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The SHAP–hyaluronan complex in serum from patients with chronic liver diseases caused by hepatitis virus infection

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Abstract

Our previous study suggested that the serum-derived hyaluronan associated protein (SHAP)–hyaluronan (HA) complex in the sera of patients with rheumatoid arthritis is useful as a marker that directly correlates with the degree of inflammation. Here, we have investigated the serum levels of the SHAP–HA complex in patients at various clinical stages of chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular carcinoma (HCC) caused by infection with the hepatitis C or hepatitis B virus. Both serum levels of the SHAP–HA complex and HA in those patients were significantly higher than those of the controls and increased in the order of CH < LC < HCC. Different from the HA levels, there was a significant difference in the SHAP–HA complex levels between the LC and HCC groups in both HBV- and HCV-infected patients. In addition, the serum level of the SHAP–HA complex correlated with the well-known biomarkers for liver injury and function such as albumin and platelet, including the HCC indicator alpha-fetoprotein. In conclusion, the present data suggest that the SHAP–HA complex level is a better indicator for the progression of the stages of liver fibrosis, and that it could be a marker for HCC, in both HBV- and HCV-infected patients.

Keywords: Hyaluronan; Chronic hepatitis; Liver cirrhosis; Hepatocellular carcinoma; Inter-α-trypsin inhibitor

1. Introduction

Hyaluronan (HA), a high-molecular weight glycosaminoglycan, is an important component of the mammalian extracellular matrix. HA is synthesized by a variety of cell types, and secreted into the extracellular matrices. Some is released from tissues by degradation, circulated into blood, and is rapidly cleared from the blood by HA receptors-bearing hepatic sinusoidal endothelial cells that take up and degrade HA [1–7]. In recent years, HA has received considerable attention because of various biological functions that have been associated with abnormal cell behaviors and impaired extracellular matrix deposition in liver diseases, including chronic and acute hepatitis [8,9]. A significant increase in serum HA

Abbreviations: SHAP, serum-derived hyaluronan associated protein; HA, hyaluronan; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; TSG-6, tumor necrosis factor-stimulated gene-6; HCV, hepatitis C virus; HBV, hepatitis B virus; B-CH, HBV-induced CH; B-LC, HBV-induced LC; B-HCC, HBV-induced HCC; C-LC, HCV-induced LC; C-CH, HCV-induced CH; C-HCC, HCV-induced HCC; ITI, inter-α-trypsin inhibitor; HRP, horseradish peroxidase; BSA, bovine serum albumin; Alb, albumin; ALT, alanine aminotransferase; AFP, α-fetoprotein; Plt, platelet count

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levels has been reported in patients with chronic liver diseases, especially liver cirrhosis. Serum HA levels may be an index of hepatic fibrosis and sinusoidal endothelial cell function [10–14].

A variety of HA-binding proteins have been reported, such as CD44, versican/PG-M, and TSG-6 (tumor necrosis factor-stimulated gene-6 product) [15–18]. Most of them bind to HA non-covalently, but there is only one exception. Serum-derived hyaluronan-associated protein (SHAP) binds to HA through a covalent bond. SHAP was originally isolated from the extracellular matrix of mouse dermal fibroblasts cultured in the presence of serum [19], and was later identified to be the heavy chains of inter-α-trypsin inhibitor (ITI) family molecules, high-molecular weight glycoproteins synthesized in liver and circulated in blood [20–22]. We previously reported that the serum SHAP–HA levels were significantly increased in patients with rheumatoid arthritis and osteoarthritis and that those levels may be an indicator for inflammation or the reparative processes in the arthritic joints [23]. We found that the SHAP–HA complex is essential for the construction of the cumulus oophorus, where the SHAP–HA complex is one of the major extracellular matrix molecules and the defect of the SHAP–HA complex leads to infertility due to incomplete oocyte maturation and ovulation [24]. Further, we have recently found that the mutant mice hardly suffered from type II collagen-induced arthritis [25], suggesting that the SHAP–HA complex may be an extracellular matrix molecule specifically functioning for inflammatory cells and greatly involved in establishing inflammation, although the mechanism is largely unknown [25,26].

Hepatitis B or C virus (HBV or HCV) infection causes slowly progressive inflammation and is often associated with scarring and architectural changes in liver, which is known as chronic hepatitis (CH). When advanced, it leads to liver cirrhosis (LC). HBV or HCV infection also increases the risk of development of hepatocellular carcinoma (HCC), which is one of the most common human cancers that lead to death [27,28].

In the present study, we measured the serum levels of SHAP–HA complex and HA in patients with chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma with infection of either HCV or HBV, and investigated whether there is any significant relationship between the diagnostic states of each disease and the serum SHAP–HA and HA levels.

2. Materials and methods

2.1. Patients

A total of 396 patients diagnosed with HCV- or HBV-related chronic liver disease (46 patients with B-CH, 100 patients with C-CH, 46 patients with B-LC, 104 patients with C-LC, 33 patients with B-HCC, and 67 patients with C-HCC) were investigated in the present study. All the patients and 27 healthy controls were recruited from Aichi Medical University Hospital, Aichi, Japan. The patients positive for both antibodies to HCV by the third-generation enzyme-linked immunosorbent assay (Lumipulse II, Ortho HCV, Ortho-Clinical Diagnostics, Tokyo, Japan) and HCV-RNA in the sera by RT-PCR assay (AMPLICOR HCV Amplification Kit, Roche Diagnostics, Neuilly, France) were diagnosed as having HCV-related liver disease. The patients positive for both the HBV surface antigen (HBsAg) and HBV DNA in the sera were diagnosed as having HBV-related liver disease. The patients were classified into CH or LC according to the histological findings of their liver biopsy specimens in case liver biopsy was performed within the period of 3 years. About 40% of patients with CH and 10% of patients with LC patients were diagnosed histologically. In case liver biopsy could not be carried out because of some reason such as the risk of bleeding, the patients were classified into these groups according to the levels of platelet counts ($<1 \times 10^5/\mu l$). Diagnosis of HCC was made according to the findings of the CT scan and/or angiography. Blood was drawn from the patients and controls after overnight fasting, and, after separation step, sera was stored in −80 °C until the measurement.

Informed consent was obtained from all patients and healthy controls under the conditions that the project was considered to be reasonable by the Committee at Aichi Medical University in terms of the ethical rules.

2.2. Sandwich ELISA for the measurement of SHAP–HA complex levels in sera

The method has been described previously [23,26]. Reagents used were purchased as follows. Rabbit anti-human ITI antibody was from Dako (Glistrup, Denmark); Maxisorp immunoassay plates, Nunc (Roskilde, Denmark); horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody, Jackson Immunoresearch Laboratories (West Grove, PA, USA); bovine serum albumin (BSA), Miles Inc. (Kankakee, IL, USA); TMB solution, Moss Inc. (Pasadena, MD); and the HA-binding region of bovine cartilage aggrecan (HABP), Seikagaku Corporation (Tokyo, Japan). A typical standard curve based on the concentrations of the SHAP–HA complex (ng protein/ml) is shown in Fig. 1A. The value for each sample was the mean of triplicate measurements.

2.3. Competitive inhibition ELISA for the measurement of HA levels in sera

Serum HA levels were measured with an IBA kit (Seikagaku Corp., Tokyo, Japan) according to the manufacturer's directions. The principle of this method is as follows: the plates are coated with BSA-conjugated HA, which competes with the soluble HA in the sample solution for the binding of the biotinylated HABP. The amount of biotinylated HABP bound to the immobilized HA was measured by using HRP-conjugated streptavidin and a subsequent color reaction, and was plotted against the HA concentrations in the standard sample solutions to give the standard curve (Fig. 1B), from

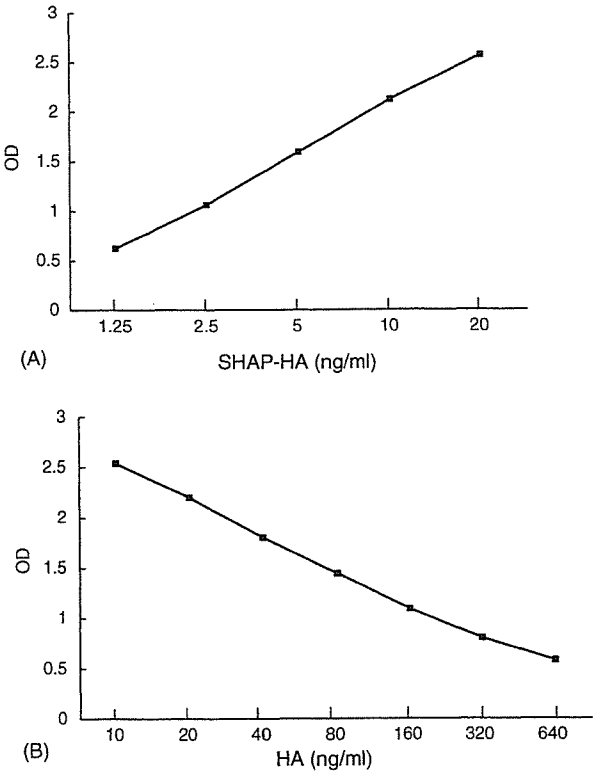

Fig. 1. Representative standard curves of the sandwich ELISA for serum SHAP–HA (A) and competitive inhibition ELISA for serum HA (B). Concentration of SHAP–HA standards (ng/ml) is expressed as the weight content of SHAP protein. Absorbance at 450 nm (OD) at each point is the mean of triplicate measurements, and the deviation of the mean absorbance value for each point was <8%.

which the HA concentrations in the patient samples were calculated. This method detects not only high-molecular weight HA but also low molecular weight HA, in contrast to the sandwich ELISA method that only detects high-molecular weight HA. The value for each sample was the mean of triplicate measurements.

