of the causes of death in liver cirrhosis is alcohol abuse or alcoholism, a situation which is quite different from Japan, where 80–90% of cause of cirrhosis is chronic viral infection. Although, many ALD patients were found to have chronic hepatitis C after discovery of hepatitis C virus (HCV), alcohol intake is an exacerbating factor of chronic hepatitis C [1], and HCV infection is an exacerbating factor of ALD [2]. Eradication of HCV by interferon therapy may result in an increase in the proportion of ALD among chronic liver diseases in Japan in the future.

The rate-limiting step in progression of ALD to liver cirrhosis is the development of alcoholic steatohepatitis, which occurs in 20–30% of heavy drinkers, and steatohepatitis may develop to liver cirrhosis if left untreated. While cytochrome P4502E1 (CYP2E1) is a major microsomal source of oxidative stress and is a candidate for the pathogenesis of alcoholic steatohepatitis [3], approximately 40–50% of the cases of alcoholic steatohepatitis in heavy drinkers have been reported to progress to liver cirrhosis within 5 years. Thus, liver cirrhosis does not occur in all heavy drinkers, and its occurrence is not correlated with the level of alcohol consumption. These observations led to the hypothesis that immunological mechanisms play a role in the development of ALD in addition to individual differences in polymorphisms of CYP2E1 or its expression levels [4], although the precise immunologic mechanisms have not been established.

Animal models are major research tools for understanding the mechanisms of ALD. The initial two reports of animal model were in baboons given a 50% calories alcohol diet [5], and in small animals, rats fed the Lieber–DeCarli diet (36% calories) [6]. Tsukamoto et al. [7] reported continuous intragastric feeding of alcohol to rats and demonstrated that Kupffer cell activation by elevated levels of sinusoidal endotoxin due to increased intestinal permeability to endotoxin caused by alcohol administration was an important event in ALD. Kupffer cells cause oxidative stress following activation of nuclear factor-kappa B (NF-kB), up-regulation of inflammatory cytokines and adhesion molecules, and, finally, inflammatory cell invasion. In rats, these changes are followed by fatty change, patchy necrosis, mild inflammation, and perivenular fibrosis; however, there have been no animal models that are histologically compatible with human ALD, nor have there been any adequate models of viral hepatitis, making it difficult to clarify the pathophysiological mechanisms of ALD.

Immunological mechanisms and immunological abnormalities in ALD have been assessed in terms of both their humoral and cellular aspects. Alcoholic drinking results in two controversial effects on the immunological system. One is that heavy drinking results in a decrease in immunological activity [8], and the other is that an alcohol intake strongly stimulates lymphocytes, leading to inflammation in the liver and a decrease in various immunological markers [9]. There have been many reports about immunological aspects of ALD, but they have not been consistent, because each report has been a reflection of the immunological experimental procedures available when the investigation was performed. For example, various pathogeneses, such as an antibody against Mallory body [10,11], an antibody against lipopolysaccharide [12], an antibody against self-antigen or alcoholic adducts [13–17], cytotoxic T cells [18–21], decrease in cellular immunity [22–24], IgA [25–27], phagocytic activity [28], and cytokines [29–31], have been considered as causes for alcoholic hepatitis in a long research history of alcoholic liver diseases [32]. We review the immunological aspects of alcoholic hepatitis in this article.

2. Role of Kupffer cells in alcoholic hepatitis

Chronic alcohol administration increases intestinal mucosal permeability and the serum lipopolysaccharide (LPS) concentration. LPS binds to LPS-binding protein (LBP), forming an LPS–LBP complex, and this complex binds to the CD14 receptor on the cell membrane of Kupffer cells (KCs). The LSP–CD14 complex reacts with toll-like receptor 4 (Tlr4), which is a membrane-penetration-type receptor, and this stimulates signal transduction and activates nuclear factor-kappa B (NF-kB). Another pathway that activates NF-kB is the oxidative stress caused by LPS itself. NF-kB up-regulates proinflammatory cytokines, tumor necrosis factor (TNF)-α, and cyclooxygenase (Cox)-2, and they induce intrahepatic inflammation. There have been many reports of animal models and human investigations supporting the above scenario as described below.

2.1. Animal models

CD14 and LBP mRNA levels have been demonstrated to correlate well with the extent of liver damage in the Tsukamoto–French model [32]. CD14 has been found to be expressed in KCs and LBP to be expressed in hepatocytes, and alcoholic liver damage has not been induced in CD14-deficient mice or Tlr4-mutant mice [33,34]. NF-kB activation in hepatocytes was demonstrated in the Tsukamoto model [35], but no TNF-α or NF-kB up-regulation or liver damage was observed after ethanol administration to $p47^{Phos}$ knock-out (k/o) mice [36]. The $p47^{Phos}$ is a central subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The findings in this k/o mouse model suggest a scenario, in which the deficiency of NADPH oxidase in KCs cannot induce free-radicals after ethanol administration, and the free-radicals up-regulate NF-kB and TNF-α which then induce liver damage. Intragastric feeding of corn oil and fish oil up-regulated expression of Cox-2 and TNF-α mainly in KCs, that induced necroinflammation in the liver [37]. A significant

role of Cox-2 in hepatic inflammation was also demonstrated in the Cox-2 k/o mice having TNF-α plus galactosamine administration [38].

2.2. Human investigation

Significantly higher levels of TNF-α production has been demonstrated by the monocytes of ALD patients than by the monocytes of healthy controls. The levels of TNF-α, interleukin (IL)-1, and IL-6 in cholangio-endothelial cells and KCs have been found to be significantly higher in ALD patients than in healthy controls [39]. Moreover, monocyte chemoattractant protein-1 and IL-8 are expressed in KCs and may facilitate invasion of the liver by other inflammatory cells.

3. Role of neutrophil invasion

One of the histological characteristics of ALD is neutrophil invasion of the liver. Major neutrophil chemoattractants are CXC chemokines (IL-8, cytokine-induced neutrophil chemoattractant (CINC), macrophage inflammatory protein (MIP)-2, KC, inducible protein (IP)-10, ENA) and RANTES (regulated upon activation, normal T cell expressed and secreted). These chemokines are produced by many different kinds of cells in the liver, including hepatocytes and KCs. On the other hand, several adhesion molecules, such as selectin, which is expressed on vascular endothelium in neutrophil rolling phase, β2 integrin (CD11b, CD18), which is expressed on neutrophils after the rolling phase, and intercellular adhesion molecule (ICAM)-1, which is then expressed on both endothelium and hepatocytes.

TNF-α and chemokine production is increased in both humans with alcoholic hepatitis and animal models [40–43]. Hirano et al. [44] demonstrated that chemokines such as RANTES, are up-regulated by stimulation with TNF-α. Oxidative stress up-regulates redox-reactive transcription factors, such as NF-kB and activator protein (AP)-1, and induces secretion of various cytokines and chemokines [45]. CXC chemokines seem to be produced by KCs, because inactivation of KCs results in attenuation of CXC chemokines and a decrease in liver damage [46]. TNF-α and IL-1 then up-regulate adhesion molecules of neutrophils, hepatocytes, and other cells, and that induces cell-to-cell interaction. The cell-to-cell interactions involve neutrophil-mediated hepatocyte damage [47] or sinusoidal endothelial damage [48,49]. The chronic neutrophil invasion of the liver observed in ALD requires prolonged chemokine production in the liver, because the half-life of neutrophils is short. The cytotoxic effect of neutrophils has been demonstrated in the ischemia–reperfusion model, the endotoxin model, the warm-shock model, the cold-shock model, and a drug toxicity model. Hepatocyte apoptosis also stimulates neutrophil invasion, which then expands inflammation by a positive feedback mechanism [50,51].

