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.

References

[1] Friedman SL, Maher JJ, Bissell DM. Mechanisms and therapy of hepatic fibrosis: report of the AASLD Single Topic Basic Research Conference. Hepatology 2000;32:1403–8.

[2] Bissell DM, Roulot D, George J. Transforming growth factor beta and the liver. Hepatology 2001;34:859–67.

[3] Chiaramonte MG, Donaldson DD, Cheever AW, Wynn T A. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. J Clin Invest 1999;104:777–85.

[4] Powell EE, Edwards-Smith CJ, Hay JL, et al. Host genetic factors influence disease progression in chronic hepatitis C. Hepatology 2000;31:828–33.

[5] Yoshiji H, Kuriyama S, Yoshii J, et al. Angiotensin-II type 1 receptor interaction is a major regulator for liver fibrosis development in rats. Hepatology 2001;34:745–50.

[6] Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. J Biol Chem 2000;275:2247–50.

[7] Oka H, Kurioka N, Kim K, et al. Prospective study of early detection of hepatocellular carcinoma in patients with cirrhosis. Hepatology 1990;12:680–7.

[8] Seeff LB, Buskell-Bales Z, Wright EC, et al. Long-term mortality after transfusion-associated non-A, non-B hepatitis. The National Heart, Lung, and Blood Institute Study Group. N Engl J Med 1992;327:1906–11.

[9] Ikeda K, Saitoh S, Suzuki Y, et al. Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. J Hepatol 1998;28:930–8.

[10] Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group. N Engl J Med 1999;340:1228–33.

[11] Yoshida H, Shiratori Y, Moriyama M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. Ann Intern Med 1999;131:174–81.

[12] Poynard T, McHutchison J, Davis GL, et al. Impact of interferon alfa-2b and ribavirin on progression of liver fibrosis in patients with chronic hepatitis C. Hepatology 2000;32:1131–7.

[13] Shiratori Y, Imazeki F, Moriyama M, et al. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. Ann Intern Med 2000;132:517–24.

[14] Holund B, Poulsen H, Schlichting P. Reproducibility of liver biopsy diagnosis in relation to the size of the specimen. Scand J Gastroenterol 1980;15:329–35.

[15] Abdi W, Millan JC, Mezey E. Sampling variability on percutaneous liver biopsy. Arch Intern Med 1979;139:667–9.

[16] Bravo AA, Sheth SG, Chopra S. Liver biopsy. N Engl J Med 2001;344:495–500.

[17] Piccinino F, Sagnelli E, Pasquale G, Giusti G. Complications following percutaneous liver biopsy. A multicentre retrospective study on 68,276 biopsies. J Hepatol 1986;2:165–73.

[18] Garcia G, Keeffe EB. Liver biopsy in chronic hepatitis C: routine or selective. Am J Gastroenterol 2001;96:3053–5.

[19] Sandrin L, Fourquet B, Hasquenoph JM, et al. Transient elastography: a new non-invasive method for assessment of hepatic fibrosis. Ultrasound Med Biol 2003;29:1719–27.

[20] Sandrin L, Tanter M, Gennisson JL, Catheline S, Fink MA. Shear elasticity for soft tissue 1-D transient elastography. IEEE Trans Ultrason Ferroelect Freq Contr 2002;49:436–46.

[21] Catheline S, Thomas J-L, Wu F, Fink MA. Diffraction field of a low frequency vibrator in soft tissues using transient elastography. IEEE Trans Ultrason Ferroelect Freq Contr 1999;46:1013–9.

[22] Ichida F, Tsuji T, Omata M, et al. New Inuyama classification new criteria for histological assessment of chronic hepatitis. Int Hepatol Commun 1996;6:112–9.

[23] Grant A, Neuberger J. Guidelines on the use of liver biopsy in clinical practice. British Society of Gastroenterology. Gut 1999;45(Suppl 4):IV1–IV11.

[24] Skelly MM, James PD, Ryder SD. Findings on liver biopsy to investigate abnormal liver function tests in the absence of diagnostic serology. J Hepatol 2001;35:195–9.

[25] Andriulli A, Festa V, Leandro G, Rizzetto M. Usefulness of a liver biopsy in the evaluation of patients with elevated ALT values and serological markers of hepatitis viral infection: an AIGO study. Dig Dis Sci 2001;46:1409–15.

[26] Saadeh S, Cammell G, Carey WD, Younossi Z, Barnes D, Easley K. The role of liver biopsy in chronic hepatitis C. Hepatology 2001;33:196–200.
[27] Fontana RJ, Lok AS. Noninvasive monitoring of patients with chronic hepatitis C. Hepatology 2002;36:S57–64.
[28] Dienstag JL. The role of liver biopsy in chronic hepatitis C. Hepatology 2002;36:S152–60.
[29] Poynard T, Ratziu V, Benmanov Y, DiMartino V, Bedossa P, Opolon P. Fibrosis in patients with chronic hepatitis C: detection and significance. Semin Liver Dis 2000;20:47–55.
[30] Bonacini M, Hadi G, Govindarajan S, Lindsay KL. Utility of a discriminant score for diagnosing advanced fibrosis or cirrhosis in patients with chronic hepatitis C virus infection. Am J Gastroenterol 1997;92:1302–4.
[31] Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. Lancet 2001;357:1069–75.
[32] Iredale JP, Benyon RC, Pickering J, et al. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. J Clin Invest 1998;102:538–49.
[33] Friedman SL, Arthur MJ. Activation of cultured rat hepatic lipocytes by Kupffer cell conditioned medium. Direct enhancement of matrix synthesis and stimulation of cell proliferation via induction of platelet-derived growth factor receptors. J Clin Invest 1989;84: 1780–5.
[34] Jarnagin WR, Rockey DC, Koteliansky VE, Wang SS, Bissell DM. Expression of variant fibronectins in wound healing: cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis. J Cell Biol 1994;127:2037–48.
[35] Louis H, Le Moine A, Quertinmont E, et al. Repeated concanavalin A challenge in mice induces an interleukin 10-producing phenotype and liver fibrosis. Hepatology 2000;31:381–90.
[36] Ma X, Zhao J, Lieber CS. Polyenylphosphatidylcholine attenuates non-alcoholic hepatic fibrosis and accelerates its regression. J Hepatol 1996;24:604–13.
[37] Aleynik SI, Leo MA, Ma X, Aleynik MK, Lieber CS. Polyenylphosphatidylcholine prevents carbon tetrachloride-induced lipid peroxidation while it attenuates liver fibrosis. J Hepatol 1997;27:554–61.
[38] Lieber CS, DeCarli LM, Mak KM, Kim CI, Leo MA. Attenuation of alcohol-induced hepatic fibrosis by polyunsaturated lecithin. Hepatology 1990;12:1390–8.
[39] Nelson DR, Lauwers GY, Lau JY, Davis GL. Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: a pilot trial of interferon nonresponders. Gastroenterology 2000;118:655–60.
[40] Qi Z, Atsuchi N, Ooshima A, Takeshita A, Ueno H. Blockade of type beta transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat. Proc Natl Acad Sci USA 1999;96:2345–9.
[41] George J, Roulot D, Koteliansky VE, Bissell DM. In vivo inhibition of rat stellate cell activation by soluble transforming growth factor beta type II receptor: a potential new therapy for hepatic fibrosis. Proc Natl Acad Sci USA 1999;96:12719–24.
[42] Genereau T, Bellin MF, Wechsler B, et al. Demonstration of efficacy of combining corticosteroids and colchicine in two patients with idiopathic sclerosing mesenteritis. Dig Dis Sci 1996;41:684–8.
[43] Paolini O, Hebuterne X, Flory P, Charles F, Rampal P. Treatment of pyoderma gangrenosum with colchicine. Lancet 1995;345:1057–8.
[44] Lin DY, Sheen IS, Chu CM, Liaw YF. A prospective randomized trial of colchicine in prevention of liver cirrhosis in chronic hepatitis B patients aliment. Pharmacol Ther 1996;10:961–6.
[45] Kershenobich D, Vargas F, Garcia-Tsao G, Perez Tamayo R, Gent M, Rojkind M. Colchicine in the treatment of cirrhosis of the liver. N Engl J Med 1988;318:1709–13.
[46] Ohishi T, Saito H, Tsusaka K, et al. Anti-fibrogenic effect of an angiotensin converting enzyme inhibitor on chronic carbon tetrachloride-induced hepatic fibrosis in rats. Hepatol Res 2001;21:147–58.
[47] Lai CL, Chien RN, Leung NW, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998;339:61–8.
[48] Dufour JF, DeLellis R, Kaplan MM. Reversibility of hepatic fibrosis in autoimmune hepatitis. Ann Intern Med 1997;127:981–5.
[49] Hammel P, Couvelard A, O'Toole D, et al. Regression of liver fibrosis after biliary drainage in patients with chronic pancreatitis and stenosis of the common bile duct. N Engl J Med 2001;344:418–23.
[50] Diehl AM, Goodman Z, Ishak KG. Alcohol like liver disease in nonalcoholics. A clinical and histologic comparison with alcohol-induced liver injury. Gastroenterology 1988;95:1056–62.
[51] Knodell RG, Ishak KG, Black WC, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 1981;1:431–5.
[52] Scheuer PJ. Classification of chronic viral hepatitis: a need for reassessment. J Hepatol 1991;13:372–4.
[53] Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology 1996;24:289–93.
[54] Batts KP, Ludwig J. Chronic hepatitis. An update on terminology and reporting. Am J Surg Pathol 1995;19:1409–17.
[55] Ishak K, Baptista A, Bianchi L, et al. Histological grading and staging of chronic hepatitis. J Hepatol 1995;22:696–9.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Hepatology Research 30 (2004) 193–198
Hepatology Research
http://www.elsevier.com/locate/hepres

Review

Recent understanding of immunological aspects in alcoholic hepatitis

Hidetsugu Saito*, Hiromasa Ishii

Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Received 9 July 2004; accepted 24 August 2004
Available online 6 November 2004

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

Contents

* Corresponding author. Tel.: +81 3 3353 1211.
E-mail address: hsaito@sc.itc.keio.ac.jp (H. Saito).

1386-6346/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.hepres.2004.08.016

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 $p47^{Phos}$ knockout (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.