2.4. Serum biochemical markers

The serum levels of albumin (Alb), alanine aminotransferase (ALT), α-fetoprotein (AFP), and platelet (Plt) were measured as diagnostic markers at the Aichi Medical University Hospital. The Alb levels in patient sera are considered to be a marker to evaluate and grade liver function. The ALT levels are known to reflect the degree of liver cell damage. The serum AFP levels are well known to be a major tumor indicator to evaluate HCC. Those marker levels in sera are not available for all the patients whose sera were served for the measurement of SHAP–HA complex and HA levels. Therefore, numbers (n) of the patients for the correlation analysis on the serum levels of SHAP–HA and HA and other biochemical parameters were indicated in the respective tables.

2.5. Statistical analysis

Values were expressed as means ± S.E.M. Statistical analysis of the differences in the serum SHAP–HA and HA levels of HBV- and HCV-infected patients among CH, LC, and HCC was performed by the Steel–Dwass method using Kyplot software, which is the one for multiple comparisons of nonparametric variables. Statistical analysis of the differences in the clinical characteristics of HBV- and HCV-infected patients among CH, LC, and HCC was also performed by the Steel method using Kyplot software. Statistical comparisons of the serum SHAP–HA and HA levels of CH, LC, and HCC between HBV and HCV-infected patients were made using the Mann–Whitney U-test. Correlations of serum levels of SHAP–HA and HA and other biochemical parameters were tested using Pearson's correlation coefficient. Probability values (p) ≤ 0.05 were considered significant.

3. Results

3.1. Clinical characteristics of the patients

Clinical characteristics of the patients were shown in Table 1. Patients at LC stage were older than those at CH stage, and those with HCC were older than those at CH and LC stage, regardless of the etiology of liver disease. Albumin, total cholesterol (T. Chol), and triglycerides (TG), which are considered to be markers to evaluate and grade liver function were decreased in parallel with the progression of the disease. Platelet count (Plt), which is considered to correlate with the degree of liver fibrosis, was also decreased in parallel with the progression of the disease. There was no significant difference in ALT levels between CH, LC, and HCC stages, which are known to reflect the degree of liver cell damage.

3.2. Serum levels of the SHAP–HA complex in patients with chronic liver diseases

We first examined the serum SHAP–HA levels in each group of patients (Fig. 2). The serum SHAP–HA levels in patients with B-CH (29 ± 18 ng/ml), B-LC (60 ± 56 ng/ml), and B-HCC (101 ± 77 ng/ml) were significantly higher than those of the controls (14.1 ± 2.7 ng/ml) ($p < 0.01$ in all cases). The SHAP–HA levels in the B-LC group were significantly higher than those in the B-CH group ($p < 0.01$), and those in B-HCC were further significantly higher than those in the B-LC group ($p < 0.01$). The serum SHAP–HA levels in patients with C-CH (36 ± 25 ng/ml), C-LC (62 ± 48 ng/ml), and C-HCC (110 ± 89 ng/ml) were also significantly higher than those of controls ($p < 0.01$ in all cases). The SHAP–HA levels in the C-LC group were significantly increased when compared with those in the C-CH group ($p < 0.01$). Similarly, the patients with C-HCC showed further higher SHAP–HA levels than those with C-LC ($p < 0.01$). A comparison among the SHAP–HA levels in those patients indicated that the lev-

Table 1
Clinical characteristics of the patients with chronic liver disease

	HBV-related			HCV-related		
	CH	LC	HCC	CH	LC	HCC
M : F	31 : 15	35 : 11	20 : 13	58 : 42	52 : 52	42 : 25
Age (years)	42±13	55±10	59±7	56±11	62±12	68±7
ALT (IU/ml)	55±50	38±26	53±49	68±78	67±40	63±32
Albumin (mg/dl)	4.3±0.5	4.2±0.5	3.9±0.7	4.4±0.3	4.0±0.5	3.7±0.5
T. Chol (mg/dl)	184±25	190±33	143±34	181±33	136±42	142±37
TG (mg/dl)	108±74	100±39	85±39	127±53	99±36	101±49
Hb (g/dl)	14.0±1.4	14.4±0.3	13.1±1.5	14.1±1.5	13.3±2.1	12.4±1.8
Plt (x10⁴/μl)	16.2±5.5	12.4±4.1	9.0±3.7	18.6±5.7	10.4±4.1	9.5±4.3

ALT, alanine aminotransferase; T. Chol, total cholesterol; TG, triglycerides; Hb, hemoglobin; Plt, platelets.
$^{*}p<0.05$, $^{**}p<0.01$.

els of SHAP–HA in the HCV-groups had a tendency to be higher than those in the HBV groups, especially at the CH stage ($p=0.05$).

Sera of patients infected with HCV or HBV have tendency that albumin, cholesterol, platelets, etc. were lowered (see Table 1). Because the patients with HCC would have problem with the lowering liver functions (see the details in the latter Section 3.5). Therefore, the increased levels of the SHAP–HA complex in sera of those patients would not simply reflect the carcinogenesis. To clarify those points, we examined and compared the serum levels of the SHAP–HA complex between the patients with HCC ($n=31$) and those with LC ($n=30$) whose albumin concentrations in sera were both higher than 4.0 g/dl. The levels showed 75.6 ± 60.4 and 47.9 ± 32.6 ng/ml, respectively, and were significantly high in sera of the HCC, compared with those of the LC ($p=0.03$). Similarly, the patients with HCC ($n=38$) and those with LC ($n=19$) whose platelet numbers in blood were at the same range (5–12 × 10^4/μl) showed the SHAP–HA complex levels of 97.8 ± 71.3 and 61.5 ± 32.4 ng/ml, respectively ($p=0.04$). Even when some of the serum conditions were similar to each other, the SHAP–HA complex levels in sera of HCC were still higher than those in sera of LC. It is very likely, therefore, that the increased levels of the SHAP–HA complex in sera could reflect the carcinogenesis.

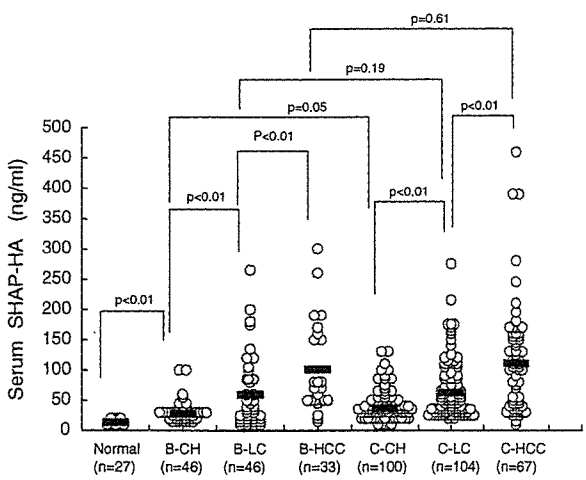

Fig. 2. Serum levels of the SHAP–HA complex in patients with B-CH, B-LC, B-HCC, C-HC, C-LC and C-HCC, and in healthy controls. The concentrations are measured by sandwich ELISA. The horizontal bars denote the mean value in each group. Standard deviations of these mean values are described in the text. The p-values between the HBV- and HCV-groups were obtained by the Mann–Whitney's test, and those among HC, LC, and HCC groups were obtained by the Steel–Dwass method using Kyplot software for multiple comparisons.