Expression of E-cadherin and vascular cell adhesion molecule (VCAM)-1 on the endothelium, in addition to constitutive expression of ICAM-1, is necessary for neutrophil-invasion of the liver parenchyma [52]. Circulating neutrophils are always activated in patients with ALD [53], and neutrophil-activation leads to production of reactive oxygen species (ROS) and Mac-1, followed by increased production of TNF-α and IL-8, leading to hepatocyte toxicity and apoptosis [54]. Serum IL-8 levels have been shown to be correlated with neutrophil-invasion levels [43], which supports the scenario described above.

There is no neutrophil invasion in either the Lieber–DeCarli model; or the Tsukamoto–French model, however, LPS administration in these models was followed by increased expression of CXC chemokines and adhesion molecules and neutrophil invasion of the liver [55–57]. This phenomenon suggests that the most important factor in the establishment of the neutrophil-invasion in the liver seen in ALD is the supply of endotoxin from the intestine.

4. Role of lymphocyte invasion

Circulating lymphocytes in ALD patients may be trapped by the sinusoidal endothelium by adhesion with VCAM-1, ICAM-1, 2, or vascular adhesion protein (VAP)-1 [58], and then they invade into hepatic parenchyma by several CXC chemokines, such as monokine induced by gamma-interferon (KIG), interferon-inducinble T cell alpha chemoattranctant (ITAC) and IL-10, which are up-regulated in hepatitis [40,42,59,60]. The CD31 molecule is thought to be responsible for invasion through inter-endothelial tight junctions [61].

Necroapoptosis in alcoholic hepatitis is thought to be caused by cytokines, such as TNF-α, and free-radicals, such as nitric oxide (NO). The precise role of lymphocytes in ALD, however, is still a matter of controversy. Chedid et al. [62] and Sakai et al. [21] showed increased numbers of intrahepatic CD8+ cells and CD44+ cells, and decreased numbers of B cells and natural killer (NK) cells. NK activity is decreased in animal models [63], but the numbers and activity of CD3–CD56+ cells (compatible with NK cells) in the peripheral blood of ALD patients are increased, while their intrahepatic numbers are decreased [62], in contrast to viral hepatitis. Summarizing the findings in many reports, TNF-α and IL-6 are up-regulated in alcoholic hepatitis patients but IFN-γ is downregulated. T cells that produce TNF-α and IFN-γ are classified as CD57+ cells and are reactive to T helper (Th)1-type cytokines [9], but there are individual differences in the production of these cytokines [8], probably because of promoter polymorphisms [64,65]. It has also been reported that T cells are activated in chronic drinkers who drink more than 80 g/day ethanol, and that the activation persists after they stop drinking [8]. Production of these cytokines has also been demonstrated in animal models.

The CD8/CD4 ratio is said to be higher in the liver than in peripheral blood, and French and co-workers [66] demonstrated that CD4+ cells are predominant in zone 3 and CD8+ cells are predominant in zone 1, although both cells are seen in the portal area. MHC class I expression correlates with levels of portal inflammation and interface hepatitis, and MHC class II expression correlates with hepatocyte necrosis and appearance of Mallory bodies. CD29, CD45 RA, and CD45 RO, which are important for recognition of allo antigens or for adhesion, are highly expressed in the necrotic area and/or in Mallory-positive liver. These findings indicate that cytotoxic T cells (CTLs) are important to the progression of alcoholic hepatitis as well as to the progression of viral hepatitis.

Fas-Fas-ligand (FasL)-mediated CTL and TNF-α play important roles in chronic hepatitis C virus (HCV) infection [67], and alcohol abuse may exaggerate the cytotoxic process [68] because alcohol increases Fas/Fas-L expression [69,70]. Expression of another important cytotoxic mediator, perforin-granzyme, requires MHC class I expression, and alcohol intake increases its expression, suggesting that alcohol drinking increases CD8+ T cell-mediated cytotoxicity in the liver [71].

5. Role of antibody-dependent cell-mediated cytotoxicity

Antibody-dependent cell-mediated cytotoxicity (ADCC) is thought to be involved in the liver damage in alcoholic hepatitis. Autoantibodies against CYP3A4 and CYP2E1 are found in 20–30% and 10–20%, respectively, of healthy individuals [72], and the titers of these autoantibodies in the Tsukamoto–French model rise to two- to three-fold above the control level 1 month after the start of feeding and they correlate with levels of liver damage. Administration of chlormethiazole, an inhibitor of CYP2E1, reduced CYP2E1 activity as well as the autoantibody titers [73]. A similar phenomenon has also been observed in regard to other autoantibodies, such as anti-hydroxyethyl adduct and anti-malondialdehyde adduct, in both animal models and humans [74]. These adducts are produced during oxidation of ethanol by CYP2E1. Accumulation of the autoantibodies to these adducts is observed on hepatocyte membranes by confocal microscopy [75], suggesting that the ADCC mechanism may operate in alcoholic hepatitis [66,76]. Vidali et al. [77] recently reported that polymorphism in the exon 1 of CTL antigen (CTLA)-4 gene induces disregulation of T-cell proliferation, leading to production of autoantibody against CYP2E1.

6. Role of monocytes

The peripheral blood monocytes of alcoholic hepatitis patients produce more TNF-α, both in the presence and absence of stimulation by LPS, than those of healthy controls [31], and TNF receptor expression is increased [30]. The expression levels are always correlated with progression to liver cirrhosis, and the same phenomenon is observed in regard IL-6 production. The production levels and serum levels of MCP-1 and MIP-1 are significantly higher in alcoholic hepatitis patients than in healthy controls [42,78]. A recent study demonstrated that acute alcoholic stimulation inhibits cytokine production from monocytes, but chronic stimulation up-regulates production of cytokines and reactive oxygen from monocytes [79], suggesting that pathophysiological mechanisms are different between acute injury and chronic injury. Thus, inhibition of monocyte activation is now going to be the target of the treatment of alcoholic hepatitis in the future.

Chronic ethanol administration to rats for 6–8 weeks makes hepatocytes more susceptible to injury by TNF-α [80], and the mechanism of the change in susceptibility is attributable to a change in the membrane permeability of mitochondria [81]. Recent studies have shown that liver injury always correlates with genomic polymorphisms of manganese superoxide dismutase [82], while a negative report appeared after then. Further studies are needed in regard to this matter [83].

7. Conclusion

We have reviewed the immunological aspects of alcoholic hepatitis. Although many factors such as hypoxia have been implicated to have a significant role in the pathogenesis of alcoholic liver disease [84], immunologic factors seem to be still important for making its individual difference. The major reason why the precise immunological mechanism has not been understood in the pathogenesis of alcoholic hepatitis is a lack of adequate animal models, the same as in viral hepatitis. Integration of human investigations and accumulated information from various animal models will gradually clarify the immunological mechanisms of alcoholic hepatitis in the future.