References

[1] Anderson S, Nevins CL, Green LK, El-Zimaity H, Anand BS. Assessment of liver histology in chronic alcoholics with and without hepatitis C virus infection. Dig Dis Sci 2001;46:1393–8.
[2] Anand BS, Velez M. Influence of chronic alcohol abuse on hepatitis C virus replication. Dig Dis 2000;18:168–71.
[3] Ishii H. Common pathogenic mechanisms in ASH and NASH. Hepatol Res 2004;28:18–20.
[4] Lieber CS. CYP2E1: from ASH to NASH. Hepatol Res 2004;28:1–11.
[5] Lieber CS, DeCarli L, Rubin E. Sequential production of fatty liver, hepatitis, and cirrhosis in sub-human primates fed ethanol with adequate diets. Proc Natl Acad Sci USA 1975;72:437–41.
[6] Lieber CS, DeCarli LM. Liquid diet technique of ethanol administration: 1989 update. Alcohol Alcohol 1989;24:197–211.
[7] Tsukamoto H, French SW, Benson N, et al. Severe and progressive steatosis and focal necrosis in rat liver induced by continuous

intragastric infusion of ethanol and low fat diet. Hepatology 1985;5:224–32.
[8] Cook RT. Alcohol abuse, alcoholism, and damage to the immune system: a review. Alcohol Clin Exp Res 1998;22:1927–42.
[9] Song K, Coleman RA, Alber C, et al. TH1 cytokine response of CD57+ T-cell subsets in healthy controls and patients with alcoholic liver disease. Alcohol 2001;24:155–67.
[10] Kanagasundaram N, Kakumu S, Chen T, Leevy CM. Alcoholic hyalin antigen (AHAg) and antibody (AHAb) in alcoholic hepatitis. Gastroenterology 1977;73:1368–73.
[11] Kehl A, Schober A, Junge U, Winckler K. Solid-phase radioimmunoassay for detection of alcoholic hyalin antigen (AHAg) and antibody (anti-AH). Clin Exp Immunol 1981;43:215–21.
[12] Mutchnick MG, Keren DF. In vitro synthesis of antibody to specific bacterial lipopolysaccharide by peripheral blood mononuclear cells from patients with alcoholic cirrhosis. Immunology 1981;43:177–82.
[13] Anthony RS, Farquharson M, MacSween RN. Liver membrane antibodies in alcoholic liver disease. II. Antibodies to ethanol-altered hepatocytes. J Clin Pathol 1983;36:1302–8.
[14] Neuberger J, Crossley IR, Saunders JB, et al. Antibodies to alcohol altered liver cell determinants in patients with alcoholic liver disease. Gut 1984;25:300–4.
[15] Barry RE, Williams AJ, McGivan JD. The detection of acetaldehyde/liver plasma membrane protein adduct formed in vivo by alcohol feeding. Liver 1987;7:364–8.
[16] Niemela O, Klajner F, Orrego H, Vidins E, Blendis L, Israel Y. Antibodies against acetaldehyde-modified protein epitopes in human alcoholics. Hepatology 1987;7:1210–4.
[17] Yokoyama H, Nagata S, Moriya S, et al. Hepatic fibrosis produced in guinea pigs by chronic ethanol administration and immunization with acetaldehyde adducts. Hepatology 1995;21:1438–42.
[18] Kakumu S, Leevy CM. Lymphocyte cytotoxicity in alcoholic hepatitis. Gastroenterology 1977;72:594–7.
[19] Cook RT, Garvey MJ, Booth BM, Gocken JA, Stewart B, Noel M. Activated CD-8 cells and HLA DR expression in alcoholics without overt liver disease. J Clin Immunol 1991;11:246–53.
[20] Burra P, Hubscher SG, Shaw J, Elias E, Adams DH. Is the intercellular adhesion molecule-1/leukocyte function associated antigen 1 pathway of leukocyte adhesion involved in the tissue damage of alcoholic hepatitis? Gut 1992;33:268–71.
[21] Sakai Y, Izumi N, Marumo F, Sato C. Quantitative immunohistochemical analysis of lymphocyte subsets in alcoholic liver disease. J Gastroenterol Hepatol 1993;8:39–43.
[22] Snyder N, Bessoff J, Dwyer JM, Conn HO. Depressed delayed cutaneous hypersensitivity in alcoholic hepatitis. Am J Dig Dis 1978;23:353–8.
[23] Mutchnick MG, Cohen IA, Elta GH. Persistent immune deficiency in patients with alcoholic hepatitis. Am J Gastroenterol 1990;85:428–34.
[24] MacGregor RR. In vivo neutrophil delivery in men with alcoholic cirrhosis is normal despite depressed in vitro chemotaxis. Alcohol Clin Exp Res 1990;14:195–9.
[25] Swerdlow MA, Chowdhury LN, Horn T. Patterns of IgA deposition in liver tissues in alcoholic liver disease. Am J Clin Pathol 1982;77:259–66.
[26] Nouri-Aria KT, Alexander GJ, Portmann BC, Hegarty JE, Eddleston AL, Williams R. T and B cell function in alcoholic liver disease. J Hepatol 1986;2:195–207.
[27] Brown WR, Kloppel TM. The liver and IgA: immunological, cell biological and clinical implications. Hepatology 1989;9:763–84.
[28] Rajkovic IA, Williams R. Abnormalities of neutrophil phagocytosis, intracellular killing and metabolic activity in alcoholic cirrhosis and hepatitis. Hepatology 1986;6:252–62.
[29] Yin M, Wheeler MD, Kono H, et al. Essential role of tumor necrosis factor alpha in alcohol-induced liver injury in mice. Gastroenterology 1999;117:942–52.
[30] Hanck C, Glatzel M, Singer MV, Rossol S. Gene expression of TNF-receptors in peripheral blood mononuclear cells of patients with alcoholic cirrhosis. J Hepatol 2000;32:51–7.
[31] Hill DB, Barve S, Joshi-Barve S, McClain C. Increased monocyte nuclear factor-kappaB activation and tumor necrosis factor production in alcoholic hepatitis. J Lab Clin Med 2000;135:387–95.
[32] Su GL, Rahemtulla A, Thomas P, Klein RD, Wang SC, Nanji AA. CD14 and lipopolysaccharide binding protein expression in a rat model of alcoholic liver disease. Am J Pathol 1998;152:841–9.
[33] Yin M, Bradford BU, Wheeler MD, et al. Reduced early alcohol-induced liver injury in CD14-deficient mice. J Immunol 2001;166:4737–42.
[34] Uesugi T, Froh M, Arteel GE, Bradford BU, Thurman RG. Toll-like receptor 4 is involved in the mechanism of early alcohol-induced liver injury in mice. Hepatology 2001;34:101–8.
[35] Nanji AA, Jokelainen K, Rahemtulla A, et al. Activation of nuclear factor kappa B and cytokine imbalance in experimental alcoholic liver disease in the rat. Hepatology 1999;30:934–43.
[36] Kono H, Rusyn I, Yin M, et al. NADPH oxidase-derived free radicals are key oxidants in alcohol-induced liver disease. J Clin Invest 2000;106:867–72.
[37] Nanji AA, Miao L, Thomas P, et al. Enhanced cyclooxygenase-2 gene expression in alcoholic liver disease in the rat. Gastroenterology 1997;112:943–51.
[38] Dinchuk JE, Car BD, Focht RJ, et al. Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. Nature 1995;378:406–9.
[39] McClain CJ, Barve S, Deaciuc I, Kugelmas M, Hill D. Cytokines in alcoholic liver disease. Semin Liver Dis 1999;19:205–19.
[40] Afford SC, Fisher NC, Neil DA, et al. Distinct patterns of chemokine expression are associated with leukocyte recruitment in alcoholic hepatitis and alcoholic cirrhosis. J Pathol 1998;186:82–9.
[41] Bautista AP. Impact of alcohol on the ability of Kupffer cells to produce chemokines and its role in alcoholic liver disease. J Gastroenterol Hepatol 2000;15:349–56.
[42] Fisher NC, Neil DA, Williams A, Adams DH. Serum concentrations and peripheral secretion of the beta chemokines monocyte chemoattractant protein 1 and macrophage inflammatory protein 1alpha in alcoholic liver disease. Gut 1999;45:416–20.
[43] Maltby J, Wright S, Bird G, Sheron N. Chemokine levels in human liver homogenates: associations between GRO alpha and histopathological evidence of alcoholic hepatitis. Hepatology 1996;24:1156–60.
[44] Hirano F, Komura K, Fukawa E, Makino I. Tumor necrosis factor alpha (TNF-alpha)-induced RANTES chemokine expression via activation of NF-kappaB and p38 MAP kinase: roles of TNF-alpha in alcoholic liver diseases. J Hepatol 2003;38:483–9.
[45] Lakshminarayanan V, Drab-Weiss EA, Roebuck KA. H_2O_2 and tumor necrosis factor-alpha induce differential binding of the redox-responsive transcription factors AP-1 and NF-kappaB to the interleukin-8 promoter in endothelial and epithelial cells. J Biol Chem 1998;273:32670–8.
[46] Mosher B, Dean R, Harkema J, Remick D, Palma J, Crockett E. Inhibition of Kupffer cells reduced CXC chemokine production and liver injury. J Surg Res 2001;99:201–10.
[47] Saito H, Fukumura D, Kurose I, et al. Visualization of oxidative processes at the cellular level during neutrophil-mediated cytotoxicity against a human hepatoma cell line, HCC-M. Int J Cancer 1992;51:124–9.
[48] Sarphie TG, D'Souza NB, Spitzer JJ, Deaciuc IV. Chronic alcohol feeding in liquid diet or in drinking water has similar effects on electron microscopic appearance of the hepatic sinusoid in the rat. Alcohol Clin Exp Res 1996;20:973–9.
[49] Sarphie TG, D'Souza NB, Deaciuc IV. Kupffer cell inactivation prevents lipopolysaccharide-induced structural changes in the rat liver sinusoid: an electron-microscopic study. Hepatology 1996;23:788–96.