3.3. Serum levels of HA in patients with chronic liver diseases

We then examined the serum HA levels in each group of patients (Fig. 3). The serum HA levels were 228 ± 229 ng/ml in the B-CH group, 841 ± 811 ng/ml in the B-LC group, and 1170 ± 1436 ng/ml in the B-HCC group, all being significantly higher than those of the controls (52.4 ± 10.5 ng/ml) ($p<0.01$ in all cases). The serum HA levels in the patients with C-CH (403 ± 485 ng/ml), C-LC (1280 ± 1421 ng/ml), and C-HCC (1374 ± 1 364 ng/ml) were also significantly higher than those of the controls ($p<0.01$ in all cases). In both the HBV- and HCV-groups, the HA levels in the LC patients were significantly higher than those in the CH group (both $p<0.01$). However, in contrast to the SHAP–HA levels,

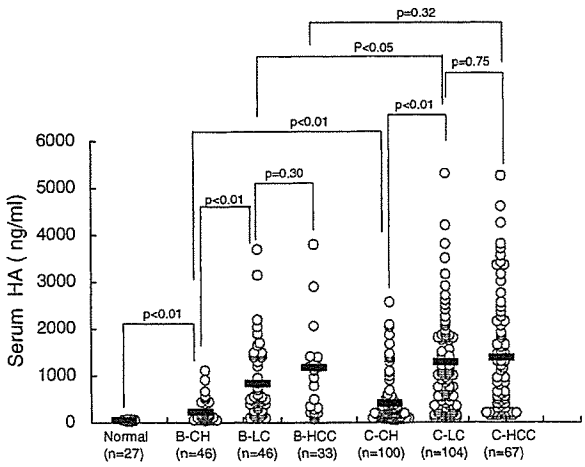

Fig. 3. Serum levels of HA in patients with B-CH, B-LC, B-HCC, C-HC, C-LC and C-HCC, and in healthy controls. The concentrations were measured with competitive inhibition ELISA. The horizontal bars denote the mean value in each group. Standard deviations of these mean values are described in the text. The p-values between the HBV- and HCV-groups were obtained by the Mann–Whitney's test, and those among HC, LC, and HCC groups were obtained by the Steel–Dwass method using Kyplot software for multiple comparisons.

there was no further significant increase of serum HA levels in the HCC groups ($p = 0.30$ and 0.75 in HBV- and HCV-groups, respectively). When a comparison between the HBV- and HCV-groups was made, HCV-infected patients showed significantly higher serum HA levels at the CH and LC stages (C-CH versus B-CH, $p < 0.01$; C-LC versus B-LC, $p < 0.05$), but not at the HCC stage (C-HCC versus B-HCC, $p = 0.32$).

3.4. Positive correlations between serum levels of SHAP–HA complex and HA in chronic liver diseases

We further investigated whether there are any correlations between the serum levels of the SHAP–HA complex and HA at each stage of chronic liver disease (Table 2). Significant positive correlations were found between the levels of the SHAP–HA complex and HA at all stages in both HBV- and HCV-groups. The r-value was highest in C-HCC ($r = 0.84$) and was lowest in C-CH ($r = 0.41$). Thus the r-value varied according to the status of the liver disease, suggesting the difference in the mechanisms in the formation of SHAP–HA complex. No significant correlation was found in the healthy control groups.

Table 2
Correlation between serum levels of SHAP–HA complex and HA

Samples	n	r	p-Value
Control	27	0.20	NS
B-CH	46	0.67	<0.0001
B-LC	46	0.73	<0.0001
B-HCC	33	0.67	<0.0001
C-CH	100	0.41	<0.0001
C-LC	104	0.70	<0.0001
C-HCC	67	0.84	<0.0001

The null hyaluronan, $r = 0$, against the alternative $r \neq 0$. NS: not significant.

The serum levels of the SHAP–HA complex and HA at each stage of chronic liver disease were also comparatively investigated. Averaged ratios of the SHAP–HA complex to HA was 0.095 ± 0.094 and 0.186 ± 0.273 for C-LC and for C-HCC, respectively, and 0.103 ± 0.075 and 0.184 ± 0.194 for B-LC and for B-HCC, respectively, although statistical significant values given by the Mann–Whitney U-test were $p < 0.05$ and $p = 0.135$ for the former and latter comparisons, respectively, and the latter high value (i.e. low significance) was probably due to the less sample number ($n = 33$). Thus, it is possible to consider ratios of the SHAP–HA to HA levels as well as their respective levels as diagnostic and prognostic indexes to monitor trends of changes in the disease state from LC to HCC.

3.5. Correlations of the serum SHAP–HA and HA levels with other biochemical parameters in patients with chronic liver diseases

The correlations of serum levels of the SHAP–HA complex and HA in each disease stage with other biochemical parameters were investigated (Tables 3 and 4). In HBV-related chronic liver diseases, significant correlations were found between the serum levels of HA, Alb, and Plt, at the CH stage. This was also the case for the serum levels of the SHAP–HA complex. At the LC stage, only the serum levels of SHAP–HA complex but not HA correlated with the levels of Alb and Plt. At the HCC stage, there was neither correlation between the serum levels of the HA and these parameters, nor

Table 3
Correlation between serum levels of HA/SHAP–HA complex and clinical variables in patients with HBV-related chronic liver diseases

	CH ($n = 46$)				LC ($n = 46$)				HCC ($n = 33$)			
	HA		SHAP–HA		HA		SHAP–HA		HA		SHAP–HA	
	r*	p**	r	p	r	p	r	p	r	p	r	p
Alb	−0.56	<0.01	−0.60	<0.01	−0.40	NS	−0.57	<0.01	−0.29	NS	−0.49	NS
ALT	0.29	NS	0.22	NS	0.35	NS	0.20	NS	0.06	NS	−0.16	NS
Plt	−0.55	<0.05	−0.58	<0.05	−0.37	NS	−0.64	<0.05	0.22	NS	0.21	NS

* r-Value.
** p-Value.

Table 4
Correlation between serum levels of HA/SHAP–HA complex and clinical variables in patients with HCV-related chronic liver diseases

	CH ($n=100$)				LC ($n=104$)				HCC ($n=67$)			
	HA		SHAP–HA		HA		SHAP–HA		HA		SHAP–HA	
	r^*	p^{**}	r	p	r	p	r	p	r	p	r	p
Alb	−0.37	<0.01	−0.39	<0.01	−0.09	NS	−0.44	<0.05	−0.21	NS	−0.37	<0.01
ALT	0.02	NS	0.08	NS	0.04	NS	0.04	NS	0.32	<0.05	0.33	<0.05
Plt	−0.05	NS	−0.44	<0.01	−0.18	NS	−0.45	<0.05	−0.17	NS	−0.24	NS

* r-Value.
** p-Value.

Table 5
Correlation of serum levels of SHAP–HA and HA with levels of AFP in patients bearing HCC

	HA		SHAP–HA	
	r-Value	p-Value	r-Value	p-Value
B-HCC ($n=33$)	−0.02	NS	0.63	<0.05
C-HCC ($n=67$)	0.07	NS	0.31	<0.05

between the serum SHAP–HA levels and these parameters. On the other hand, in HCV-related chronic liver diseases, the serum levels of the SHAP–HA complex correlated significantly with the levels of Alb and Plt at the CH stage, while serum HA levels correlated only with serum levels of Alb. At the LC stage, serum levels of the SHAP–HA complex still had a significant correlation with the levels of Alb and Plt. At the HCC stage, serum levels of both HA and SHAP–HA complex correlated with that of ALT, and the serum SHAP–HA levels still had a significant correlation with the levels of Alb.

3.6. Correlations of the serum SHAP–HA and HA levels with AFP levels in patients bearing HCC

Since the patients with HCC showed further higher serum SHAP–HA levels than those with LC regardless of the difference of viruses infected, we investigated whether the serum levels of SHAP–HA complex correlate with AFP, one of the well-known marker for HCC. Serum levels of SHAP–HA complex had a significant correlation with AFP levels, but those of HA did not (Table 5).

4. Discussion

The SHAP–HA complex is the modified form of the HA molecule. In contrast to the daily turnover of considerable amounts of HA, the SHAP–HA complex apparently occurs only under unusual circumstances, especially during inflammatory responses. It is already known that this situation occurs in the inflamed synovia of rheumatoid arthritis patients [23,26]. We have shown that the formation of the SHAP–HA complex requires, in addition to HA and ITI family molecules as substrates, enzyme factor(s) that are also present in plasma [19,24]. The enzyme factors catalyze the transfer of the heavy chains from the chondroitin sulfate chain of the bikunin portion to HA, accompanied by the release of bikunin [24,25].