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Benefit of Hepatitis C Virus Core Antigen Assay in Prediction of Therapeutic Response to Interferon and Ribavirin Combination Therapy

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A highly sensitive second-generation hepatitis C virus (HCV) core antigen assay has recently been developed. We compared viral disappearance and first-phase kinetics between commercially available core antigen (Ag) assays, Lumipulse Ortho HCV Ag (Lumipulse-Ag), and a quantitative HCV RNA PCR assay, Cobas Amplicor HCV Monitor test, version 2 (Amplicor M), to estimate the predictive benefit of a sustained viral response (SVR) and non-SVR in 44 genotype 1b patients treated with interferon (IFN) and ribavirin. HCV core Ag negativity could predict SVR on day 1 (sensitivity = 100%, specificity = 85.0%, accuracy = 86.4%), whereas RNA negativity could predict SVR on day 7 (sensitivity = 100%, specificity = 87.2%, accuracy = 88.6%). None of the patients who had detectable serum core Ag or RNA on day 14 achieved SVR (specificity = 100%). The predictive accuracy on day 14 was higher by RNA negativity (93.2%) than that by core Ag negativity (75.0%). The combined predictive criterion of both viral load decline during the first 24 h and basal viral load was also predictive for SVR; the sensitivities of Lumipulse-Ag and Amplicor-M were 45.5 and 47.6%, respectively, and the specificity was 100%. Amplicor-M had better predictive accuracy than Lumipulse-Ag in 2-week disappearance tests because it had better sensitivity. On the other hand, estimates of kinetic parameters were similar regardless of the detection method. Although the correlations between Lumipulse-Ag and Amplicor-M were good both before and 24 h after IFN administration, HCV core Ag seemed to be relatively lower 24 h after IFN administration than before administration. Lumipulse-Ag seems to be useful for detecting the HCV concentration during IFN therapy; however, we still need to understand the characteristics of the assay.

Hepatitis C virus (HCV) infection causes a slowly progressive disease which can lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (28, 30). Successful interferon (IFN) therapy for HCV leads to persistently undetectable serum viral levels and histological improvement (9, 11) and improves the survival of chronic hepatitis C patients by preventing liver-related deaths (36). A meta-analysis study including data from randomized trials showed that retreatment of nonresponders with a combination of IFN-α2b and ribavirin (RBV) for 24 weeks was associated with only 14% sustained virological response (SVR) in genotype 1-infected patients (4). When a combination of pegylated interferon (IFN) and RBV was used as retreatment for 48 weeks, a 46% SVR rate was reached; however, patients infected with genotype 1 still had a limited chance of achieving SVR (12).

Studies aimed at understanding the predictive value of SVR and non-SVR in the absence or presence of serum RNA during IFN therapy within 2 days (32), 1 week (18), 2 weeks (17), 1 month (3, 6, 13, 15, 29, 39), or 3 months (23) have been reported. The biphasic or triphasic initial decline in the level of serum HCV RNA after IFN therapy has also been characterized and analyzed mathematically (8, 14, 21, 27, 40). The exponential and dose-dependent first phase is a 0.5- to 2.5-log decline in the serum virus concentration within 24 h. The first-phase response is attributed to IFN blocking viral production or release and a rapid free virion clearance rate. A slower second-phase decline in serum viral levels is observed after the first phase and may reflect the rate of clearance of infected hepatocytes and the effectiveness of IFN in blocking viral production.

Viral kinetic studies have been analyzed predominantly by the HCV RNA assay using the PCR method. Although the PCR assay is very sensitive in detecting the serum HCV RNA, the results sometimes have problems of specificity and precision, and blind comparisons have significant error rates (37). Methods for detecting viral antigens (Ag) were developed by applying a monoclonal antibody to the HCV core Ag (19, 33, 35); however, the assays have been insufficient for clinical application because of their low sensitivity and the requirement for complicated specimen pretreatment. An accurate and specific new HCV core Ag detection assay system (total HCV core Ag assay) (2) has recently been developed and is commercially available in European countries (*trak-C* assay) (7, 20, 24, 25, 31); it has a lower detection level limit of 1.5 pg/ml, which is equivalent to 20 KIU/ml. More recently, Lumipulse Ortho HCV Ag (Lumipulse-Ag), with a lower detection level limit of 50 fmol/liter (equivalent to 1.0 pg/ml), was developed in Japan (1, 34). The dynamic range of Lumipulse-Ag (from 50 to 50,000 fmol/liter, equivalent to 1.0 to 1,000 pg/ml) is broader than that of the *trak-C* assay (1.0 to 100 pg/ml) and the PCR quantitative assay, Cobas Amplicor HCV Monitor test (Am-

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plicor-M). Because HCV core Ag is stable, no extra precautions are needed for storing samples. An easy, simple, low-cost new HCV core Ag detecting system seems to be useful for assessing and monitoring IFN treatment of HCV. Only a few predictive studies for IFN-RBV combination therapy (24, 25) and no studies of first-phase viral kinetics have been established for the HCV core Ag assay so far. In the present study, we compared the viral disappearance rates and kinetic data between Lumipulse-Ag and Amplicor-M to estimate the predictive benefit of SVR and non-SVR with IFN-RBV combination therapy.

MATERIALS AND METHODS

Patients and treatment regimen. Adult patients with consecutive genotype 1b HCV-RNA positive, abnormal alanine aminotransferase (ALT) levels and histologically consistent chronic hepatitis C were eligible for this study. All patients gave written informed consent before treatment, and the protocol was approved by the ethics committee of each participating center. Patients with cirrhosis or patients coinfected with HBV were excluded. All patients received 6 MU of IFN-α2b every day for 2 weeks and then thrice weekly for 22 weeks. RBV was given orally for a total dose of 600 mg (body weight, <60 kg) or 800 mg (body weight, ≥60 kg) per day for 24 weeks. An SVR was defined as a normal serum ALT level and undetectable serum HCV RNA and HCV core Ag 24 weeks after discontinuation of therapy. A non-SVR was defined as the response in patients who did not show SVR.

Determination of serum HCV RNA, HCV genotypes, and HCV core Ag. The HCV genotype was determined using a commercially available probe assay which can distinguish genotypes 1a, 1b, 2a, 2b, and 3a of HCV (Monitor Genotype; Roche Diagnostics, Tokyo, Japan). Serum HCV RNA levels were determined by a Cobas Amplicor HCV Monitor test, version 2 (Amplicor-M) (Roche Diagnostics). For quantification of serum HCV core Ag, Lumipulse Ortho HCV Ag (Lumipulse-Ag) (Ortho Clinical Diagnostics, Tokyo, Japan) was used as specified by the manufacturer. The lower detection limit for each assay kit was 0.5 KIU/ml in Amplicor-M and50 fmol/liter in Lumipulse-Ag.

Viral kinetic parameters. Viral load at baseline (V_0) and on day 1 (V_1) and the viral load decline 24 h after IFN administration (ε) were calculated using HCV core Ag and HCV RNA data by nonlinear fitting as described elsewhere (27). Whenever HCV core Ag and HCV RNA levels were below the sensitivity of each assay, a value of 50 fmol/liter and 0.5 KIU/ml were arbitrarily used for calculation because those were the lower limit for each assay.