[50] Lawson JA, Fisher MA, Simmons CA, Farhood A, Jaeschke H. Parenchymal cell apoptosis as a signal for sinusoidal sequestration and transendothelial migration of neutrophils in murine models of endotoxin and Fas-antibody-induced liver injury. Hepatology 1998;28:761–7.
[51] Jaeschke H, Fisher MA, Lawson JA, Simmons CA, Farhood A, Jones DA. Activation of caspase 3 (CPP32)-like proteases is essential for TNF-alpha-induced hepatic parenchymal cell apoptosis and neutrophil-mediated necrosis in a murine endotoxin shock model. J Immunol 1998;160:3480–6.
[52] Lawson JA, Burns AR, Farhood A, et al. Pathophysiologic importance of E- and L-selectin for neutrophil-induced liver injury during endotoxemia in mice. Hepatology 2000;32:990–8.
[53] Taieb J, Mathurin P, Elbim C, et al. Blood neutrophil functions and cytokine release in severe alcoholic hepatitis: effect of corticosteroids. J Hepatol 2000;32:579–86.
[54] Ziol M, Tepper M, Lohez M, et al. Clinical and biological relevance of hepatocyte apoptosis in alcoholic hepatitis. J Hepatol 2001;34:254–60.
[55] Bhagwandeen BS, Apte M, Manwarring L, Dickeson J. Endotoxin induced hepatic necrosis in rats on an alcohol diet. J Pathol 1987;152:47–53.
[56] Pennington HL, Wilce PA, Worrall S. Chemokine and cell adhesion molecule mRNA expression and neutrophil infiltration in lipopolysaccharide-induced hepatitis in ethanol-fed rats. Alcohol Clin Exp Res 1998;22:1713–8.
[57] Yamada S, Iida T, Tabata T, et al. Alcoholic fatty liver differentially induces a neutrophil-chemokine and hepatic necrosis after ischemia-reperfusion in rat. Hepatology 2000;32:278–88.
[58] Salmi M, Jalkanen S. Human vascular adhesion protein 1 (VAP-1) is a unique sialoglycoprotein that mediates carbohydrate-dependent binding of lymphocytes to endothelial cells. J Exp Med 1996;183:569–79.
[59] Narumi S, Yoneyama H, Inadera H, et al. TNF-alpha is a potent inducer for IFN-inducible protein-10 in hepatocytes and unaffected by GM-CSF in vivo, in contrast to IL-1beta and IFN-gamma. Cytokine 2000;12:1007–16.
[60] Tamaru M, Nishioji K, Kobayashi Y, et al. Liver-infiltrating T lymphocytes are attracted selectively by IFN-inducible protein-10. Cytokine 2000;12:299–308.
[61] Berman ME, Xie Y, Muller WA. Roles of platelet/endothelial cell adhesion molecule-1 (PECAM-1. CD31) in natural killer cell transendothelial migration and beta 2 integrin activation. J Immunol 1996;156:1515–24.
[62] Chedid A, Mendenhall CL, Moritz TE, et al., Veterans Affairs Cooperative Study Group 275. Cell-mediated hepatic injury in alcoholic liver disease. Gastroenterology 1993;105:254–66.
[63] Meadows GGBS, Duncan DA. Modulation of natural killer immunomodulation and AIDS. In: Seminara DWR, Pawlowski A, editors. Alcohol, Immunology, and AIDS, Proceedings of the Alcohol-Immunology AIDS Conference. New York Alan Liss: 1989; p. 181.
[64] Grove J, Daly AK, Bassendine MF, Day CP. Association of a tumor necrosis factor promoter polymorphism with susceptibility to alcoholic steatohepatitis. Hepatology 1997;26:143–6.
[65] Grove J, Daly AK, Bassendine MF, Gilvarry E, Day CP. Interleukin 10 promoter region polymorphisms and susceptibility to advanced alcoholic liver disease. Gut 2000;46:540–5.
[66] French SW. Mechanisms of alcoholic liver injury. Can J Gastroenterol 2000;14:327–32.
[67] Chisari FV. Cytotoxic T cells and viral hepatitis. J Clin Invest 1997;99:1472–7.
[68] Pianko S, Patella S, Ostapowicz G, Desmond P, Sievert W. Fas-mediated hepatocyte apoptosis is increased by hepatitis C virus infection and alcohol consumption, and may be associated with hepatic fibrosis: mechanisms of liver cell injury in chronic hepatitis C virus infection. J Viral Hepat 2001;8:406–13.
[69] Deaciuc IV, Fortunato F, D'Souza NB, et al. Modulation of caspase-3 activity and Fas ligand mRNA expression in rat liver cells in vivo by alcohol and lipopolysaccharide. Alcohol Clin Exp Res 1999;23:349–56.
[70] Natori S, Rust C, Stadheim LM, Srinivasan A, Burgart LJ, Gores GJ. Hepatocyte apoptosis is a pathologic feature of human alcoholic hepatitis. J Hepatol 2001;34:248–53.
[71] Slukvin II, Boor PJ, Jerrells TR. Initiation of alcoholic fatty liver and hepatic inflammation with a specific recall immune response in alcohol-consuming C57Bl/6 mice. Clin Exp Immunol 2001;125:123–33.
[72] Lytton SD, Helander A, Zhang-Gouillon ZQ, et al. Autoantibodies against cytochromes P-4502E1 and P-4503A in alcoholics. Mol Pharmacol 1999;55:223–33.
[73] Gouillon Z, Lucas D, Li J, et al. Inhibition of ethanol-induced liver disease in the intragastric feeding rat model by chlormethiazole. Proc Soc Exp Biol Med 2000;224:302–8.
[74] Albano E. Free radical mechanisms in immune reactions associated with alcoholic liver disease. Free Radic Biol Med 2002;32:110–4.
[75] Albano E, French SW, Ingelman-Sundberg M. Hydroxyethyl radicals in ethanol hepatotoxicity. Front Biosci 1999;4:533–40.
[76] Clot P, Parola M, Bellomo G, et al. Plasma membrane hydroxyethyl radical adducts cause antibody-dependent cytotoxicity in rat hepatocytes exposed to alcohol. Gastroenterology 1997;113:265–76.
[77] Vidali M, Stewart SF, Rolla R, et al. Genetic and epigenetic factors in autoimmune reactions toward cytochrome P4502E1 in alcoholic liver disease. Hepatology 2003;37:410–9.
[78] Devalaraja MN, McClain CJ, Barve S, Vaddi K, Hill DB. Increased monocyte MCP-1 production in acute alcoholic hepatitis. Cytokine 1999;11:875–81.
[79] Zhang Z, Bagby GJ, Stoltz D, Oliver P, Schwarzenberger PO, Kolls JK. Prolonged ethanol treatment enhances lipopolysaccharide/phorbol myristate acetate-induced tumor necrosis factor-alpha production in human monocytic cells. Alcohol Clin Exp Res 2001;25:444–9.
[80] Pastorino JG, Hoek JB. Ethanol potentiates tumor necrosis factor-alpha cytotoxicity in hepatoma cells and primary rat hepatocytes by promoting induction of the mitochondrial permeability transition. Hepatology 2000;31:1141–52.
[81] Higuchi H, Adachi M, Miura S, Gores GJ, Ishii H. The mitochondrial permeability transition contributes to acute ethanol-induced apoptosis in rat hepatocytes. Hepatology 2001;34:320–8.
[82] Degoul F, Sutton A, Mansouri A, et al. Homozygosity for alanine in the mitochondrial targeting sequence of superoxide dismutase and risk for severe alcoholic liver disease. Gastroenterology 2001;120:1468–74.
[83] Brind A, Fryer A, Hurlstone A, Fisher N, Pirmohamed M. The role of polymorphism in manganese superoxide dismutase in susceptibility to alcoholic liver disease. Gastroenterology 2003;124:2000–2.
[84] French SW. The role of hypoxia in the pathogenesos of alcoholic liver disease. Hepatol Res 2004;29:69–74.

Gene expression associated with the decrease in malignant phenotype of human liver cancer cells following stimulation with a histone deacetylase inhibitor

KANJI WAKABAYASHI[1,2], HIDETSUGU SAITO[1], FUMIHIKO KANEKO[3], NOBUHIRO NAKAMOTO[1], SHINICHIRO TADA[1] and TOSHIFUMI HIBI[1]

[1]Department of Internal Medicine, School of Medicine, Keio University, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582; [2]Department of Internal Medicine, Tokyo Electric Power Company Hospital, 9-2 Shinano-machi, Shinjuku-ku, Tokyo 160-0016; [3]Liver Center, Kitasato Institute Hospital, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8642, Japan

Received August 2, 2004; Accepted October 24, 2004

Abstract. Sodium butyrate is a short-chain fatty acid produced by fermentation in the gastrointestinal tract. It induces differentiation of several kinds of cancer by inhibiting histone deacetylase activity. We have reported that butyrate stimulates hepatocellular carcinoma cells into their normal phenotype. Since sodium butyrate affects both differentiation and apoptosis, we investigated expression of *bcl*-2-related genes in a human hepatocellular carcinoma cell line HCC-T. The expression of anti-apoptotic Bcl-2 and Mcl-1/EAT was up-regulated 4 h after the treatment, while pro-apoptotic Bax expression did not change. Gene expressions in the early stage of butyrate-stimulation were investigated by the differential display assay and the cDNA expression array. Laminin and keratin 18 were increased 6 h after the stimulation with sodium butyrate. The results of cDNA expression array revealed up-regulation of cell cycle inhibitory genes such as cyclin-dependent kinase 4 inhibitor, and interferon-related genes such as STAT2 and 3, while down-regulation of cyclin-dependent kinase 2 and cyclin E. Up-regulated production of $p21^{WAF-1}$ and Mcl-1/EAT was also confirmed by Western blotting. The cytoskeletal change indicated by up-regulation of laminin and keratin 18 may be an important factor in the decrease in malignant phenotype of cancer cells. Up-regulation of interferon-related genes indicated that butyrate-treatment might induce a similar phenotypic change to that induced by type I interferons. This study suggests several target genes for the future gene therapy of cancer or genes preventing cancer development from pre-malignant tissues.