Considering the above characteristics of the SHAP–HA complex formation, the following circumstances could give rise to the high levels of the complex in sera from patients with the liver diseases. An abnormally high amount of HA is synthesized and accumulates in tissues of the diseased liver such as LC and HCC [29,30], and there are the abnormal development and organization of blood circulation systems [31]. Such conditions make it possible to supply the ITI family molecules and enzyme factor(s) directly to the tissue HA. Consequently, the abnormal formation and accumulation of the SHAP–HA complex likely occur within the diseased liver and some of the complex leaks into the blood circulation. Under normal conditions, HA is released from tissues into the circulation and cleared in the liver. Although the HA encounters the ITI family molecules and enzyme factor(s) in the circulation, the formation reaction proceeds slowly [20,24] and the clearance rate of the HA is too fast to receive the modification to form the SHAP–HA complex [4]. However, when the liver function is impaired in the diseased liver, the degradation of HA slows down, which results in the accumulation of HA. Consequently, the HA has more chance of undergoing the modification.

Marked increases of serum HA levels in chronic liver disease have been reported before [8,12–14,32–39]. Several studies have evaluated the measurement of the serum HA level as a useful indicator for assessing the severity of liver injury and fibrosis [3,12,32–34]. McHutchison et al. [12] reported not only significant elevation of the serum HA levels in patients with C-CH but also a correlation with the degree of fibrosis in the liver. Pontinha et al. [8] confirmed that the serum HA levels in patients with B-CH are useful for assessing and monitoring time trends in the disease state. Das et al. [31] reported that the serum HA levels are useful to estimate the functional hepatic reserve as preoperative risk factors in patients with LC. Frebourg et al. [34] suggested that the increase of serum HA levels in chronic liver disease is due to the excessive production of HA, while Engstrom et al. [35] claimed that liver fibrosis changes the tissue structures which cause a reduction in the HA uptake and degradation activity in hepatic sinusoidal endothelial cells. Although it is still under question as to which possibility is more likely,

increased levels of HA in the sera have an effect on the serum levels of the SHAP–HA complex.

The major aim of this study is to examine the change of serum levels of the SHAP–HA complex and, if any, the correlations with those of HA along with the progression of hepatitis virus infection-induced chronic liver diseases (from CH to LC, then to HCC), and explore its potential to serve as a marker for assessing the severity of liver injury and fibrosis.

Our present results of the serum HA levels confirmed the significant elevation in the patients with virus-induced chronic liver diseases (Fig. 3). As expected, elevation was also found in the serum levels of the SHAP–HA complex (Fig. 2), and it showed significant but different correlation with the HA levels at each stage of chronic liver disease (Table 2), which supports our hypothesis that the complex formation depends on the availability of HA and also suggests that the serum SHAP–HA levels are more preferential as a marker for the diagnosis of chronic liver disease as discussed below.

In both HBV- and HCV-infected patients, a significant elevation of serum HA levels was found compared with the healthy controls and CH group, more strikingly between the CH and LC groups, but not between the LC and HCC groups, indicating that the serum HA level reflects the inflammatory and cirrhotic processes, but not the tumorigenic process (Fig. 3). In contrast, there is a significant elevation of the serum levels of the SHAP–HA complex between the LC and HCC groups (Fig. 2). The finding suggests that the SHAP–HA complex levels relate more closely than the HA levels to the tumorigenic progress. This is also confirmed in Table 5 to show significant correlation of the serum AFP levels with the serum SHAP–HA levels but not with the serum HA levels. Recent reports have shown that the serum level of PIVKA-II (protein induced by Vitamin K absence or antagonists II)/DCP (des-γ-carboxy prothrombin) is the more useful predisposing clinical parameter than that of AFP for the development of portal venous invasion in patients with HCC [40,41]. Therefore, it should be worthy examining the correlation between DCP and the SHAP–HA complex levels in sera of those patients. In relation to the increase of the SHAP–HA complex levels, TSG-6 has recently been observed to enhance the formation reaction or be itself involved in the reaction as an enzyme [42,43]. We previously observed that PG-M/versican enhanced the formation reaction in vitro by binding to both ITI and HA to form a tertiary complex where the transesterification reaction may be accelerated [44,45]. PG-M/versican is often found as a component of the tumorigenic extracellular matrix [46]. Therefore, those factors may contribute to the increase in the efficiency of the formation of the complex and result in a higher ratio of the SHAP–HA complex to HA as seen in the case of HCC. Thus, it is possible to consider ratios of the SHAP–HA to HA levels as well as their respective levels as diagnostic and prognostic indexes to monitor trends of changes in the disease state from LC to HCC.

We then investigated the correlation of the serum levels of the SHAP–HA complex and HA with other biomarkers which represent liver functions and injury. Both the levels negatively correlate with Alb and Plt in the most serum samples, indicating that the serum levels of the SHAP–HA complex and HA do reflect the decreased liver function and degree of liver fibrosis. It is of note that a better correlation with these biomarkers was frequently found in the serum levels of the SHAP–HA complex than in the HA levels, particularly in LC stage. Interestingly, no significant correlation was found between these biomarkers and serum levels of SHAP–HA complex in B-HCC group, whereas both the serum levels of HA and SHAP–HA had a weak correlation with ALT levels in C-HCC. A different panel of correlations with the biomarkers observed between the HBV- and HCV-infected patients may be explained at the present time by possible differences in the mechanisms and processes for the infection and the induction of liver diseases between hepatitis C and B viruses.

Although we have shown the positive correlation between the SHAP–HA complex level and the hepatocellular carcinogenesis, sera of patients infected with HCV or HBV have significant tendency that albumin, cholesterol, platelets, etc. were lowered as shown in Table 1. Those would have been caused by the lowering liver functions and the decrease of the platelets of the patients with HCC. Therefore, the increased levels of the SHAP–HA complex in sera of those patients might not simply reflect the carcinogenesis. However, even if we compared the serum levels of the SHAP–HA complex between the patients with HCC and those with LC whose albumin concentrations in sera were above 4.0 g/dl and also whose platelet numbers in blood were almost at the same range, the SHAP–HA complex levels in sera of HCC were still higher than those in sera of LC. Thus, we could consider that the increased levels of the SHAP–HA complex in sera could reflect the carcinogenesis. If we had the patients with the progression from LC to HCC, it should be worthy to follow-up the changes of the SHAP–HA complex levels during the processes.

Considering those results together, the serum levels of the SHAP–HA complex may be a useful index not only for monitoring functional changes of the liver in patients with chronic hepatitis and liver cirrhosis, but also for the diagnosis of progression to hepatocellular carcinoma, where it is superior to using the serum level of HA.

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Alteration of serum cytokine balances among different phases of chronic hepatitis B virus infection

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Abstract

To understand the pathogenesis of chronic hepatitis B virus (HBV) infection, we examined the serum levels of IL-10, TNF-α IL-12 p70, and IL-12 p40 in 77 patients chronically infected with HBV and 19 controls. The patients were classified into four groups: asymptomatic carriers (ASC), patients with chronic hepatitis (CH), patients with liver cirrhosis (LC), and patients with hepatocellular carcinomas (HCC). The cytokine values among these groups were compared and their relations to clinical parameters were investigated. All these cytokine values were higher in the patient groups than in controls. IL-10 and TNF-α became higher in accordance with the progress of the disease phases, from ASC to LC, and lowest when the patients had HCC. IL-12 p40 was also lowest in HCC, however, the group with highest levels was CH. IL-12 p70 was unchanged among ASC, CH, and LC, but were raised in HCC. Serial analyses for the cytokine values in the same patients showed the similar tendencies. Regression analysis showed the significant correlations between ALT and IL-10. Serum cytokine values well reflected the pathological differences of the individual disease phases, and may become useful indices to understand the pathogenesis of chronic HBV infection.

Keywords: HBV; IL-10; IL-12 p70; IL-12 p40; TNF-α

1. Introduction

Hepatitis B virus (HBV) is an infectious agent, causing chronic infection, leading infected patients to various clinical conditions from asymptomatic status to liver cirrhosis, and sometimes to the condition complicating hepatocellular carcinoma (HCC). Since HBV is a noncytolytic virus, the most part of the pathology in HBV infection is thought to be mediated by the host immune response against the viral products [1–3].

Cytokines are produced by the various cell types including lymphocytes, macrophages, dendritic cells (DC), etc., and have been claimed to play pivotal roles in the pathogenesis of chronic HBV infection [1,4]. Inflammation in HBV-infected liver is proved to be mediated by cytokines, like IFN-γ and TNF-α, and the elimination of the virus is also caused by these cytokines [1,5–9]. Fibrosis in the liver is thought to be caused or accelerated by the cytokines, like TGF-β [10,11], and development or enlargement of HCC is suggested to be accompanied by the abnormality in immunity like cytokine profiles [12–16]. As mentioned, cytokines are believed to be important factors that organize pathology of the disease, however, the relation of cytokine levels to sequence of the disease phases or clinical factors are not well understood.