Statistical analysis. The statistical significance of differences between groups was analyzed using the Mann-Whitney U-test, Fisher's exact test, and the Wilcoxon signed-rank test. Sensitivity was calculated as the ratio of the number of patients who gave SVR to the number of patients predicted to give SVR. Specificity was defined as the ratio of the number of patients who became non-SVR to the number of patients predicted to give non-SVR by our criteria. The positive predictive value (PPV) was defined as the proportion of patients with the factor who responded to treatment. The negative predictive value (NPV) was defined as the proportion of patients without the factor who did not respond to treatment. Accuracy was defined as the ratio of the number of patients correctly predicted to be SVR or non-SVR to the total number of patients.

RESULTS

Patient characteristics. Forty-nine genotype 1b-infected patients were enrolled, of whom 44 completed the study as per protocol. The demographic characteristics of the patients are shown in Table 1. There was no statistical significance in the 6-month IFN-RBV combination therapy between SVR and non-SVR patients with respect to age, gender, body weight, baseline ALT levels, and platelet counts. We also observed no significance with respect to baseline viral loads detected by HCV core Ag or HCV RNA (Table 1).

Disappearance of serum HCV core Ag or HCV RNA during therapy. There were 10 SVR patients (22.7%) who remained HCV RNA negative and HCV core Ag negative throughout the follow-up period and had sustained normalization of serum ALT levels. Four patients were HCV core Ag negative on day 1, whereas no patients were negative for HCV RNA on day 1. All patients (four of four) who were negative for HCV core Ag on day 1 achieved SVR (sensitivity = 100%, specificity = 85.0%, accuracy = 86.4%) (Table 2). None of the patients achieved negative results for HCV RNA by Amplicor-M on day 1, while five patients were HCV RNA negative on day 7; all patients (five of five) who were negative for HCV RNA on day 7 achieved SVR (sensitivity = 100%, specificity = 87.2%, accuracy = 88.6%). The specificity of Lumipulse-Ag increased on day 7 and reached 100% on day 14. The specificity was 77.3% on day 1 and also reached 100% on day 14 by Amplicor-M. None of the patients who had detectable serum core Ag or RNA on day 14 achieved SVR (specificity = 100%). All 10 SVR patients were both core Ag and RNA negative on day 14 (PPV = 100%). Core Ag and RNA negativity on day 14 could predict 23 and 31 non-SVR patients out of 34 (NPV = 67.6 and 91.2%, respectively) (Tables 2 and 3).

Viral kinetics assay. We analyzed the decline in the viral load within the initial 24 h (ε) and found that a >90% decline (1.00-log decline) in core Ag and an 87% decline (0.89 log decline) in RNA levels gave a specificity of 100% and sensi-

TABLE 1. Baseline clinical and virological characteristics of the comparison of SVR and non-SVR

Characteristic	SVR (n = 10)	Non-SVR (n = 34)	P
Age (yr)[a]	60.5 (29–66)	60.0 (21–70)	1.000
Gender (female/male)[b]	4/6	10/24	0.701
Body weight (kg)[a]	61.85 (47.0–75.5)	66.35 (40.4–86.4)	0.712
ALT (IU/liter)[a]	74.5 (17–402)	75.5 (28–206)	0.906
Platelet (×10/liter)[a]	16.95 (11.6–25.3)	14.75 (10.1–28.7)	0.114
Liver histology			
Activity (A0/A1/A2/A3)	1/1/8/0	2/11/21/0	0.350
Fibrosis (F0/F1/F2/F3)	1/3/6/0	0/9/19/6	0.132
HCV RNA (kIU/ml)[a]	290 (90.1–4,100)	660 (81–40,000)	0.130
HCV core Ag (fmol/liter)[a]	4,800 (207–16,829)	6,154 (726.2–50,000)	0.245

[a] Values are expressed as medians (range); groups were compared by the Mann-Whitney U-test except for gender.
[b] Fisher's exact test.

TABLE 2. Treatment results and disappearance of serum viral load

Ag or RNA and time	Presence of Ag or RNA	No. giving SVR (n = 10)	No. giving non-SVR (n = 34)	Fisher exact P-value
HCV core Ag				
Day 1	−	4	0	0.00150
	+	6	34	
Day 7	−	8	6	0.000551
	+	2	28	
Day 14	−	10	11	0.000141
	+	0	23	
HCV RNA				
Day 1	−	0	0	NA[a]
	+	10	34	
Day 7	−	5	0	0.000232
	+	5	34	
Day 14	−	10	3	<0.0001
	+	0	31	

[a] P value was not calculable because none of the patients had negative HCV RNA on day 1.

TABLE 3. Viremia at different time points after IFN therapy as a predictor of treatment response

Negativity and time	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
HCV core Ag					
Day 1	100	85.0	40.0	100	86.4
Day 7	57.1	93.3	80.0	82.4	81.8
Day 14	47.6	100	100	67.6	75.0
HCV RNA					
Day 1	0	77.3	0	100	77.3
Day 7	100	87.2	50.0	100[a]	88.6
Day 14	76.9	100	100	91.2[a]	93.2[a]

[a] $P < 0.05$.

tivities of 35.7 and 33.3%, respectively. Thus, failure to drop 1.00 log in core Ag and 0.89 log in RNA predict non-SVR. The thresholds of the baseline viral load (V_0), which gave 100% specificity for the prediction of SVR in Lumipulse-Ag and Amplicor-M, were 17,000 fmol/liter and 5,000 kIU/ml, respectively. A total of 10 of 37 with Lumipulse-Ag and 10 of 36 with Amplicor-M achieved SVR under those criteria (the sensitivities were 27.0 and 27.8%, respectively). When we assessed viral load after 24 h (V_1), the thresholds that gave 100% specificity for the prediction of non-SVR were 850 fmol/liter with Lumipulse-Ag and 120 kIU/ml with Amplicor-M, and the sensitivities were 34.5 and 38.5%, respectively. The viral-load decline during the initial 24 h of IFN treatment (ε), basal viral loads (V_0), and viral loads on day 1 (V_1) detected by Lumipulse-Ag and Amplicor-M and the correlation between the three values were investigated. When we combined the criteria of both ε (1.00-log decline in the core Ag assay and 0.89-log decline in the RNA assay) and V_0 (17,000 fmol/liter in Lumipulse-Ag and 5,000 kIU/ml in Amplicor-M), the sensitivities with Lumipulse-Ag and Amplicor-M were 45.5% (accuracy = 72.7%) and 47.6% (accuracy = 75.0%), respectively, while the specificities were both 100%. The predictive efficiencies did not improve when we assessed the data in combination with ε and V_1 instead of ε and V_0 with both Lumipulse-Ag and Amplicor-M.

Correlation between HCV core Ag and HCV RNA before and after IFN administration. We found a good correlation between HCV core Ag and HCV RNA levels not only for basal levels but also for those on day 1 ($r = 0.870$, $P < 0.05$ and $r = 0.794$, $P < 0.05$, respectively). However, we found that the correlation coefficients evaluated before and after IFN administration were different (Fig. 1). HCV core Ag levels were relatively higher in the basal viral loads than 1 day after IFN therapy compared with the levels of HCV RNA.