Correspondence to: Dr Hidetsugu Saito, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan
E-mail: hsaito@sc.itc.keio.ac.jp

Key words: sodium butyrate, differentiation, histone deacetylase inhibitor, STAT, laminin

Introduction

Hepatocellular carcinoma (HCC) often develops as a complication of chronic hepatitis B and C, or liver cirrhosis. It has been reported that HCC occurs in the liver chronically infected by hepatitis B virus (HBV) or hepatitis C virus (HCV) in 90% of cases in Japan (1). Although the incidence of HCC is expected to be reduced by the widespread adoption of preventive measures against HBV and HCV infection, at present, HCC remains one of the most common malignant tumors of the digestive system not only in Japan but also in other Asian countries. Various methods have been employed to treat HCC, including partial hepatectomy, transarterial (chemo)embolization [TA(C)E], and local therapies, such as radiofrequency ablation, microwave coagulation, cryoablation and percutaneous ethanol injection therapy. However, radical treatment using these methods remains difficult to achieve, unless they are instituted at an early stage of the disease. Chemotherapy and radiotherapy have been administered for the treatment of HCC, but neither has been reported to yield satisfactory therapeutic results in cases with advanced HCC. In addition, deterioration of liver function due to progression of underlying chronic liver disease often adversely interferes with the treatment of HCC. In cases of chronic hepatitis C, attempts have been made to suppress the progression of liver fibrosis by interferon (IFN) therapy, with the ultimate goal of preventing liver cirrhosis and HCC. However, in those cases where the virus load is high and HCV genotype 1b is the causative pathogen, the response to IFN therapy is quite poor (2), and it is often not possible to prevent progression of hepatitis C into liver cirrhosis or HCC in these difficult-to-treat cases. Although IFN therapy has been useful for preventing HCC recurrence after surgery, this agent also has been shown to have many significant adverse effects, such as fever, leucopenia, thrombocytopenia, depression, alopecia, and so on. It would therefore be desirable to develop other effective strategies for preventing HCC.

Sodium butyrate (SB) is a short-chain fatty acid, which has been shown to be constitutively produced in the digestive tract *in vivo* (3). Following ingestion of edible fiber that is not degraded by amylase in the small intestine, it is degraded

by enterobacteria in the ileocecum to yield butyrate (4). It has been reported that butyrate possesses the ability to induce apoptosis or differentiation of various cancer cells, such as leukemic, colorectal cancer and breast cancer cells (5-8). For the case of colorectal cancer, it has been reported that butyrate formed from edible fiber in rats led to the prevention of colorectal cancer (9), however, the precise mechanism of SB is not yet understood. Butyrate is partially transported from the intestine, via the portal vein, to the liver. SB, served as a histone deacetylase inhibitor, alters histone into a protein of relatively high level of acetylation (10), and causes a change in DNA methylation status or disaggregation of chromatin, resulting in elevation of the transcriptional activity for various genes (11). On the other hand, there remain many unresolved questions about the effects of SB on genes; e.g., which genes are induced, whether or not SB stimulates the expression of some particular genes depending on the cell type, etc. We previously studied the effects of SB on cultured HCC cells. Based on the results of these studies, we reported that SB at least reduces the malignant potential of HCC significantly, and that it is a promising agent for the chemoprevention of cancer (12-17). However, it remained unknown whether or not these effects of SB on HCC were identical to its reported effect of 'inducing cancer differentiation' in other tumors.

Identification of the genes that are altered after a dose of SB and clarification of the protein types induced by butyate may be expected to facilitate the evaluation of the clinical applicability of SB for cancer prevention, and to also allow us to examine whether or not the effect of SB in reducing the malignant potential of liver cancer is identical with its effect of inducing cellular differentiation, both these effects have been reported previously. In the present study, a human HCC cell line, HCC-T (18), was cultured with SB at a concentration too low to induce apoptosis, and the protein expression and gene alterations were analyzed by means of the differential display assay and the cDNA expression array analysis in the early stages of incubation.

Materials and methods

Reagents. SB, purchased from Sigma Chemical (Tokyo), was dissolved in distilled water at a concentration of 1 M to yield the stock solution. The stock solution was stored at -80°C and diluted with the culture medium immediately before each use.

HCC cell line. The human HCC cell line, HCC-T (18), established at our laboratory, was used for this study. The cell was cultured in RPMI 1640 supplemented with 5% FCS. The medium was changed twice every week. When the density of the cells reached to be confluent, the cells were harvested by treatment with trypsin. The cells were subsequently passaged for multiple generations.

Western blot analysis. The harvested cells were subjected to protein extraction in a lysis buffer (1% Triton X-100, 10 mM Tris pH 7.4, 150 mM NaCl, 5 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulfonylfluoride, 18 ng protein/ml aprotinin, 50 ng/ml leupeptin, 1 mM benzamidine, 0.7 ng/ml pepstatin, and 1% NP-40). The extracted proteins were processed into samples containing equal amounts (20 μg) of protein. The samples were electrophoresed on 12% sodium dodecylsulfate (SDS) polyacrylamide gel and transferred on to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences). After being blocked at room temperature for 1 h using 2.5% BSA (bovine serum albumin), the samples were incubated overnight with monoclonal antibodies against $p21^{WAF-1}$, Bcl-2, Bcl-XL, BAX, Mcl-1/EAT, laminin and keratin 18 (purchased from Calbiochem-Novabiochem, Nottingham, UK) diluted to a concentration of 1 μg/ml with PBS (phosphate-buffered saline). The samples were then reacted to secondary antibody (anti-rabbit IgG horseradish peroxidase) diluted 1:1500 with PBS for 1 h at room temperature. X-ray film was then developed after the membrane was reacted with a Western blotting detection kit (ECL; Amersham Biosciences, Tokyo, Japan).

Table I. Anchor primers used for differential display analysis.

No. 1	GTVA-ROX	5'-GTTTTTTTTTTTTTTTVA-3'
No. 2	GTVC-ROX	5'-GTTTTTTTTTTTTTTTVC-3'
No. 3	GTVG-ROX	5'-GTTTTTTTTTTTTTTTVG-3'
No. 4	GTVT-ROX	5'-GTTTTTTTTTTTTTTTVT-3'

V, mixture of A, C, G.

Differential display. SB was added to the HCC-T culture at a concentration of 2 mM, that is not toxic and apoptotic to the cell according to the previous studies. The cells were harvested immediately before, and 1, 2, 4 and 8 h after stimulation with SB. RNA was extracted from the cells by the acid guanidine thiocyanate phenol chloroform (AGPC) method reported elsewhere (19). Then, the samples were subjected to the differential display assay, using a partial modification of the method of Liang *et al* (20,21). Reverse transcription was performed to prepare cDNA, using 4 anchor primers prepared from 12 anchor primers, with a G, A and C mix serving as the V of GT15VN (V=G, A, C, N=G, A, T, C) (Table I). PCR was performed using the same anchor primers, as well as 29 arbitrary primers (Table II). The PCR products were electrophoresed, and the results were analyzed with an image analyzer (FMBIO II, Takara, Ohtsu, Japan). Bands that were different from those obtained before butyrate-stimulation of the samples were cut out, and the PCR products were extracted. Second PCR was performed using a combination of the same anchor primers and arbitrary primers to cut out targeted bands and extract the PCR products. The extracted PCR products were ligated to the pCR2.1 vector (Invitrogen, Carlsbad, CA) using a DNA Ligation Kit, Ver. 2 (Takara). The ligation mixture was incubated at 16°C for 2 h and was transfected to a competent cell line, JM109 (Takara), and incubated for 30 min. The transfectant received a heat shock at 42°C for 45 sec, and then the cell was immediately incubated under the ice-cooled condition for 2 min. The sample was then incubated under shaking in LB medium at 37°C for 1 h. It was then incubated in LB plate containing 0.1 mM X-Gal, 0.1 mM IPTG, and 50 μg/ml ampicillin. Single colonies were harvested and amplified by overnight culture in LB medium

Table II. Arbitrary primer used for differential display analysis.

AP 1	5'-ROX-CTGATCCATG-3'
AP 2	5'-ROX-ACTGCTCTCA-3'
AP 3	5'-ROX-CTTGATTGCC-3'
AP 4	5'-ROX-TACAACGAGG-3'
AP 5	5'-ROX-TGGATTGGTC-3'
AP 6	5'-ROX-CTTTCTACCC-3'
AP 7	5'-ROX-TTTTGGCTCC-3'
AP 8	5'-ROX-GGAACCAATC-3'
AP 9	5'-ROX-AAACTCCGTC-3'
AP 10	5'-ROX-TCGATACAGG-3'
AP 11	5'-ROX-TGGTAAAGGG-3'
AP 12	5'-ROX-TCGGTCATAG-3'
AP 13	5'-ROX-GGTACTAAGG-3'
AP 14	5'-ROX-TACCTAAGCG-3'
AP 15	5'-ROX-CTGCTTGATG-3'
AP 16	5'-ROX-GTTTTCGCAG-3'
AP 17	5'-ROX-GATCAAGTCC-3'
AP 18	5'-ROX-GATCCAGTAC-3'
AP 19	5'-ROX-GATCACGTAC-3'
AP 20	5'-ROX-GATCTGACAC-3'
AP 21	5'-ROX-GATCTCAGAC-3'
AP 22	5'-ROX-GATCATAGCC-3'
AP 23	5'-ROX-GATCAATCGC-3'
AP 24	5'-ROX-GATCTAACCG-3'
AP 25	5'-ROX-GATCGCATTG-3'
AP 26	5'-ROX-GATCTGACTG-3'
AP 27	5'-ROX-GATCATGGTC-3'
AP 28	5'-ROX-GATCATAGCG-3'
AP 29	5'-ROX-GATCTAAGGC-3'

(containing ampicillin 50 μg/ml) under shaking. DNA was extracted from the culture and was fluorescence-labeled using a DNA Sequencing Kit and a BigDye Terminator Cycle Sequencing Reaction Kit (ABI, Tokyo, Japan). Sequencing was then performed using an ABI PRISM™ 377 sequencer for subsequent homology search on a database (GenBank).

cDNA microarray. SB was added to the HCC-T culture at a concentration of 2 mM, and cells were harvested before and 2 h after stimulation with SB. mRNA was extracted by the AGPC method and subsequently analyzed using a cDNA expression array (Human Cancer 1.2 k, Clontech, Palo Alto, CA). The nylon membrane of the Human Cancer 1.2 k used in this assay had spots of cDNA for 1185 cancer-related human genes. RNA was labeled with an Atlas™ Pure Total RNA Labeling System. After hybridization (Fig. 4), the sample was analyzed using the AtlasImage™ software. This analysis was performed twice and the consensus result was confirmed.