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In the present study, to elucidate the detailed roles of cytokines on the disease pathogenesis of HBV infection, we measured IFN-γ, IL-2, IL-4, IL-10, IL-12 p40, IL-12 p70, TNF-α, and TGF-β. IL-12 is known to be composed of two chains, a heavy chain (p40), and a light chain (p35), forming a disulfide-linked heterodimer (p70), and IL-12 p40 is thought to be a negative regulator of IL-12 p70, an active form of IL-12 [17]. Serum levels of these cytokines were measured in the chronically HBV-infected patients with various disease phases. They were compared among patient groups divided by the disease phases, and their relations to clinical parameters were investigated. The results suggested the deep association of these cytokines to inflammation, viral load, viral antigen load, fibrotic changes of the liver, and the complication of HCC in chronic HBV infection as described below.

2. Materials and methods

2.1. Patients and control subjects

Seventy-seven patients with chronic HBV infection and 19 healthy control individuals were tested for their serum levels of cytokines. Diagnosis for chronic HBV infection was done by detecting serum HBsAg and HBcAb. Serum ALT (IU/l), HBV-DNA (pg/ml), HBeAg titer (cut off index), and HBeAb titer (percent inhibition) were also measured in these patients. According to the activity of inflammation and the stage of fibrosis, patients were subdivided to four groups: ASC—asymptomatic carriers, CH—the patients with chronic hepatitis, LC—the patients with liver cirrhosis, and HCC—the patients with hepatocellular carcinoma. The patients consisted of 23 ASC, 34 CH, 12 LC, and 8 HCC (Table 1a). The diagnosis of each patient category was made by assessing the changes of biochemical tests, such as serum aminotransferase levels, and/or analyzing the shape of liver and spleen by ultrasonography (US) or computed tomography (CT), and/or examining the histology by liver biopsy. The diagnosis of ASC was made when the serum aminotransferase levels of the patients were persistently normal at least for six months. When the patients showed abnormalities in serum aminotransferase levels persistently or at least in two different time points during six months period, they were diagnosed as CH. Liver biopsies were done in 24 patients, and 22 of them were proved to have CH, 2 of them were diagnosed as LC. The extent of inflammation and fibrosis in the liver were evaluated according to the criteria suggested by Ludwig [18] and Ichida et al. [19] (Table 1b). The diagnosis of LC was made by the clinical parameters (albumin, prothrombin time, platelet count, etc.), morphological changes of liver and spleen (by US or CT), and clinical manifestation (esophageal varices, jaundice, acites, etc.), except for two cases by liver biopsies. The occurrence of HCC was diagnosed by US or CT, then, the tumor stages were classified by the TNM staging system suggested by the Liver Cancer Study Group of Japan (Table 1c). All had liver cirrhosis as background liver disease. Two of eight patients were operated, and both were diagnosed as moderately differentiated hepatocellular carcinoma. In the case of CH and LC, the blood was collected when the antiviral drugs, such as interferon or lamivudine were not used. In the case of HCC, the blood was drawn before the treatment for HCC had started. In the patients whose sera were available, the serum cytokine values at different phases of the

Table 1b
Histological findings in patients with chronic hepatitis

	Mild	Moderate	Severe
Grade[a]	4	14	4
Stage[b]	5	10	7

Liver biopsies were executed in 22 of 34 patients with chronic hepatitis. Histological findings were evaluated according to the criteria suggested by Ludwig [18] and Ichida et al. [19].

[a] Grade, activity of necro-inflammatory reaction in the liver; mild, mild limiting plate necrosis and/or focal lobular necrosis; moderate, moderate limiting plate necrosis and/or severe focal cell damage; severe, severe limiting plate necrosis and/or bridging necrosis.

[b] Stage, extent of liver fibrosis; mild, fibrous portal expansion; moderate, bridging fibrosis but intact architecture; severe, bridging fibrosis with architectural distortion. Numbers listed are the numbers of the patients categorized to each grade or stage.

Table 1a
Profiles of the patients with chronic HBV infection and the control subjects

	Control ($n = 19$)[a]	ASC[b] ($n = 23$)	CH[c] ($n = 34$)	LC[d] ($n = 12$)	HCC[e] ($n = 8$)
Age (years)	34 ± 2	34 ± 3	37 ± 2	53 ± 2	56 ± 3*
Gender (M/F)	13/6	10/13	19/15	10/2	6/2
ALT[f] (IU/l)	–	23 ± 1	153 ± 11	104 ± 47	61 ± 12*
$HBeAg^+/HBeAb^+$	–	9/14	22/12	6/6	2/6
HBV-DNA (pg/ml)	–	417 ± 193	783 ± 287	67 ± 48	2 ± 1

All the data are expressed as mean ± standard errors (S.E.).

[a] n, number of the patients.

[b] ASC: asymptomatic HBV carrier with persistently normal serum aminotransferase levels.

[c] CH: patient with chronic active hepatitis.

[d] LC: patient with liver cirrhosis.

[e] HCC: patients with hepatocellular carcinoma.

[f] ALT: alanine aminotransferase (normal range: <45 IU/l).

* $p < 0.001$ (ANOVA).

Table 1c
TNM stages of hepatocellular carcinomas

TNM stage	Numbers of the patients
I	2
II	5
III	1

Patients with hepatocellular carcinoma were classified by TNM staging system suggested by the Liver Cancer Study Group of Japan. Numbers indicated are the numbers of the patients categorized to each stage.

disease were analyzed (ASC and CH: 6 patients; CH and LC: 3 patients; LC and HCC: 6 patients). All the healthy control subjects were negative for hepatitis viral markers including HBsAg, HBcAb, and anti-HCV antibody. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the human research committee of Aichi Medical University School of Medicine. The profiles of the patients were summarized in Tables 1a–1c.

2.2. Detection of cytokines

Blood samples were collected under informed consent, and sera were stored at −80 °C until the measurement of cytokines. Commercially available ELISA kits (IFN-γ, IL-12 p70 + p40, and TGF-β by Amersham Pharmacia Biotech, U.S.A., IL-2, IL-4, IL-10, IL-12 p70, and TNF-α by BD Biosciences Pharmingen, U.S.A.) were used for the detection of serum cytokines, and assays were done according to the manufacturer's instruction. Briefly, the serum samples thawed just before the assay were applied to assay plate (96-well plate) that was precoated with monoclonal antibody (first antibody) for each cytokine. Then the plate was incubated at room temperature for 2–4 h, washed three times with 0.05% Tween 20 in PBS, and another monoclonal antibody for each cytokine conjugated with horseradish-peroxidase (second antibody) was applied to the wells. After 2 h incubation at room temperature, the plates were washed three times, and the enzyme reaction was performed with tetramethylbenzidine dissolved in substrate buffer and 33% H_2O_2. After 30 min of incubation at room temperature, the reaction was stopped with 1.8 M H_2SO_4, and the light absorbance at 450 nm was measured with an ELISA spectrophotometer. IL-12 p40 levels were calculated by subtracting the values of IL-12 p70 from the values detected by the kit for IL-12 p40 + p70. The assays for all the cytokines were done simultaneously to avoid frequent freeze-thawing of the serum samples.

2.3. Statistics

The data were presented as means ± standard errors (S.E.). The differences in clinical parameters among the patient groups and control group were analyzed by one-way analysis of variance between groups (ANOVA) or chi-square for independence test. The relationship of the cytokine levels among the controls and patient groups were analyzed by Student's *t*-test, paired *t*-test, and ANOVA. The regression analysis with clinical parameters and cytokine values were done using commercially available JMP software by SAS Institute Inc., U.S.A. The *p*-value below 0.05 was regarded as statistically significant.

3. Results

3.1. Clinical parameters in patient groups and control group

Since the patient groups were subdivided by the disease phases that show different activities of inflammation and the different fibrotic changes of the liver, there were significant differences in ALT levels and ages among the groups ($p < 0.001$, respectively, by ANOVA). The ages were almost equivalent among control, ASC, and CH, and higher in LC and HCC. The ages between the latter two groups were almost equivalent. Balances of gender and $HBeAg^+/HBeAb^+$ status were not significantly different among groups (by chi-square for independence test, Table 1a).

3.2. Serum cytokine levels of the patients and controls

Since serum levels of IL-2, IL-4, and IFN-γ were almost always under detection levels both in the patient groups and the controls, they were excluded from the analysis. Serum TGF-β levels were measured in the limited number of the patients, however, they were also excluded from the detailed analysis because of the instability of the data. The use of sera instead of plasma and the condition of preparing sera might cause the problem.

As shown in Fig. 1, all the detected cytokine values (IL-10, TNF-α, IL-12 p70, and IL-12 p40) in the patient groups were higher compared to those in the healthy controls.