DISCUSSION

It is better to differentiate between non-SVR and SVR patients as fast as possible during IFN-RBV combination therapy because this therapy is expensive and is associated with several severe adverse reactions. If we can distinguish SVR from non-SVR patients on the basis of viral kinetics in the early stages, we can avoid unnecessary IFN therapy. Early clearance of viremia (3, 6, 13, 15, 17, 18, 29, 32, 39) has been reported to be a strong predictor of SVR, using multivariate analysis of IFN monotherapy. Several predictions of therapeutic response on the basis of early viral clearance have also been reported for IFN-RBV combination therapy. The sensitivity and specificity were said to estimate the predictive benefit in these reports; however, they seemed to depend on the procedure of the HCV detection assay.

We found 100% sensitivity in the prediction of SVR by the HCV RNA assay after 1 week of IFN-RBV combination therapy and 100% sensitivity by the HCV core Ag assay on day 1. The different time points of HCV disappearance between Lumipulse-Ag and Amplicor-M could be due mainly to the different sensitivities of the assay systems. Amplicor-M has higher sensitivity for HCV detection, resulting in an HCV-positive result on day 1 after initial IFN administration in some SVR patients given the 6-month combination therapy. Since the sensitivity of Lumipulse-Ag was lower than that of Amplicor-M, this system could precisely predict SVR earlier than could the use of HCV RNA measurements.

The response to IFN-RBV combination therapy is so much better than the response to IFN monotherapy that some SVR still existed in patients in whom the HCV RNA level was positive 4 weeks after the start of therapy (26); however, early viral clearance was generally associated with SVR in IFN-RBV combination therapy (10). Brouwer et al. reported that the disappearance of serum HCV RNA at week 4 was highly predictive of non-SVR (specificity = 100%) and that SVR was achieved in 46% of patients in whom HCV RNA was cleared by week 4 (sensitivity = 46%) of the 6-month combination therapy (3). In the present study, we also found that the specificity was 100% at week 2 with both Lumipulse-Ag and Amplicor-M and the sensitivities were 47.6 and 76.9%, respectively. Because of its higher sensitivity, Amplicor-M could detect more non-SVR on day 14 than could Lumipulse-Ag.

On the other hand, the decrease in viral load within 24 h of the initial IFN injection has been used for the prediction of SVR in combination therapy. Jessner et al. reported that the reduction in viral load within 24 h following a single injection of IFN could be a good predictor of non-SVR in 6-month combination therapy (16). They administered an initial 10 MU followed by a secondary 5 MU of IFN 1 week later. They achieved 100% specificity for non-SVR prediction in patients who did not show more than a 70% reduction in viral load within 24 h of receiving the initial 10 MU of IFN, with 61% sensitivity; 83% sensitivity was obtained after the later 5-MU administration. We found that none of the patients gave SVR in whom the viral load declined less than 90% (1.00-log decline) of the initial load within 24 h by the core Ag assay, whereas the viral load declined less than 87% (0.89-log decline) by the RNA assay, with 35.7 and 33.3% sensitivity, respectively. The sensitivity of predicting SVR in our data was calculated to be lower than that in Jessner's report. This difference may be due to the patient distribution. We investigated 44 genotype 1b-infected patients, while Jessner et al. included 29 genotype 1a- and 1b-infected patients. Although changes in viral load seem to be important for therapeutic prediction, further study is required before we can understand the predictive benefit of a viral decline within 24 h of the initial injection.

Furthermore, the combination of viral decline within 24 h (ε) and the viral load on day 1 (V_1) was reported to have a good predictive value (22). We also obtained better sensitivity along

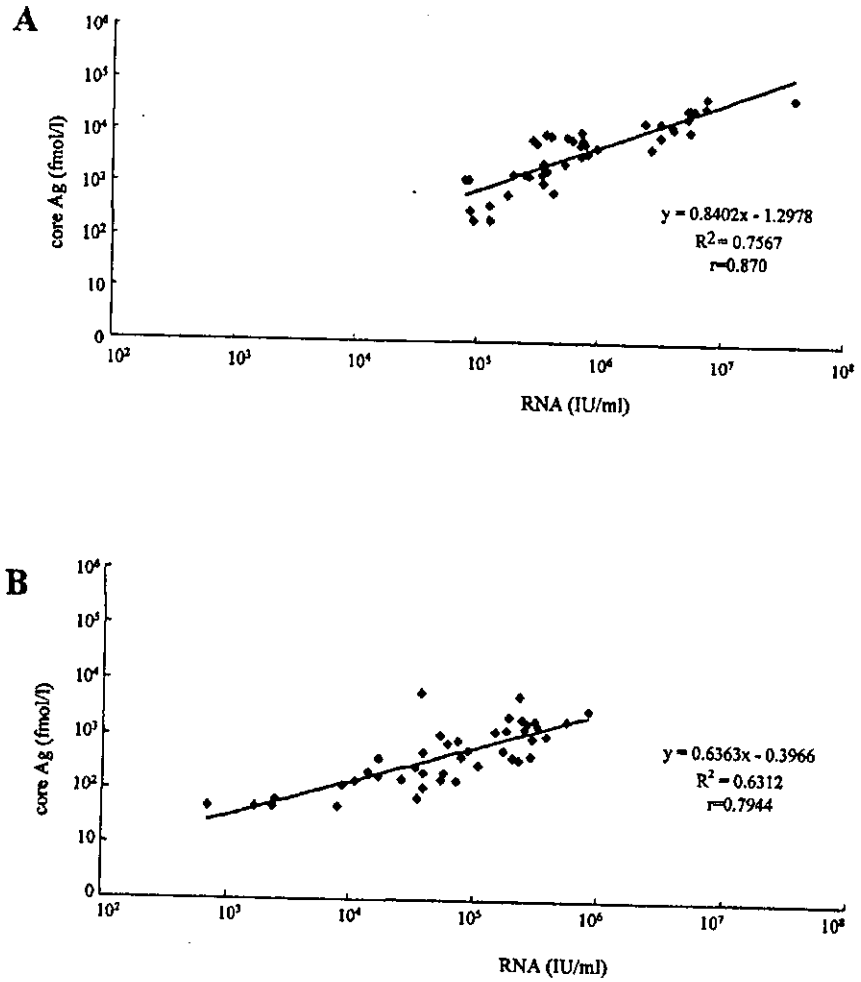

FIG. 1. Correlation between HCV core Ag and HCV RNA before (A) and 24 h after (B) therapy. Correlations were good both before and 24 h after therapy (r = 0.870 and 0.794, respectively), although the coefficient was smaller 24 h after therapy than before therapy ($P < 0.05$). The HCV RNA level was relatively higher than that of core Ag 24 h after therapy.

with 100% specificity when using this combination of criteria (sensitivities were 45.5% with the core Ag assay and 47.6% with the RNA assay) than using single criteria. These authors reported better prediction when using a combination of ε and V_1 than using ε and V_0 in studies of IFN monotherapy. In the present study, we observed that sensitivity and accuracy did not improve with the combination of ε and V_1 compared to ε and V_0 in IFN-RBV combination therapy. It seemed that the prediction of the criteria would be different according to the combination with RBV.