Results

Changes in protein expression following treatment with SB. Following stimulation with SB, the cell cycle of the HCC-T cells was stopped at the G0/G1 phase (12). This phenomenon was accompanied by an increase in the expression of $p21^{WAF-1}$, a protein known to regulate the cell cycle. In view of a previous report of the ability of SB to induce apoptosis, we also checked for changes in the expression of the apoptosis-related proteins in the HCC-T cells. The expression of Bcl-2 and Bcl-XL, which are anti-apoptotic proteins, was up-regulated 2 h after stimulation with SB. The expression of Mcl-1/EAT, another anti-apoptotic protein, and which has a close relationship with cellular differentiation, was also increased. On the other hand, the expression of BAX, a pro-apoptotic protein, remained unchanged during first 8 h after the SB stimulation (Fig. 1).

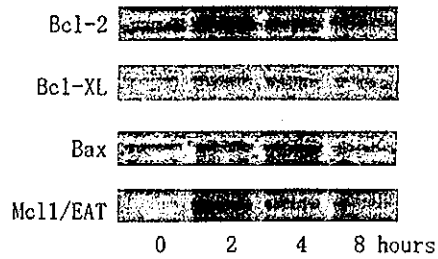

Figure 1. HCC-T cells were harvested immediately before, and 2, 4, 6, and 8 h after stimulation with 2 mM of sodium butyrate. Cell lysate was prepared to contain equal amount of protein (20 μg). Samples were electrophoresed on an 12% SDS-polyacrylamide gel and analized by Western blotting with Bcl-2, Bcl-XL, BAX, and Mcl-1/EAT monoclonal antibodies.

Differential display. To investigate the early gene expression in HCC cells after SB stimulation, we conducted the differential display assay using HCC-T cells. Totally 96 bands, showing enhanced or reduced gene expression, were found by the differential display. Nucleic acid was extracted from the bands, and was incorporated into the vector for cloning. Sequencing of the cloned genes revealed that many up-regulated genes were mitochondria-related genes and mRNA for ribosomal proteins. Several bands were not successfully cloned and could not be analyzed. Some cytoskeleton-related genes such as laminin or keratin 18 were specifically up-regulated (Fig. 2). Other up- and down-regulated genes are summarized in Table III. The significant down-regulated gene was cyclophilin, which is a key target of an immunosuppressant cyclosporin A, which sepcifically inhibit calcineurin activation. It was confirmed by Western blotting that the expression of laminin and keratin 18 were rapidly increased at 6 h after stimulation with SB (Fig. 3).

cDNA expression array. An analysis of the cDNA expression array revealed enhanced expression of $p21^{WAF-1}$ and cyclin-dependent kinase (CDK) 4 inhibitor D in the HCC-T cells, as shown in Table IV. On the other hand, the expression of CDK2 and G1/S-specific cyclin E was decreased (Table V). Of the genes associated with the cytoskeleton, the expression of Rho E and hepatocyte growth factor (HGF)/scatter factor (SF) receptor was found to be enhanced, while that of collagen 18AI and MIF/GIF was decreased. Enhanced expression of the IFN-associated genes, interferon regulatory factor (IRF)-1, STAT2 and STAT3, and of ELF1 and Mcl-1/EAT was also

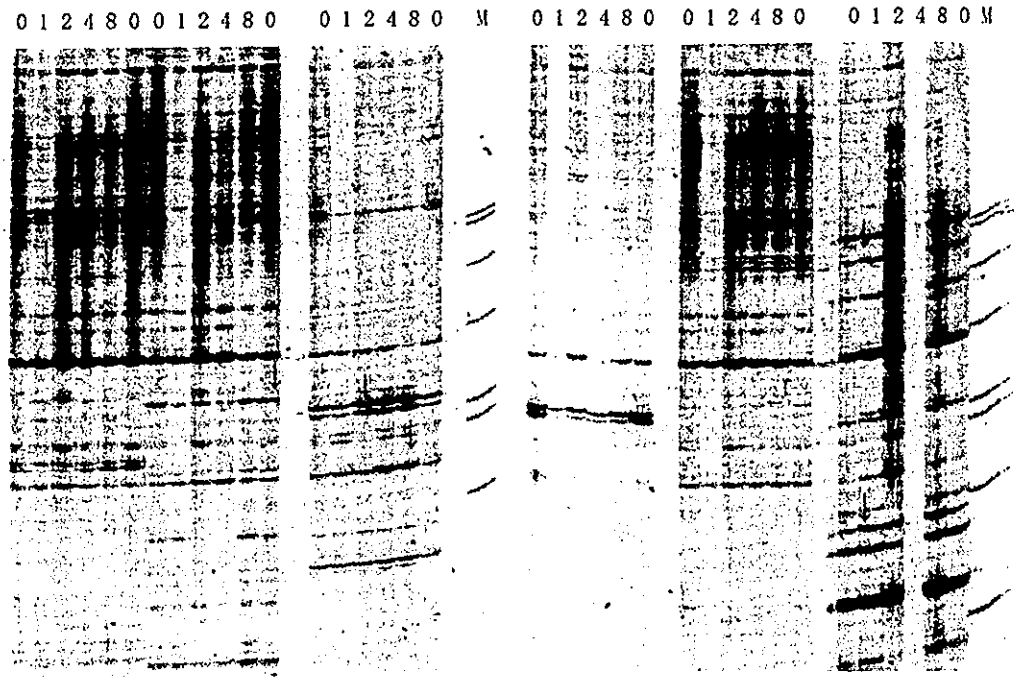

Figure 2. A typical photograph of the image analyzer after electrophoresis of PCR products obtained by the differential display assay. The bands indicated with arrows were mRNA up-regulated by sodium butyrate compared with those of control. Ninety-six bands were cut out and PCR products were extracted. M, maker; 0, before stimulation with sodium butyrate. Numbers indicate hours after stimulation with sodium butyrate.

noted, as shown in Table III. The finding of enhanced expression of Mcl-1/EAT and $p21^{WAF-1}$ was consistent with the results of the Western blot analysis (Fig. 1).

Discussion

Several agents that powerfully induce differentiation have been identified, and SB has been recognized to be one such agent. In recent years, even more powerful inducers of differentiation, such as trichostatin A (22) and trapoxin (23), have also been reported. Like SB, trichostatin A belongs to the class of histone deacetylase inhibitors, and has been reported to induce apoptosis and differentiation of liver cancer cells (24). This agent, however, has not yet been introduced for clinical use in liver malignancies. SB at high concentrations induces apoptosis of some liver cancer cells, while at concentrations below 5 mM, it induced differentiation without inducing apoptosis in our previous studies (12). Cells which remained viable following treatment with SB showed a decrease in the production of α-fetoprotein (AFP), an increase in the production of albumin (13,25), and an enhanced expression of liver-specific antigens (14). AFP is a fetal protein and albumin is a protein in mature liver, and the shift of protein production from fetal to mature suggests that this alteration is a kind of normal differentiation. These findings indicate that SB change HCC cells into those with mature phenotype (14,15). When SB-treated HCC cells are cultured in soft agar, the cells lose their anchorage-independent proliferation, lose their implantability into nude mice, and become non-malignant (26). Furthermore, a high level expression of E-cadherin and ß-catenin was induced along the cell membrane by SB-treatment, and this result indicates that SB-treatment enriches a force of cell-to-cell adhesion and reduces a chance of metastasis (16). The invasion ability of HCC cells through a matrix-coated membrane, which indicates invasion ability of HCC, was also reduced by SB-treatment (27). SB also reduced the telomerase activity of HCC cells (17). These findings suggest that treatment with SB induces HCC cells to be differentiated and HCC cells become closer to the non-malignant phenotype.

However, the genes responsible for the change of the HCC cells into their non-malignant phenotype have not been identified. In the present study, we analyzed the Bcl-2 family gene expression, considering previous results of our studies that SB induced apoptosis of HCC cells at high concentrations, whereas it induced differentiation instead of apoptosis at low concentrations. Bcl-2 family proteins have an important role in regulation of apoptosis (28,29). Bcl-2, Bcl-XL, and Mcl-1/EAT are anti-apoptotic proteins, while Bax, Bcl-Xs, and Bad are pro-apoptotic proteins. In the present study, the enhanced expression of Bcl-2 and Bcl-XL was observed soon after the induction of non-malignant phenotype of HCC cells by SB, and the expression of Mcl-1/EAT, a protein previously reported to be expressed in the early stages of cellular differentiation (32), was also enhanced. On the other hand, expression of the pro-apoptotic protein BAX remained unchanged (Fig. 1). SB also increased $p21^{WAF-1}$ and CDK4 inhibitor D transcription, which induces cell-cycle arrest at the G0/G1 phase, not at G1/S phase. These findings suggest that treatment of HCC cells with SB achieve the cell-cycle arrest at the G0/G1 phase, and the further induction into G0 phase is conducted without inducing apoptosis.

Table III. Results of cloned genes obtained from the differential display assay.[a]

Genes up-regulated (results of homology research)
Cctg mRNA for chaperonin
Chromosome 14 DNA (BAC C-3104H21)
Chromosome 14 DNA (BAC R-681H18)
Chromosome 14 DNA (BAC R-97N10)
Clone AMF151xc3
Clone DJ0635B05
Clone DJ076B20
DNA sequence clone 99E18 on chromosome 6q25
DNA sequence from clone RP1-144C9 on chromosome 1p34.3-36.11
DNA sequence from clone RP5-1185K9 on chromosome 20
Genomic sequence for Arabidopsis thaliana BAC F22C12 from chromosome I
Germinal center kinase related protein kinase mRNA
Keratin 18 (KRT18) mRNA
Laminin
Laminin receptor
MHC protein homologous to chicken B complex protein mRNA
NADH
PAC clone RP5-1048B16 from 7q34-q36
Signal sequence receptor
Staufen (Dorosophia, RNA-binding protein) mRNA
Sui 1iso 1 mRNA
Genes down-regulated
BAC clone GS1-5K18 from 7p15-p21
Clone DKFZp564E10882
Cyclophilin
DNA-binding protein, TAXREB107
mRNA for imogen 38

[a]Up- and down-regulated genes by sodium butyrate stimulation of HCC-T cells were summarized.