IL-10 levels got higher as the disease phases progressed from ASC to LC (ASC: 11.2 ± 0.9 pg/ml; CH: 14.2 ± 0.9 pg/ml; LC: 17.0 ± 2.5 pg/ml; mean ± S.E., respectively), and declined when the patients complicated with HCC (9.4 ± 0.7 pg/ml). TNF-α levels showed similar tendency. They were lowest in HCC (89 ± 20 pg/ml), but in the patients without HCC, they went up with the advancement of the disease phases (ASC: 168 ± 37 pg/ml; CH: 193 ± 20 pg/ml; LC: 268 ± 39 pg/ml). IL-12 p40 levels were also lowest in HCC (89 ± 28 pg/ml), however, the group that had highest levels of the cytokine was CH (ASC: 138 ± 28 pg/ml; CH: 166 ± 31 pg/ml; LC: 115 ± 16 pg/ml). IL-12 p70 levels showed different tendency compared to the other cytokines. The changes of IL-12 p70 among the patient groups were not obvious, while their levels in HCC were tended to be higher than the other groups (ASC: 5.8 ± 1.4 pg/ml; CH: 6.2 ± 0.3 pg/ml; LC: 6.2 ± 0.3 pg/ml; HCC: 7.8 ± 0.6 pg/ml).

By ANOVA, significant differences in the individual cytokine levels were observed among all the groups including control group, and also among the patient groups, except in IL-12 p40 among the patient groups (Fig. 1).

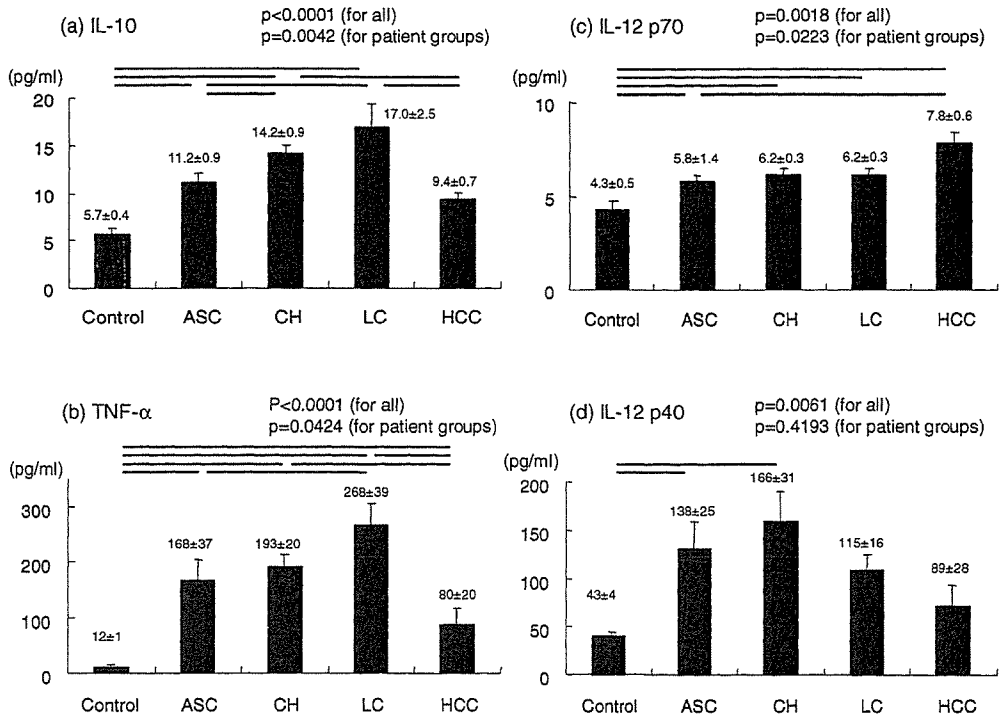

Fig. 1. Serum cytokine levels of the patient groups with chronic HBV infection. Serum cytokine levels in controls and patients were measured and calculated as described in Section 2. Numbers at the upper part of the vertical bars indicate mean ± standard error (S.E.) of the cytokine values in the individual groups. Statistical analysis was done using ANOVA and unpaired Student's *t*-test. *p*-Values calculated by ANOVA are described on top of the individual graphs. Horizontal bars on the upper part of the graphs indicate the existence of significant differences between the groups of both ends of the bars by *t*-test ($p < 0.05$). ASC, asymptomatic carriers with persistently normal serum aminotransferase levels; CH, patients with chronic active hepatitis; LC, patients with liver cirrhosis; HCC, patients with hepatocellular carcinomas.

3.3. Influence of HBeAg⁺/HBeAb⁺ status on cytokine balances

To evaluate the influence of HBeAg⁺/HBAb⁺ status on cytokine values and clinical parameters, the patients were subdivided into HBeAg⁺ and HBeAb⁺ within each patient group.

In ASC, the patients with HBeAg⁺ were younger than the patients with HBeAb⁺ ($p = 0.0468$), and HBV-DNA levels of HBeAg⁺ group were higher than those of HBeAb⁺ group ($p = 0.0013$). With regard to cytokine values, TNF-α levels of HBeAg⁺ group were significantly higher than those of HBeAb⁺ group ($p = 0.0231$). IL-12 p40 levels showed similar tendency but without statistical significance ($p = 0.0786$, Table 2).

Different from ASC, the ages of the patients were almost equivalent between HBeAg⁺ and HBeAb⁺ in CH. However, HBV-DNA levels of HBeAg⁺ group were higher than those of HBeAb⁺ group ($p = 0.0478$). Although none of cytokine values showed significantly differences between two groups, TNF-α and IL-12 p40 levels seemed to be high in HBeAg⁺ group (Table 3). These results may suggest that HBeAg⁺/HBAb⁺ status and/or the amount of HBV-DNA influence the serum levels of these two cytokines in ASC and CH.

There were no statistical differences in clinical parameters and cytokine values between HBeAg⁺ and HBeAb⁺ when analyzed in LC and HCC (data not shown).

3.4. Influence of fibrotic changes of the liver on cytokine balances

The patients who received liver biopsy were subdivided according to the extent of fibrosis. Then, the influence of liver fibrosis on cytokine values was evaluated among these groups and the group with LC. The significant difference was seen

Table 2
Comparison of clinical parameters and cytokine profiles between HBeAg⁺ and HBeAb⁺ group in asymptomatic carriers

Parameters	HBeAg⁺ ($n = 8$)[a]	HBeAb⁺ ($n = 15$)	*p*-Value
Age (years)	27 ± 4	39 ± 3	0.0468
ALT (IU/l)	26 ± 1	22 ± 1	0.0326
HBV-DNA (pg/ml)	1198 ± 452	0.8 ± 0.8	0.0013
IL-10 (pg/ml)	12.7 ± 1.7	10.3 ± 1.1	0.2252
TNF-α (pg/ml)	280 ± 89	109 ± 21	0.0231
IL-12 p70 (pg/ml)	5.6 ± 0.3	7.9 ± 1.9	0.3792
IL-12 p40 (pg/ml)	205 ± 69	102 ± 19	0.0786

All the data are expressed as mean ± S.E. *p*-Values lower than 0.05 were considered to be significant (Student's *t*-test).
[a] *n*, number of the patients.

Table 3
Comparison of clinical parameters and cytokine profiles between $HBeAg^+$ and $HBeAb^+$ group in the patients with chronic hepatitis

Parameters	$HBeAg^+$ ($n=22$)[a]	$HBeAb^+$ ($n=12$)	p-Value
Age (years)	37 ± 3	39 ± 3	0.6053
ALT (IU/l)	163 ± 11	136 ± 25	0.2717
HBV-DNA (pg/ml)	1120 ± 420	21.4 ± 10.4	0.0478
IL-10 (pg/ml)	15.3 ± 1.2	12.4 ± 1.2	0.1271
TNF-α (pg/ml)	208 ± 28	167 ± 23	0.3446
IL-12 p70 (pg/ml)	6.0 ± 0.2	6.6 ± 0.6	0.2976
IL-12 p40 (pg/ml)	194 ± 45	114 ± 28	0.2244

All the data are expressed as mean ± S.E. p-Values lower than 0.05 were considered to be significant (Student's t-test).
[a] n, number of the patients.

in TNF-α levels between the group with mild or moderate fibrosis and the one with LC ($p<0.05$). And TNF-α levels seemed to show sequential changes in accordance with the progression of fibrosis. The changes of other cytokine values among the groups seemed to be similar to those between CH and LC (Table 4).