The predictive efficiency of viral kinetics for IFN therapy has been achieved mainly by using the RNA PCR assay. Application of a commercially available quantification system for determination of HCV RNA levels may be limited by underestimation of highly viremic sera as described for the Amplicor-M (5), although a Cobas TaqMan HCV assay, which has an increased upper cutoff limit, is being developed. The upper limit of the Cobas Amplicor HCV test version 2 is 850 kIU/ml, which is not always enough to assess the viral levels before IFN treatment, especially in genotype 1-infected patients. In our series, 16 of 44 patients had viral loads above 850 kIU/ml, which required dilution of the sera. In contrast, the dynamic range of Lumipulse-Ag is broader than that of Amplicor-M, and all the samples in our series could be assayed without dilution.

When we compared core Ag and RNA PCR assays in first-phase viral kinetics for predicting SVR and non-SVR, none of the patients achieved SVR with a 90% (1.00 log) decline in core Ag levels, which was similar to the results of PCR. The sensitivities were also similar in the two detection systems in the criteria giving 100% specificity. Amplicor-M, however, achieved a better predictive accuracy than did the core Ag assay when evaluated 2 weeks after the start of therapy because this assay is more sensitive. On the other hand, the cost of Lumipulse-Ag is 73% lower than that of Amplicor-M. Because of its stability and low cost, along with the advantages described above, the HCV core Ag assay system seems to have an advantage in detecting HCV concentrations in large clinical trials in many countries including developing countries, but we need to understand the characteristics of these assays to select the assay system suitable for the aim of each study. The Ag assay may have utility, but further studies are needed to de-

termine when therapy can be discontinued based on a failure to observe a drop in Ag levels. To our knowledge, this is the first study in which patients with chronic HCV infection were monitored by HCV core Ag in the first phase of IFN-RBV therapy.

Another novel finding of this study was the different HCV RNA core Ag ratio before and 24 h after IFN therapy. Although the correlation between the levels with the core Ag assay and the RNA PCR assay was good both in basal viral load (V_0) and viral load 24 h after IFN administration (V_1), the correlation coefficients were different (Fig. 1). The results of the core Ag assay were relatively lower for V_1 than for V_0. An exponential first-phase viral decline, which was attributed to the direct effects of IFN in blocking HCV production or release and very rapid free virion clearance based on mathematical modeling (21, 27), began within 24 h of IFN administration. We had speculated that the serum core Ag level might be greater in V_1 than in V_0 because destroyed HCV particles without RNA would appear in the serum. However, a different result was obtained. Although the exact reason was unclear, these findings might be valuable in our understanding of the mechanism of rapid viral decline within 24 h of IFN administration. Further precise determination of first-phase viral kinetics by both assay systems might clarify further the mechanisms of the antiviral effects of IFNs.

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Synergistic Effect of Basic Fibroblast Growth Factor and Vascular Endothelial Growth Factor in Murine Hepatocellular Carcinoma

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The growth of any solid tumor depends on angiogenesis. Among the known angiogenic factors, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), are potent and representative factors involved in tumor development. It has been reported that bFGF and VEGF showed a synergistic effect in both *in vitro* and *in vivo* angiogenesis. However, the interaction of these factors on tumor development and angiogenesis, including hepatocellular carcinoma (HCC), has not yet been elucidated. In this study, we examined the combined effect of bFGF and VEGF overexpression by means of a combination of a retroviral tetracycline (tet)–regulated (Retro-Tet) gene expression system, which can manipulate the gene expression *in vivo* by providing tet in the drinking water, and a conventional plasmid gene expression system. In an allograft study, bFGF and VEGF overexpression synergistically increased tumor growth and angiogenesis in the murine HCC cells. This synergistic effect also was found in established tumors. VEGF messenger RNA (mRNA) expression in the tumor was increased 3.1-fold by bFGF-overexpression, and the bFGF-induced tumor development was significantly attenuated by treatment with KDR/Flk-1 neutralizing monoclonal antibody. In conclusion, these results suggest that bFGF synergistically augments VEGF-mediated HCC development and angiogenesis at least partly by induction of VEGF through KDR/Flk-1. (HEPATOLOGY 2002;35:834-842.)

In solid tumors, neovascularization is a prerequisite for growth and metastasis. Without angiogenesis, tumors cease to grow beyond a few cubic mm in size and remain in a state of tumor dormancy.[1-5] To date, many angiogenic factors that contribute to tumor development have been identified. It has been shown that the tumor produces several types of angiogenic factors and the interaction of these plays an important role in tumor angiogenesis. Among these, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are the most potent and best-characterized factors.[6-8] bFGF is the prototype member of 13 structurally related, heparin-binding growth factors, and is known as a mitogen for several types of cells, including vascular endothelial cells (EC) and fibroblasts.[9-11] Using a transgenic mouse model, it has been reported that angiogenic switch in bovine papilloma virus-induced fibrosarcoma correlates with the export of bFGF from tumor cells.[12] VEGF, originally identified as a vascular permeability factor, is a specific mitogen for vascular EC *in vitro* and is secreted abundantly in several human surgical specimens.[6-8] Its expression in several tumors has been correlated with high vascularity, distant metastasis, and a poorer prognosis than VEGF-negative tumors.[6-8,13-17] The effects of VEGF are mediated mainly through 2 distinct receptors, namely flt-1 and KDR/Flk-1.[6-8] It has been suggested that flt-1 and KDR/Flk-1 serve different roles in angiogenesis and signal transduction pathways and that KDR/Flk-1 is a major regulator of angiogenesis both *in vitro* and *in vivo*.[18-20]

Abbreviations: bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; EC, endothelial cell; KDR/Flk-1, kinase insert domain-containing receptor/fetal liver kinase; HCC, hepatocellular carcinoma; cDNA, complementary DNA; BNL-HCC, BNL 1.7R1 HCC; tet, tetracycline; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(-4,5-diethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction.

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Several *in vitro* studies have shown that VEGF and bFGF possessed a synergistic effect in the induction of angiogenesis, and bFGF increased VEGF and KDR/Flk-1 expression in the EC and several other types of cells.[21-27] It has been revealed that the angiogenesis enhanced by bFGF was significantly inhibited by VEGF suppression, such as VEGF-neutralizing antibody and antisense oligonucleotide.[22,27] Combined *in vivo* administration of VEGF and bFGF showed a synergistic effect in an animal model of hind limb ischemia and in mouse cornea angiogenesis.[22,28] In animal experiments, single-gene overexpression of bFGF and VEGF significantly augmented tumor growth and angiogenesis, whereas suppression of each factor inhibited tumor growth in several types of tumors.[9,10,29-33] However, the combined effect and the possible interaction of VEGF and bFGF in tumor development and angiogenesis, including hepatocellular carcinoma (HCC), have not yet been elucidated.

HCC is one of the most common malignancies in the world. An estimated 350,000 new cases per year are found worldwide. The prognosis of HCC is still poor because it is found in conjunction with cirrhosis in the majority of cases and develops multicentrically.[34,35] One of the characteristic features of HCC in clinical practice is hypervascularity; therefore, HCC is likely to produce angiogenic factors. As expected, it has been reported that human HCC surgical specimens expressed several angiogenic factors. Abundant expression of VEGF and bFGF has been shown in human HCC samples, and the serum levels of these factors were significantly increased in patients with HCC.[13,15,17,36-38] We previously reported that VEGF tightly regulated experimental murine HCC development, and this effect was mediated mainly through the KDR/Flk-1 receptor.[39,40] However, the role of bFGF in HCC development and the interaction with VEGF have not been clarified yet.