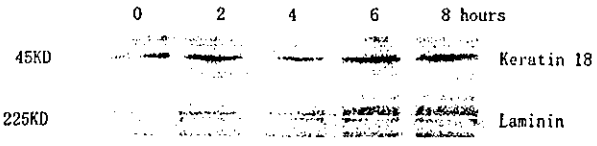

Figure 3. HCC-T cells were harvested immediately before, and 2, 4, 6, and 8 h after stimulation with 2 mM of sodium butyrate. The expression of laminin and keratin 18 were analyzed by Western blotting. It was confirmed that the expression of laminin and keratin 18 increases 6 h after stimulation with sodium butyrate.

Table IV. Genes up-regulated more than twice compared with those of control in the cDNA expression array (Human Cancer 1.2 k).

Category	Gene	Ratio vs control
Cell cycle-related	p21/WAF-1	2.0
	CDK4 inhibitor D	4.0
Cytoskeleton-related	Rho E	2.5
	HGF/SF-receptor	2.5
Interferon-related	IRF-1	2.3
	STAT2	2.7
	STAT3	3.5
Differentiation-related	ELF1	2.8
	Mcl-1/EAT	3.3

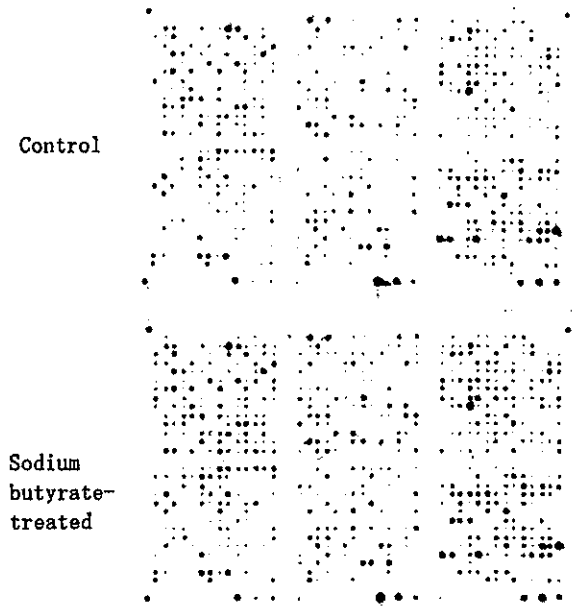

Figure 4. An image of the cDNA expression array (Human Cancer 1.2 k) after hybridization.

Table V. Genes down-regulated less than half compared with those of control in the cDNA expression array (Human Cancer 1.2 k).

Category	Gene	Ratio vs control
Cell cycle-related	CDK2	0.20
	G1/S-specific cyclin E	0.20
Cytoskeleton-related	Collagen 18A1	0.48
	MIF/GIF	0.50

Regarding the morphological changes of the cells, it has been reported that treatment with SB results in the appearance of membrane ruffling or microspikes on the cell membrane (16). In the present study, consistent with the previous report, the differential display assay revealed changes in the expression of proteins constituting the cytoskeleton, such as laminin, one of the extracellular matrix components, and keratin 18, one of the proteins lining the intermediate fibers, during the course of phenotypic changes. This finding is also consistent with a previous report that incubation of liver cancer cells with SB in a laminin-coated container reduced AFP and increased albumin production (33). Because laminin expression increases during the early stages of differentiation, it seems that an increase in laminin expression is indispensable for differentiation, and contrarily, that morphological change is also important during differentiation. According to a previous report, the increase in keratin 18 expression was associated with the SP1 binding site located upstream of the promoter, and that the promoter activity was significantly stimulated by a high level of acetylation of histone (34). This finding is also compatible with that of the present study.

Hung and Chuang reported that the expression of STAT1 in a HCC cell line, PLC/PRF/5, is increased by 48 h after stimulation with SB, and that the cellular sensitivity to IFN-α also increased (35). In the present study, no increase of STAT1 expression was seen by 2 h after stimulation with SB, although the expression of IRF-1, STAT2 and STAT3 was found to be enhanced. These findings suggest that stimulation with SB increases the expression of IFN-related transcription factor, IRF-1, via the Jak-STAT pathway, and that SB has a biological activity similar to that of type I IFN. In other words, there may be cross-talk between the SB-activated and IFN-stimulated pathways, and SB may induce tyrosine phosphorylation of STAT, similar to the effect of IFN, leading to an increased transcriptional activity of IRF-1 due to ICSBP (36). In our previous study, several phenotypic changes of HCC cells were similarly induced by both IFN-α and SB in concordance with this study (16,27).

In the present study, there was a difference between results of the differential display assay and cDNA microarray analysis. PCR in the study was conducted using 4 anchor primers and 29 arbitrary primers, allowing PCR with 116 combinations, and the temperature for annealing was set at a very low level (40˚C). It was expected that almost all human mRNA was amplified under this set of experimental conditions. However, in the differential display assay, the number of bands was limited. Most of the bands probably represented a portion of mitochondrial DNA. Differential display assay has been reported to be inferior to microarray analysis, in that the former requires more time and is less accurate. On the other hand, differential display assay is expected to allow the detection of unknown genes, as in the present study, several unknown genes not contained in the existing database were detected by the differential display assay. The data obtained from both differential display assay and microarray assay were confirmed by Western blotting in the present study.

Thus, SB reduces malignant potential of HCC cells, but it will not be easy to use SB clinically, although it has already been used in the treatment of patients with acute leukemia (37,38). A possible method of putting the beneficial effects of SB to use clinically with the goal of preventing HCC may be the administration of a substance (e.g., oligosaccharide) which stimulates gastrointestinal fermented production of SB. In addition to direct administration of SB, gene therapy targeting the genes shown to undergo alterations by SB administration can also serve as means of achieving the beneficial effects of SB. Considering previous reports that the expression of c-*myc* was elevated in HCC cells infected with HBV or HCV (39-41), and that differentiation or apoptosis of HCC cells were induced when c-*myc* expression was inhibited by antisense oligodeoxynucleotide or use of the Cre/loxP switching system (42,43), the therapy targeted at this gene appears to be promising. Recent studies demonstrated that cyclosporine A suppresses replication of HCV genome in a cultured system (44), and this topic is another interest in relation to the present study, because cyclophilin was down-regulated by SB-stimulation. Therefore the gene detected in this study may be a future target of treatment. Epigenetic alteration may be another strategy for cancer prevention (45,46). Histone acetylation and DNA demethylation are key events in the normal cellular differentiation, and they have been reported to activate gene expressions, and the regulation of epigenetic status appears to be promising in cancer prevention, because many progenitor cells, which should normally differentiate, have been discovered in cancer of every organ.

References

1. Ikai I, Itai Y, Okita K, Kojiro M, Kobayashi K, Nakanuma Y, Futagawa S, Makuuchi M and Yamaoka Y: Report of the 15th follow-up survey of primary liver cancer. Hepatol Res 28: 21-29, 2004.
2. Hadziyannis SJ and Koskinas JS: Differences in epidemiology, liver disease and treatment response among HCV genotypes. Hepatol Res 29: 129-135, 2004.
3. Kruh J: Effects of sodium butyrate, a new pharmacological agent, on cells in culture. Mol Cell Biochem 42: 65-82, 1982.
4. Le Blay G, Michel C, Blottiere HM and Cherbut C: Enhancement of butyrate production in the rat caecocolonic tract by long-term ingestion of resistant potato starch. Br J Nutr 82: 419-426, 1999.
5. Tsao D, Morita A, Bella A Jr, Luu P and Kim YS: Differential effects of sodium butytare, dimethyl sulfoxide and retinoic acid on membrane-associated antigen, enzymes, and glycoproteins of human rectal adenocarcinoma cells. Cancer Res 42: 1052-1058, 1982.
6. Abe M and Kufe DW: Sodium butyrate induction of milk-related antigens in human MCF-7 breast carcinoma cells. Cancer Res 44: 4574-4577, 1984.
7. Gleave ME, Sato N, Sadar M, Yago V, Brunchovsky N and Sullivan L: Butyrate analogue, isobutyramide, inhibits tumor growth and time to androgen-independent progression in the human prostate LNCaP tumor model. J Cell Biochem 69: 271-281, 1998.
8. McBain JA, Eastman A, Nobel CS and Mueller GC: Apoptotic death in adenocarcinoma cell lines induced by butyrate and other histone deacetylase inhibitors. Biochem Pharmacol 53: 1357-1368, 1997.
9. McIntyre A, Gibson PR and Young GP: Butyrate production from dietary fiber and protection against large bowel cancer in a rat model. Gut 34: 386-391, 1993.
10. Sealy L and Chalkley R: The effect of sodium butyrate on histone modification. Cell 14: 115-121, 1978.
11. Grunstein M: Histone acetylation in chromatin structure and transcription. Nature 389: 349-352, 1997.
12. Saito H, Ebinuma H, Takahashi M, Kaneko F, Wakabayashi K, Nakamura M and Ishii H: Loss of butyrate-induced apoptosis in human hepatoma cell lines HCC-M and HCC-T having substantial Bcl-2 expression. Hepatology 27: 1233-1240, 1998.