3.5. Serial analysis of cytokine values in the same patients with different disease phases

In some patients whose sera at different phases of the disease were available, the cytokine values through the two phases were measured and the serial changes of them were analyzed (Fig. 2). There were six CH patients who were previously diagnosed as ASC, three LC patients previously diagnosed as CH, and six HCC patients previously diagnosed as HCC, respectively. The numbers of the patients were limited, however, the serial changes of cytokine values in the same patients showed similar tendencies to the changes observed among the patient groups of different phases (Figs. 1 and 2). IL-10 levels showed significant increase when the disease progressed from ASC to CH ($p=0.0001$), and significant decrease when HCC were complicated ($p=0.0169$). TNF-α levels of the LC patients who complicated HCC also showed significant decrease ($p=0.0009$). TGF-β levels were measured in the HCC patients before and after the complication of HCC. TGF-β levels in HCC phase (38.8 ± 21.5 ng/ml) seemed to be higher than those in LC phase (51.6 ± 20.9 ng/ml), but the difference between two phases was not significant ($p=0.0905$).

Table 4
Comparison of clinical parameters and cytokine profiles in patient groups with different severity of liver fibrosis

Parameters	Extent of liver fibrosis		
	≤Moderate[a] ($n=15$)	Severe[b] ($n=7$)	Cirrhosis ($n=12$)
Age (years)	31 ± 3[c,d]	45 ± 4[c]	53 ± 2[d]
ALT (IU/l)	156 ± 21	115 ± 17	104 ± 47
HBV-DNA (pg/ml)	1380 ± 640	658 ± 508	67 ± 48
IL-10 (pg/ml)	14.8 ± 2	14.3 ± 1.1	17.0 ± 2.5
TNF-α (pg/ml)	149 ± 27[e]	229 ± 46	268 ± 39[e]
IL-12 p70 (pg/ml)	5.7 ± 0.3	6.0 ± 0.7	6.2 ± 0.3
IL-12 p40 (pg/ml)	119 ± 33	155 ± 33	115 ± 16

All the data are expressed as mean ± S.E.
[a] ≤Moderate, mild to moderate fibrosis, fibrous expansion of portal areas, with or without short fibrous septa and occasional bridging.
[b] Severe, fibrous expansion of portal areas, with marked bridging and architectural distortion.
[c] $p<0.01$.
[d] $p<0.0001$.
[e] $p<0.05$.

3.6. Regression analysis of clinical parameters and cytokine levels

The results above indicated that the cytokine levels were elevated even in ASC group without biochemical inflammation in the liver, and suggested that these cytokine levels might be influenced by the activity of the disease, the fibrotic changes of the liver, viral load, viral antigen load, and the complication of malignancy. Since the patterns of cytokine profiles were different among the patient groups, it was suggested that the production of the individual cytokines might be affected by the different factors. Thus, to analyze the relation of clinical parameters to cytokine levels, the regression analysis was done using laboratory data, such as ALT, HBV-DNA, and HBeAg levels as clinical parameters.

According to the analysis in all the patients, significant correlations were observed between ALT and IL-10 ($r=0.4095$, $p=0.0002$, Table 5; Fig. 3). In ASC, the significant correlations were observed between HBeAg and IL-12 p40/TNF-α (HBeAg and IL-12 p40: $r=0.4718$, $p=0.0230$; HBeAg and TNF-α: $r=0.4238$, $p=0.0439$). The correlation between HBV-DNA and IL-10 was observed when analyzed in CH ($r=0.4308$, $p=0.0110$) and LC ($r=0.6468$, $p=0.0230$). The correlation between HBV-DNA and IL-10 disappeared, when the patients had HCC. On the other hand, there was tendency of negative correlation between HBV-DNA and IL-12 p70 ($r=-0.6881$, $p=0.0592$) that was not seen in CH or LC (Table 5).

Thus, the analysis suggested that IL-10 levels closely related to liver inflammation and viral load, and that IL-12

Table 5
Regression analysis between clinical parameters and cytokine values in the patients with chronic HBV infection

	Patient group	n	r	p-Value
ALT vs. IL-10	All	77	0.4095	0.0002
HBeAg vs. IL-12 p40	ASC	23	0.4718	0.0230
HBeAg vs. TNF-α	ASC	23	0.4238	0.0439
HBV-DNA vs. IL-10	CH	34	0.4308	0.0110
HBV-DNA vs. IL-10	LC	12	0.6468	0.0230
HBV-DNA vs. IL-12 p70	HCC	8	−0.6881	0.0592

The combination of clinical parameter and cytokine value, with significant correlation within the indicated patient group, are listed. ASC: asymptomatic HBV carrier with persistently normal serum aminotransferase levels; CH: patient with chronic active hepatitis; LC: patient with liver cirrhosis; HCC: patients with hepatocellular carcinoma; n: number of the subjects; r: correlation coefficient.

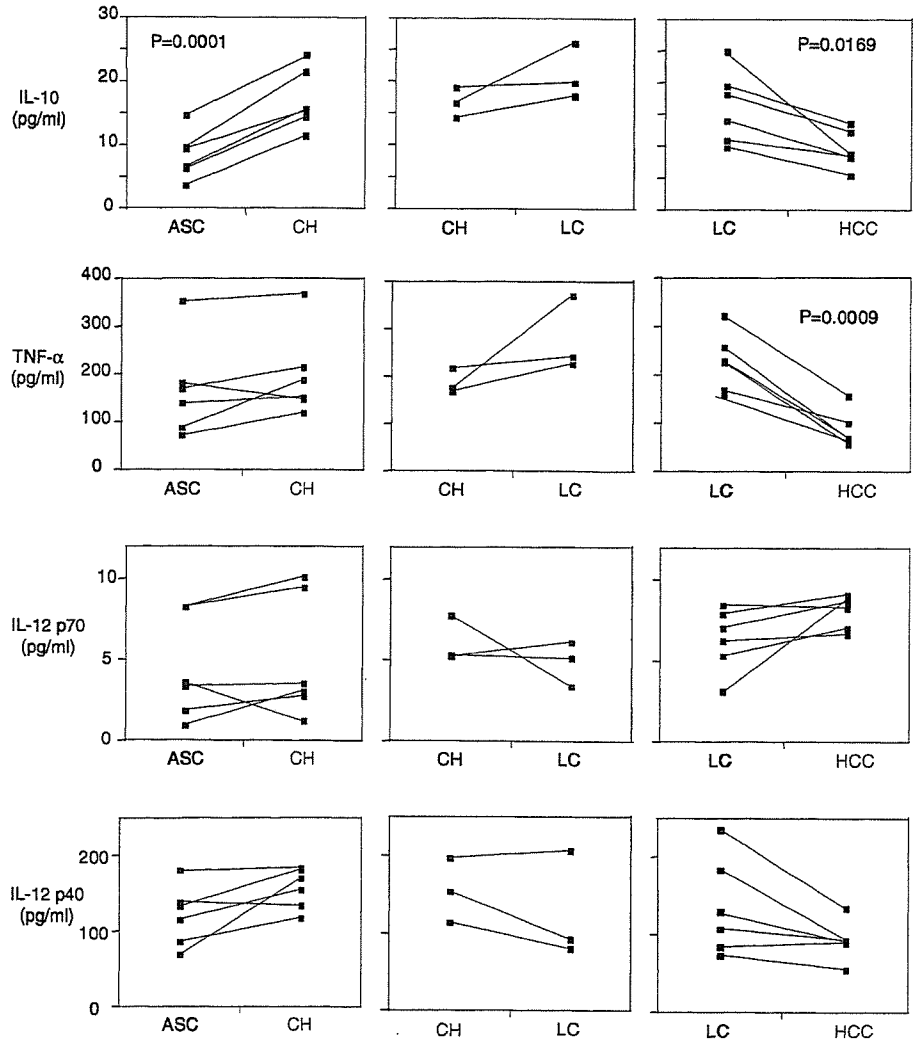

Fig. 2. Serial changes of cytokine values in the same patients in the different phases of chronic HBV infection. The patients analyzed were six with CH previously diagnosed as ASC (graphs in the right row), three with LC previously diagnosed as CH (graphs in the middle row), and six with HCC previously diagnosed as LC (graphs in the left row), respectively. The cytokine values of the same patient were connected by the lines. For statistical analysis, paired Student's *t*-test was applied. *p*-Values under 0.05 were considered to be significant and the actual numbers ($p < 0.05$) are described on the upper part of the graphs.

p40 and TNF-α levels were strongly affected by the amount of viral antigen.

4. Discussion

Liver inflammation caused by HBV infection is immune-mediated, and both acquired and innate immune responses are thought to play important roles on the pathogenesis of HBV-related hepatitis [1–3]. In the current study, we measured the serum levels of the cytokines in patients with chronic HBV infection in order to analyze the roles of the immune responses on the disease pathogenesis.