The Tet system is a novel tetracycline (tet)-regulated gene expression system.[41] This system allows manipulation of the gene of interest in an on-and-off manner *in vivo*, which the conventional gene expression system cannot achieve either overexpression or suppression. In this study, we used a retrovirus-mediated modified vector in which 2 components of the Tet systems have been organized within the same vector in the opposite direction, resulting in a decrease in the basal gene expression level.[39]

In this study, we elucidated the combined biologic effect of bFGF and VEGF on HCC development and angiogenesis at different stages by means of combination of Retro-Tet and conventional plasmid gene expression in the murine HCC model. We also examined the interaction of bFGF and VEGF and the role of KDR/Flk-1 in bFGF-induced HCC development.

Materials and Methods

Construction of the Retroviral and Plasmid Vectors. The tet aspect of the retroviral vector is conferred by the response and regulator units of the previously described Tet-inducible promoter system. A complete description of the construction of this Retro-Tet system vector has been reported previously.[39] It has been shown that transfection of the wild bFGF gene without a signal peptide sequence revealed remarkably less biologic activity than the modified bFGF gene with a signal sequence.[42] It has been shown also that the secreted protein was more relevant to the rate of tumor neovascularization than the intracellular concentration of protein.[43] We conducted a preliminary study to examine the effect of bFGF overexpression with or without a peptide sequence in BNL-HCC cells. In agreement with the previous reports, we found that the bFGF overexpression without a signal sequence showed much less *in vitro* invasion activity of BNL-HCC cells (data not shown). Thus, we fused the mouse immunoglobulin κ-chain signal peptide with the human bFGF complementary DNA (cDNA) in this study. This modified bFGF cDNA was cloned into the multicloning site of the parent PBSTR-1 vector at a *Bam* H1 site, forming the Tet-bFGF vector (Fig. 1A). For VEGF gene expression, we used a Pcl-neo plasmid vector (Promega, Forward, WI). Human VEGF cDNA was cloned into the *Eco* R1, Not 1 site of the Pcl-neo expression vector, forming Pcl-neo-VEGF. Because the Tet-bFGF vector and Pcl-neo expression vector were selected by different drugs (puromycin and geneticin, respectively), both expression systems can be introduced into the same cells.

Cell Culture and Stable Clone Production. BALB/c mice–derived BNL.1.7R.1 HCC (BNL-HCC) and BOSC 23 retrovirus packaging cells were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and American Type Culture Collection (Rockville, MD), respectively. Because BNL-HCC cells showed very low levels of endogenous bFGF and VEGF, we used this cell line for the current study.[39] The BNL-HCC cells were grown in a supplier-recommended medium as described previously.[39] First, we introduced the Tet-bFGF vector into BNL-HCC cells. A stable Tet-bFGF expressing clone was obtained by the same method as described previously.[39] The bFGF expression level in condition media were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). For further analysis, we used the clones, which showed highest and lowest bFGF in the absence or presence of tet (tet: 1 μg/mL: Sigma, St. Louis, MO), respectively (bFGF-HCC). We next introduced Pcl-neo-VEGF into the

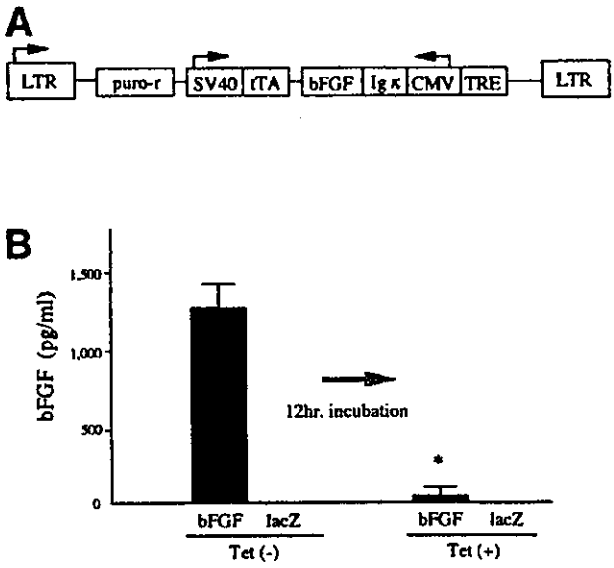

Fig. 1. (A) Construction of the tet-regulated retrovirus-mediated bFGF expression vector. The vector contains a tet-controlled transcriptional activator and tet-responsive element in the opposite direction. Human bFGF cDNA was fused with mouse immunoglobulin κ-chain signal peptide sequence. This modified bFGF was cloned into the multicloning site of the response unit. The transcriptional direction is indicated by an **arrow**. (B) Regulation of bFGF production by tet *in vitro*. (**Left**) Human bFGF levels in 24-hour culture supernatant of BNL-HCC cells. *LacZ*-HCC cells did not produce any human bFGF, whereas bFGF-HCC cells secreted a high level of bFGF. (**Right**) The tet (1 μg/mL)-mediated effect on bFGF production by the same cells reversed 12-hour incubation with tet in the culture medium. Each **bar** represents the mean ± SD of 3 separate experiments (n = 6 each). *Statistically significant difference compared with tet (−) ($P < .01$).

bFGF-HCC cells with lipofectamin (GIBCO, Rockville, MD), selected with geneticin (1 mg/mL) as described previously.[44] The VEGF expression level also was measured by ELISA (R&D Systems), and high-VEGF–expressing clones (1,160 ± 167.4 pg/mL, n = 6) were used for further analysis (bFGF-VEGF-HCC). Pcl-neo vector also was transfected into the bFGF-HCC cells to avoid the nonspecific effect of vector introduction. For the control group, the Tet-*lacZ* and Pcl-neo vectors were introduced (*lacZ*-HCC).

In Vitro *Proliferation Assay.* The *in vitro* proliferation was determined by 3-(-4,5-diethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.[39] Briefly, cell proliferation was quantified by the conversion of tetrazolium, MTT by cells cultured on 96-well plates. The absorbance with a 540-nm filter represents conversion to formazan, which is directly proportional to the number of living cells. The absorbance was read with an ELISA plate recorder (n = 6 per group). We performed the *in vitro* proliferation assay in the presence or absence of tet.