13. Saito H, Morizane T, Watanabe T, Kagawa T, Miyaguchi S, Kumagai N and Tsuchiya M: Differentiating effect of sodium butyrate on human hepatoma cell lines PLC/PRF/5, HCC-M and HCC-T. Int J Cancer 48: 291-296, 1991.
14. Saito H, Tada S, Ebinuma H, Tsunematsu S, Kagawa T, Kumagai N, Inagaki Y, Watanabe T, Tsuchimoto K, Morizane T and Tsuchiya M: Changes of antigen expression on human hepatoma cell lines caused by sodium butyrate, a differentiation inducer. J Gastroenterol 29: 733-739, 1994.
15. Saito H, Tada S, Ebinuna H, Atsukawa K, Masuda T, Inagaki Y, Tsuchimoto K, Morizane T and Ishii H: Cloning of variable regions of an antibody that reacts with the soluble fraction of human liver cells and its possible value in chronic liver disease. Hepatology 23: 1498-1506, 1996.
16. Masuda T, Saito H, Kaneko F, Atsukawa K, Morita M, Inagaki H, Kumagai N, Tsuchimoto K and Ishii H: Up-regulation of E-cadherin and I-catenin in human hepatocellular carcinoma cell lines by sodium butyrate and interferon-alpha. In Vitro Cell Dev Biol Anim 36: 387-394, 2000.
17. Nakamura M, Saito H, Ebinuma H, Wakabayashi K, Saito Y, Takagi T, Nakamoto N and Ishii H: Reduction of telomerase activity in human liver cancer cells by a histone deacetylase inhibitor. J Cell Physiol 187: 392-401, 2001.
18. Saito H, Morizane T, Watanabe T, Kagawa T, Iwabuchi MN, Kumagai N, Inagaki Y, Tsuchimoto K and Tsuchiya M: Establishment of a human cell line (HCC-T) from a patient with hepatoma bearing no evidence of hepatitis B or A virus infection. Cancer 64: 1054-1060, 1989.
19. Tada S, Saito H, Tsunematsu S, Ebinuma H, Wakabayashi K, Masuda T and Ishii H: Interferon regulatory factor-1 gene abnormality and loss of growth inhibitory effect of interferon-alpha in human hepatoma cell lines. Int J Oncol 13: 1207-1216, 1998.
20. Liang P and Pardee AB: Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257: 967-971, 1992.
21. Liang P, Averboukh L, Keyomarsi K, Sager R and Pardee AB: Differential display and cloning of messenger RNAs from human breast cancer versus mammary epithelial cells. Cancer Res 52: 6966-6968, 1992.
22. Yoshida M, Nomura S and Beppu T: Effects of trichostatins on differentiation of murine erythroleukemia cells. Cancer Res 47: 3688-3691, 1987.
23. Yoshida M, Horinouchi S and Beppu T: Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. Bioassays 17: 423-430, 1995.
24. Yamashita Y, Shimada M, Harimoto N, Rikimaru T, Shirabe K, Tanaka S and Sugimachi K: Histone deacetylase inhibitor trichostatin A induces cell-cycle arrest/apoptosis and hepatocyte differentiation in human hepatoma cells. Int J Cancer 103: 572-576, 2003.
25. Tsutsumi T, Ido A, Nakao K, Hamasaki K, Kato Y, Ohtsuru A, Nakata K, Tamaoki T and Nagataki S: Reciprocal regulation of α-fetoprotein and albumin gene expression by butyrate in human hepatoma cells. Gastroenterol 107: 499-504, 1994.
26. Yamamoto H, Fujimoto J, Okamoto E, Furuyama J, Tamaoki T and Hashimoto-Tamaoki T: Suppression of growth of hepatocellular carcinoma by sodium butyrate *in vitro* and *in vivo*. Int J Cancer 76: 897-902, 1998.
27. Kaneko F, Saito H, Saito Y, Wakabayashi K, Nakamoto N, Tada S, Suzuki H, Tsunematsu S, Kumagai N and Ishii H: Down-regulation of matrix-invasive potential of human liver cancer cells by type I interferon and a histone deacetylase inhibitor sodium butyrate. Int J Oncol 24: 837-845, 2004.
28. Mandal M and Kumar R: Bcl-2 expression regulates sodium butyrate-induced apoptosis in human MCF-7 breast cancer cells. Cell Growth Differ 7: 311-318, 1996.
29. Takahashi M, Saito H, Okuyama T, Miyashita T, Kosuga M, Sumisa F, Yamada M, Ebinuma H and Ishii H: Overexpression of Bcl-2 protects human hepatoma cells from Fas-antibody-mediated apoptosis. J Hepatol 31: 315-322, 1999.
30. Umezawa A, Maruyama T, Inazawa J, Imai S, Takano T and Hata J: Induction of mcl1/EAT, bcl-2 related gene, by retinoic acid or heat shock in the human embryonal carcinoma cells, NCR-G3. Cell Struct Funct 21: 143-150, 1996.
31. Sano M, Umezawa A, Abe H, Akatsuka A, Nonaka S, Shimizu H, Fukuma M and Hata J: EAT/mcl-1 expression in the human embryonal carcinoma cells undergoing differentiation or apoptosis. Exp Cell Res 266: 114-125, 2001.
32. Okita H, Umezawa A, Suzuki A and Hata J: Up-regulated expression of murine Mcl1/EAT, bcl-2 related gene, in the early stage of differentiation of murine embryonal carcinoma cells and embryonic stem cells. Biochim Biophys Acta 1398: 335-341, 1998.
33. Tokiwa T, Endo A and Sato J: Butyrate decreases AFP and increases albumin in combination with laminin in a human hepatoma cell line. In Vitro Cell Dev Biol Anim 26: 220-221, 1990.
34. Prochasson P, Gunther M, Laithier M, Fossar N, Lavialle C and Brison O: Transcriptional mechanisms responsible for the overexpression of the keratin 18 gene in cells of a human colon carcinoma cell line. Exp Cell Res 248: 243-259, 1999.
35. Hung WC and Chuang LY: Sodium butyrate enhances STAT 1 expression in PLC/PRF/5 hepatoma cells and augments their responsiveness to interferon-alpha. Br J Cancer 80: 705-710, 1999.
36. Holtschke T, Lochler J, Kanno Y, Fehr T, Giese N, Rosenbauer F, Lou J, Knobeloch KP, Gabriele L, Waring JF, Bachman MF, Zinkernagel RM, Morse HC III, Ozato K and Horak I: Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. Cell 87: 307-317, 1996.
37. Warrell RP Jr, De The H, Wang ZY and Degos L: Acute promyelocytic leukemia. N Engl J Med 329: 177-189, 1993.
38. Gozzini A, Rovida E, Dello Sbarba P, Galimbert S and Santini V: Butyrates, as a single drug, induce histone acetylation and granulocytic maturation: possible selectivity on core binding factor-acute myeloid leukemia blasts. Cancer Res 63: 8955-8961, 2003.
39. Ray RB, Lagging LM, Meyer K, Steele R and Ray R: Transcriptional regulation of cellular and viral promoters by the hepatitis C virus core protein. Virus Res 37: 209-220, 1995.
40. Caselmann WH: Transactivation of cellular gene expression by hepatitis B virus proteins. J Hepatol 22: 34-37, 1995.
41. Arcinas M, Sizer KC and Boxer LM: Activation of c-myc expression by c-Abl is independent of both the DNA binding function of c-Abl and the c-myc EP site. J Biol Chem 269: 21919-21924, 1994.
42. Ebinuma H, Saito H, Saito Y, Wakabayashi K, Nakamura M, Kurose I and Ishii H: Antisense oligodeoxynucleotide against c-myc mRNA induces differentiation of human hepatocellular carcinoma cells. Int J Oncol 15: 991-999, 1999.
43. Ebinuma H, Saito H, Kosuga M, Wakabayashi K, Saito Y, Takagi T, Nakamoto N, Okuyama T and Ishii H: Reduction of c-myc expression by an antisense approach under Cre/loxP switching induces apoptosis in human liver cancer cells. J Cell Physiol 188: 56-66, 2001.
44. Watashi K, Hijikata M, Hosaka M, Yamaji M and Shimotohno K: Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. Hepatology 38: 1282-1288, 2003.
45. Saito Y, Kanai Y, Nakagawa T, Sakamoto M, Saito H, Ishii H and Hirohashi S: Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas. Int J Cancer 105: 527-532, 2003.
46. Saito Y, Kanai Y, Sakamoto M, Saito H, Ishii H and Hirohashi S: Expression of mRNA for DNA methyltransferases and methyl-CpG-binding proteins and DNA methylation status on CpG islands and pericentromeric satellite regions during human hepatocarcinogenesis. Hepatology 33: 561-568, 2001.

JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2005, p. 186–191 Vol. 43, No. 1
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.1.186–191.2005

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

Masahiko Takahashi,[1,2] Hidetsugu Saito,[1]* Makiko Higashimoto,[3] Kazuhiro Atsukawa,[1] and Hiromasa Ishii[1]

Department of Internal Medicine, School of Medicine, Keio University,[1] and Department of Internal Medicine[2] and Department of Clinical Laboratory,[3] Hiro-o Metropolitan Hospital, Tokyo, Japan

Received 26 March 2004/Returned for modification 22 May 2004/Accepted 13 September 2004

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-

* Corresponding author. Mailing address: Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Phone: 81-3-3353-1211 (ext. 62894). Fax: 81-3-3351-8705. E-mail: hsaito@sc.itc.keio.ac.jp.

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

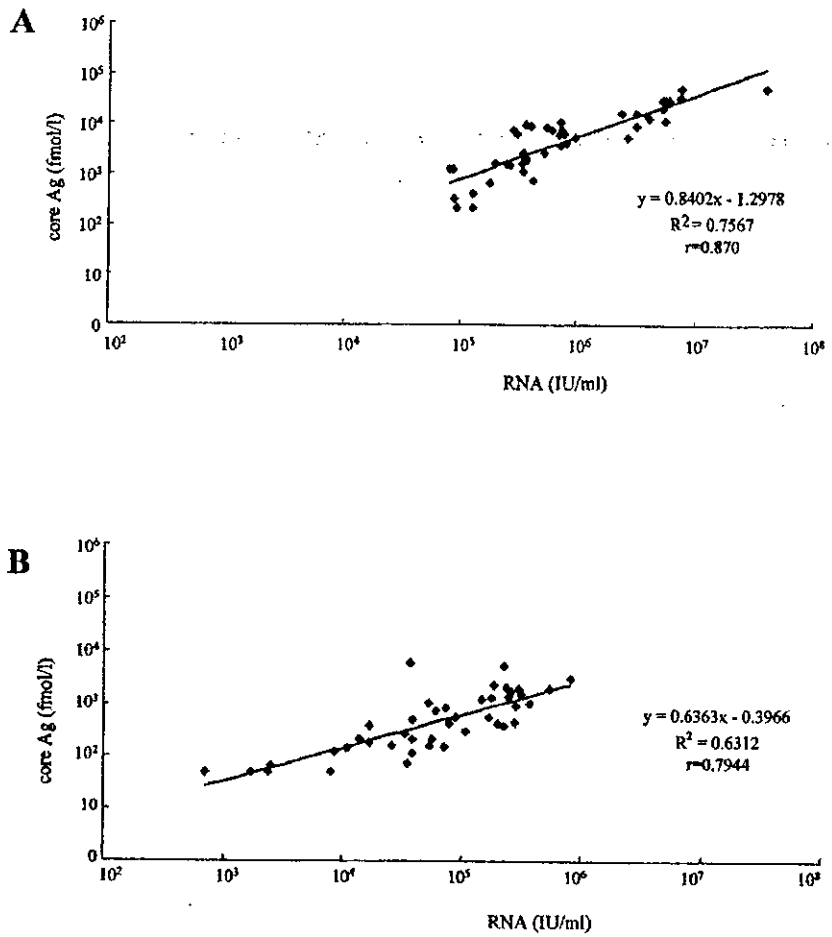

FIG. 1. Correlation between HCV core Ag and HCV RNA before (A) and 24 h after (B) therapy. Correlations were good both before and 24 h after therapy ($r = 0.870$ and 0.794, respectively), although the coefficient was smaller 24 h after therapy than before therapy ($P < 0.05$). The HCV RNA level was relatively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

ACKNOWLEDGMENTS

We are grateful to the participants in the Keio Study Group on Treatment of Chronic Hepatitis C, who contributed to the database used for the present report: S. Tanaka, National Tokyo Medical Center, Tokyo; J. Nishida, Tokyo Dental College Ichikawa General Hospital, Chiba; N. Tsukada, Saiseikai Central Hospital, Tokyo; T. Yoshida, Saitama Social Insurance Hospital, Saitama; K. Kashiwazaki, Kyosai Tachikawa Hospital, Tokyo; and H. Yoshida, Eiju General Hospital, Tokyo.

REFERENCES

1. **Aoyagi, K., C. Ohue, K. Iida, T. Kimura, E. Tanaka, K. Kiyosawa, and S. Yagi.** 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J. Clin. Microbiol. 37:1802–1808.
2. **Bouvier-Alias, M., K. Patel, H. Dahari, S. Beaucourt, P. Larderie, L. Blatt, C. Hezode, G. Picchio, D. Dhumeaux, A. U. Neumann, J. G. McHutchison, and J. M. Pawlotsky.** 2002. Clinical utility of total HCV core antigen quantification: a new indirect marker of HCV replication. Hepatology 36:211–218.
3. **Brouwer, J. T., B. E. Hansen, H. G. Niesters, and S. W. Schalm.** 1999. Early prediction of response in interferon monotherapy and in interferon-ribavirin combination therapy for chronic hepatitis C: HCV RNA at 4 weeks versus ALT. J. Hepatol. 30:192–198.
4. **Cheng, S. J., P. A. Bonis, J. Lau, N. Q. Pham, and J. B. Wong.** 2001. Interferon and ribavirin for patients with chronic hepatitis C who did not respond to previous interferon therapy: a meta-analysis of controlled and uncontrolled trials. Hepatology 33:231–240.
5. **Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina-Selby, P. J. Barr, et al.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA 88:2451–2455.
6. **Civeira, M. P., and J. Prieto.** 1999. Early predictors of response to treatment in patients with chronic hepatitis C. J. Hepatol. 31(Suppl. 1):237–243.
7. **Cividini, A., A. Cerino, A. Muzzi, M. Furlone, C. Rebucci, L. Segagni, M. Gatti, V. Barnaba, and M. U. Mondelli.** 2003. Kinetics and significance of serum hepatitis C virus core antigen in patients with acute hepatitis C. J. Clin. Microbiol. 41:2144–2146.
8. **Cotler, S. J., J. E. Layden, A. U. Neumann, and D. M. Jensen.** 2003. First phase hepatitis C viral kinetics in previous nonresponders patients. J. Viral Hepat. 10:43–49.
9. **Davis, G. L., L. A. Balart, E. R. Schiff, K. Lindsay, H. C. Bodenheimer, Jr., R. P. Perrillo, W. Carey, I. M. Jacobson, J. Payne, J. L. Dienstag, et al. for the Hepatitis Interventional Therapy Group.** 1989. Treatment of chronic hepatitis C with recombinant interferon alfa. A multicenter randomized, controlled trial. N. Engl. J. Med. 321:1501–1506.
10. **Davis, G. L., R. Esteban-Mur, V. Rustgi, J. Hoefs, S. C. Gordon, C. Trepo, M. L. Shiffman, S. Zeuzem, A. Craxi, M. H. Ling, and J. Albrecht for the International Hepatitis Interventional Therapy Group.** 1998. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. N. Engl. J. Med. 339:1493–1499.
11. **Di Bisceglie, A. M., P. Martin, C. Kassianides, M. Lisker-Melman, L. Murray, J. Waggoner, Z. Goodman, S. M. Banks, and J. H. Hoofnagle.** 1989. Recombinant interferon alfa therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. N. Engl. J. Med. 321:1506–1510.
12. **Fried, M. W., M. L. Shiffman, K. R. Reddy, C. Smith, G. Marinos, F. L. Goncales, Jr., D. Haussinger, M. Diago, G. Carosi, D. Dhumeaux, A. Craxi, A. Lin, J. Hoffman, and J. Yu.** 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N. Engl. J. Med. 347:975–982.
13. **Gavier, B., M. A. Martinez-Gonzalez, J. I. Riezu-Boj, J. J. Lasarte, N. Garcia, M. P. Civeira, and J. Prieto.** 1997. Viremia after one month of interferon therapy predicts treatment outcome in patients with chronic hepatitis C. Gastroenterology 113:1647–1653.
14. **Herrmann, E., A. U. Neumann, J. M. Schmidt, and S. Zeuzem.** 2000. Hepatitis C virus kinetics. Antiviral Ther. 5:85–90.
15. **Izopet, J., J. L. Payen, L. Alric, K. Sandres, J. P. Charlet, J. P. Vinel, M. Duffaut, J. P. Pascal, and J. Puel.** 1998. Baseline level and early suppression of serum HCV RNA for predicting sustained complete response to alpha-interferon therapy. J. Med. Virol. 54:86–91.
16. **Jessner, W., M. Gschwantler, P. Steindl-Munda, H. Hofer, T. Watkins-Riedel, F. Wrba, C. Mueller, A. Gangl, and P. Ferenci.** 2001. Primary interferon resistance and treatment response in chronic hepatitis C infection: a pilot study. Lancet 358:1241–1242.
17. **Kagawa, T., K. Hosoi, S. Takashimizu, K. Kawazoe, K. Mochizuki, M. Wasada, N. Nagata, J. Uchiyama, A. Nakano, Y. Nishizaki, N. Watanabe, and S. Matsuzaki.** 1998. Comparison of two interferon alfa treatment regimens characterized by an early virological response in patients with chronic hepatitis C. Am. J. Gastroenterol. 93:192–196.
18. **Karino, Y., J. Toyota, M. Sugawara, K. Higashino, T. Sato, T. Ohmura, T. Suga, Y. Okuuchi, and T. Matsushima.** 1997. Early loss of serum hepatitis C virus RNA can predict a sustained response to interferon therapy in patients with chronic hepatitis C. Am. J. Gastroenterol. 92:61–65.
19. **Kashiwakuma, T., A. Hasegawa, T. Kajita, A. Takata, H. Mori, Y. Ohta, E. Tanaka, K. Kiyosawa, T. Tanaka, S. Tanaka, N. Hattori, and M. Kohara.** 1996. Detection of hepatitis C virus specific core protein in serum of patients by a sensitive fluorescence enzyme immunoassay (FEIA). J. Immunol. Methods 190:79–89.
20. **Lagging, L. M., C. E. Garcia, J. Westin, R. Wejstal, G. Norkrans, A. P. Dhillon, and M. Lindh.** 2002. Comparison of serum hepatitis C virus RNA and core antigen concentrations and determination of whether levels are associated with liver histology or affected by specimen storage time. J. Clin. Microbiol. 40:4224–4229.
21. **Lam, N. P., A. U. Neumann, D. R. Gretch, T. E. Wiley, A. S. Perelson, and T. J. Layden.** 1997. Dose-dependent acute clearance of hepatitis C genotype 1 virus with interferon alfa. Hepatology. 26:226–231.
22. **Layden, J. E., T. J. Layden, K. R. Reddy, R. S. Levy-Drummer, J. Poulakos, and A. U. Neumann.** 2002. First phase viral kinetic parameters as predictors of treatment response and their influence on the second phase viral decline. J. Viral Hepat. 9:340–345.
23. **Lee, W. M., K. R. Reddy, M. J. Tong, M. Black, D. J. van Leeuwen, F. B. Hollinger, K. D. Mullen, N. Pimstone, D. Albert, and S. Gardner for The Consensus Interferon Study Group.** 1998. Early hepatitis C virus-RNA responses predict interferon treatment outcomes in chronic hepatitis C. Hepatology 28:1411–1415.
24. **Lunel, F., P. Veillon, I. Fouchard-Hubert, V. Lonstaud-Ratti, A. Abergel, C. Silvain, H. Rifflet, A. Bianchi, X. Causse, Y. Bacq, and C. Payan.** 2003. Antiviral effect of ribavirin in early non-responders to interferon monotherapy assessed by kinetics of hepatitis C virus RNA and hepatitis C virus core antigen. J. Hepatol. 39:826–33.
25. **Maynard, M., P. Pradat, P. Berthillon, G. Picchio, N. Voirin, M. Marthnot, P. Marcellin, and C. Trepo.** 2003. Clinical relevance of total HCV core antigen testing for hepatitis C monitoring and for predicting patients' response to therapy. J. Viral Hepat. 10:318–323.
26. **McHutchison, J. G., S. C. Gordon, E. R. Schiff, M. L. Shiffman, W. M. Lee, V. K. Rustgi, Z. D. Goodman, M. H. Ling, S. Cort, and J. K. Albrecht for the Hepatitis Interventional Therapy Group.** 1998. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N. Engl. J. Med. 339:1485–1492.
27. **Neumann, A. U., N. P. Lam, H. Dahari, D. R. Gretch, T. E. Wiley, T. J. Layden, and A. S. Perelson.** 1998. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science 282:103–107.
28. **Niederau, C., S. Lange, T. Heintges, A. Erhardt, M. Buschkamp, D. Hurter, M. Nawrocki, L. Kruska, F. Hensel, W. Petry, and D. Haussinger.** 1998. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. Hepatology 28:1687–1695.
29. **Orito, E., M. Mizokami, K. Suzuki, K. Ohba, T. Ohno, M. Mori, K. Hayashi, K. Kato, S. Iino, and J. Y. Lau.** 1995. Loss of serum HCV RNA at week 4