***Growth of bFGF- and VEGF-Transduced HCC Cells* In Vivo.** In an allograft model, 1×10^6 of bFGF, bFGF-VEGF, and *lacZ*-HCC cells were syngenetically transplanted into the flank of BALB/c mice (Japan SLC, Hamamatsu, Japan). Group 1 (G1) was a *lacZ*-HCC cell group, which served as the control. The animals in group 2 (G2) were bFGF-HCC transplanted mice. This group drank tet-free, normal drinking water to maintain bFGF overexpression. Groups 3 (G3) and 4 (G4) were bFGF-VEGF-HCC transplanted mice. The G3 and G4 groups first drank tet-containing drinking water (1 mg/mL) to suppress bFGF expression under constitutive VEGF overexpression, then G4 was changed to tet-free, normal water from day 28 (mean tumor volume was 1,487 mm^3) for the rest of the experiment to switch on bFGF expression. G3 received tet-containing drinking water throughout the experiment without bFGF overexpression. The mice in group 5 (G5) also received transplanted bFGF-VEGF-HCC cells, and drank tet-free, normal water throughout the experiment to maintain both bFGF and VEGF overexpression. The tumor was measured twice per week with calipers, and the tumor volume was calculated as described previously.[39] To confirm that the effect of these angiogenic factors on the tumor growth was consistent, we performed independently the same animal experiments 3 times (n = 16, the total in each experimental group). To examine whether bFGF protein expression also was regulated tightly by tet *in vivo*, 5 mice from G4 were killed 3 days after changing the drinking water. The level of bFGF protein expression of their tumor lysate was examined by ELISA. After the animals were killed, half of each tumor was snap-frozen and the reminder was used for histologic analysis. Another experiment was performed to examine the effect of KDR/Flk-1 on bFGF-induced tumor development. The mice in group 6 (G6) received transplanted bFGF-HCC cells, and drank tet-free water to bFGF overexpression. Group 7 (G7) also received transplanted bFGF-HCC cells, and the anti-KDR/Flk-1–specific neutralizing monoclonal antibody (KDR/Flk-1 mAb; 400 μg/mouse) was administered intraperitoneally twice per week as described previously.[40] The control immunoglobulin G was injected bFGF-overexpressed and *lacZ* control groups.

Immunohistochemistry. For the determination of *in vivo* angiogenesis, we employed immunohistochemical detection of platelet/EC adhesion molecule, which is widely used as a marker of neovascularization in frozen sections of tumors as described previously.[45,46] We used tumor with the same size to avoid the necrotic effect of hypoxia as described previously.[44] The immunostained microvessel length was assessed under 200× magnification. In each tumor tissue, the 5 areas showing the highest

density of staining were selected for counting. In counting, the large vessels with a thick muscular wall or with a lumen greater than 50 μm in diameter were excluded. These immunopositive vessels were evaluated with Adobe Photoshop (Adobe System Inc., Tokyo, Japan) and National Institutes of Health image software as described previously.[45]

RNA Expression of VEGF. For the evaluation of VEGF RNA alteration in the tumor by bFGF overexpression, we employed the real-time PCR analysis as described previously,[47,48] with specific primer sequence of mouse VEGF (CGCATCAGGGGCACACAGGAT, complementary to nt 290-310; and GTCAACGGTGACGATGATGG, complementary to nt 765-784). The tumor samples were prepared from 5 bFGF-HCC mice with or without tet (bFGF expression was in the off and on states, respectively) and snap-frozen into the liquid nitrogen immediately, then the mRNA was extracted from the tumor pools. For cDNA synthesis, Taqman reverse transcription reagents were used as described in the manufacturer's manual of the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA), which was used for real-time polymerase chain reaction (PCR) amplification after the Taqman Universal PCR Master Mix Protocol (PE Applied Biosystems). Relative quantification of gene expression was performed as described in the manual with glyceraldehyde-3-phosphate dehydrogenase as an internal control. The threshold cycle and the standard curve method were used for calculating the relative amount of the target RNA as described for PE. To prevent the genomic DNA contamination, all RNA samples were subjected to deoxyribonuclease (DNase) I digestion and checked by 40 cycles of PCR to confirm the absence of amplified DNA.

Statistical Analysis. To assess the statistical significance of intergroup differences in the quantitative data, Bonferroni's multiple comparison test was performed after 1-way ANOVA. This was followed by Barlett's test to determine the homology of variance.

Results

***Regulation of bFGF by Tet-Mediated Retroviral Vector* In Vitro.** First, we examined *in vitro* bFGF regulation in the bFGF-HCC cells. The *lacZ*-HCC control cells did not show evidence of human bFGF production. In the absence of tet, marked secretion of human bFGF was seen in the supernatant (1,229.4 ± 194 pg/mL, n = 6). When tet (1 μg/mL) was added to the medium and incubated for an additional 12-hour period, bFGF was drastically reduced (16.6 ± 4.6 pg/mL) ($P < .01$) (Fig. 1B). In the bFGF-VEGF-HCC cells, bFGF regulation appeared to be the same as in the bFGF-HCC cells, and in the presence or absence of tet, VEGF expression was not altered. Western blotting revealed that the molecular weight of the main product was about 18 kd, which corresponded to the secreted short form of bFGF (data not shown). We termed this tet-treated bFGF-VEGF-HCC cells as VEGF-HCC. Next, the *in vitro* proliferation rates of HCC cells from day 1 to day 9 after harvest were examined in the presence or absence of tet. In keeping with a previous report,[39] there were no differences between *lacZ*-HCC, VEGF-HCC, and bFGF-HCC cells, indicating that VEGF and bFGF overexpression did not affect the *in vitro* proliferation rates (Fig. 2). We also performed the *in vitro* proliferation assay by 3-(4,5-dimethylthizol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) method, and we obtained similar results (data not shown).

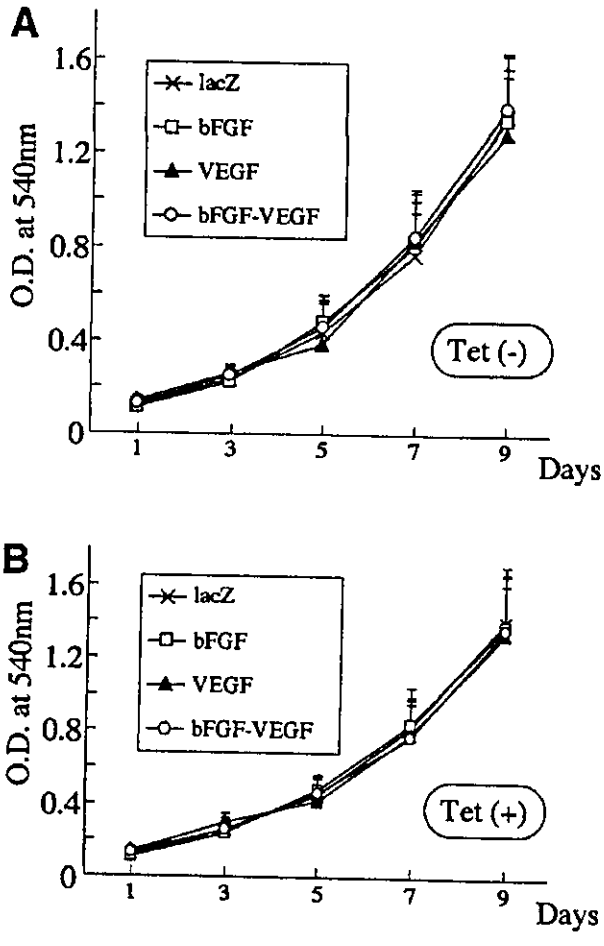

Fig. 2. *In vitro* proliferation of *lacZ*-HCC, bFGF-HCC, VEGF-HCC, and bFGF-VEGF-HCC cells in culture in the absence (A) or presence (B) of tet. The cell proliferation was measured by the MTT assay after harvest from day 1 to day 9 as described in Materials and Methods. Each **bar** represents the mean ± SD of 3 separate experiments (n = 6 each).