In the study, we could detect IL-10, TNF-α, IL-12 p70, IL-12 p40, and TGF-β, but not IFN-γ, IL-2, or IL-4 in the sera of the patients. There are some reports that could detect IL-2 [19], or the one that could detect only low levels of IL-10 [20]. Our data seem to be contradictory to these reports, however, we think that the differences are not substantial and that the sensitivity of the detection methods might be the main cause of this problem.

The serum levels of the cytokines were varied among the groups which represented the different phases of chronic HBV infection, and the changing patterns of the individual cytokines seemed to be peculiar to them (Fig. 1). The serial analysis of cytokine levels in the same patients through the different phases of HBV infection, showed the similar patterns seen in the analysis among the groups (Fig. 2). This suggests that the analysis among the groups well reflected the actual phenomena seen in the individual patients.

It was suggested from the analysis that the cytokine levels were influenced by various factors, such as inflammation, fibrosis, viral load, viral antigen load, and the occurrence of malignancy in the liver (Tables 2–5; Fig. 3). And it was also suggested that the factors relating to the changes of individual cytokine levels differed with the disease phases (Table 5). For example, IL-10 had correlation to ALT when analyzed in all the patients, but it had correlation to HBV-DNA in CH and in LC. HBV-DNA seemed to correlate to IL-12 p70 negatively in HCC, while no correlation was found between them in other phases of the disease. With regard to the relation of IL-10 to inflammation and viral load of HBV, there were the similar reports by others [19,21–23], however, the actual reasons for these correlations are difficult to explain. They might be indirect correlations, which were regulated by the unknown factors. Nonetheless, it may be important to know the changes of cytokine balances among the disease phases and the responsible factors for these changes, in order to understand the pathogeneses in the individual disease phases. These analyses may lead to the prediction for the transition of disease phase, for example, from LC to HCC, the complication of HCC.

We have to note that the levels of the measured cytokines except for IL-12 p70 were raised in ASC without visible inflammation in the liver. This clearly means that immune system recognizes and reacts against HBV even from immune tolerant phase.

ASC were usually regarded as 'healthy' because of the lack of biochemical abnormalities, however, our data suggest the reconsideration of this idea. It is well known that the occurrence of HCC is 100 times higher in HBV carriers than in healthy individuals without hepatitis virus infection [24], and HCC sometimes develops in the patients without cirrhosis or even in the patients with early stage of chronic hepatitis [25,26]. It is suggested that the development of HCC is caused by the accumulation of oxidative DNA damage in human study and in HBV transgenic mice model [27–30]. Our study may explain the high risk for HCC in the patients with chronic HBV infection, since it showed possible evidence of the activation of macrophages, the source of oxidant radicals, from early stage of infection. However, to confirm the speculation above, the liver biopsy in the patients including ASC to examine the DNA damage and the long-time follow-up of the patients, may be necessary.

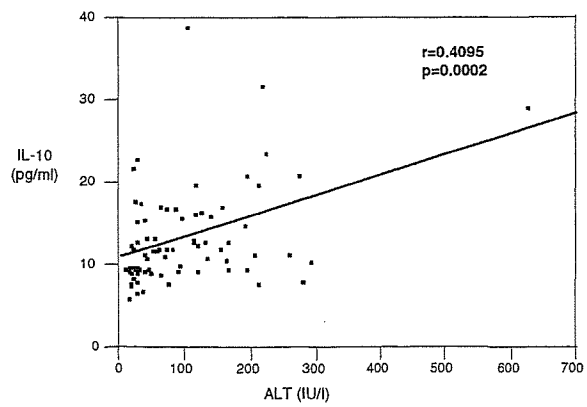

Fig. 3. Scatter plots with ALT and IL-10 in all the patients. ALT and IL-10 values of 77 patients were plotted. The line on the graph is the regression line. Correlation coefficient (r) and p-value are described on the graph.

The complication of HCC affected the cytokine levels dramatically. IL-10, TNF-α, and IL-12 p40 levels became lowest in HCC among the patient groups. It is reported that the patients with HCC are put in immuno-suppressive conditions and that the cancer cells themselves may contribute to the conditions by producing immuno-suppressive agents, such as TGF-β [13–15]. In the present study, TGF-β levels seemed to be raised when the patients complicated HCC, however, the data were not reliable because of the use of sera instead of plasma for the assay. More detailed study including the re-measurement of TGF-β, histological examination of TGF-β expression in HCC tissue, is necessary to elucidate the role of TGF-β and the conditions of immuno-suppression in HCC.

By measuring serum cytokine levels, we got the evidence that they well reflect the clinical manifestations, such as the activity of the inflammation, viral load, viral antigen load, the extent of fibrosis, complication of the cancer. The analysis of cytokine levels is helpful to understand the pathogenesis of the disease, and may become useful tool to predict the transition of the disease phase. However, more detailed analysis will be necessary for the purpose above.

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ORIGINAL ARTICLE

Dendritic cell therapy with interferon-α synergistically suppresses outgrowth of established tumors in a murine colorectal cancer model

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Both dendritic cell (DC)-based immunotherapy and interferon (IFN)-α therapy have been proved to have potent long-lasting antitumor effects. In anticipation of synergistic antitumor effects, we performed combination therapy with DCs and IFN-α gene-transduced murine colorectal cancer MC38 cells (MC38-IFN-α). DCs incubated with MC38-IFN-α, but not neomycin-resistance gene-transduced MC38 cells (MC38-Neo), effectively enhanced proliferation of allogeneic splenocytes in vitro. *In 12 of 17 mice, DCs in combination with MC38-IFN-α prevented the development of a parental tumor, while DCs and MC38-Neo did in only three of 17 mice (P = 0.008). In a therapeutic model of an established parental tumor, inoculation of DCs and MC38-IFN-α suppressed the growth of the established parental tumors significantly compared with the administration of DCs with MC38-Neo or naive splenocytes with MC38-IFN-α (P = 0.016 and 0.024, respectively). Analyses of immunohistochemistry and tumor-infiltrating mononuclear cells showed that CD8⁺, CD11c⁺, and NK1.1⁺ cells markedly infiltrated the established tumors of mice treated with DCs and MC38-IFN-α. From the results of observation of parental tumor outgrowth in immune cell-depleted mice, CD8⁺ cells, and asialo-GM-1⁺ cells were thought to contribute to the antitumor effects induced by the combination therapy. Furthermore, MC38-specific cytolysis was detected when splenocytes of mice inoculated with DCs and MC38-IFN-α cells were stimulated with MC38-IFN-α cells* in vitro. *Since DC-based immunotherapy in combination with IFN-α-expressing tumor cells induces potent antitumor cellular immune responses, it should be considered for clinical application.*
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Keywords: *dendritic cell; maturation; interferon-α; immunotherapy*

Introduction

Although therapies for malignant tumors including operation, chemotherapy, and radiation therapy have developed remarkably, the complete conquest of malignant tumors has not been achieved yet. In addition, in many cases, patients suffer from side effects of these therapies such as fatigue, anorexia, pyrexia, infection, and suppression of bone marrow. Thus, new anticancer therapies that have more efficacy and fewer side effects are required. The cellular immune response is considered not to work functionally and sufficiently in patients with advanced malignant tumors, and it has been reported that some tumors escape immune surveillance by several mechanisms.[1] To overcome immune suppression or immune escape in patients with advanced malignant tumors, many biologic therapies that aim at inducing potent antitumor immune responses have been tried.

Dendritic cells (DCs) are potent antigen-presenting cells that can elicit primary and secondary immune responses to foreign antigens.[2,3] Immature DCs express low levels of major histocompatibility complex (MHC) class I, class II, and costimulatory molecules (CD80, 86) that play important roles in T cell stimulation.[4] When immature DCs capture and take up antigens, DCs downregulate the function of antigen acquisition, but upregulate MHC and costimulatory molecules. As DCs mature, they will express higher levels of MHC and costimulatory molecules and present antigens to antigen-specific T cells and induce immune responses.[4–8] Since DCs are playing a crucial role in controlling immunity, the use of DCs may be ideal for cancer therapy.[4] Recently, various DC-based therapies have been tried to elicit antitumor responses,[6,8–15] and DCs have been used for therapy in patients with some malignant tumors such as malignant melanoma, lymphoma, renal cell carcinoma, pancreatic, and gastric cancer.[16]

Interferon (IFN)-α has been used to treat patients with not only viral infections such as hepatitis B and C but also some malignant tumors such as melanoma, renal cell carcinoma, and leukemia. The effects of type I IFN include antiviral function, enhancement of IFN-α/β production,[17,18] and inhibition of cell growth and angiogenesis.[19] In addition, IFN-α plays a crucial role in the immune system. IFN-α upregulates the expression of MHC class I on the cell surface and enhances the proliferation of Th1-lymphocytes.[20] Previous studies emphasized the importance of IFN-α for the generation of cytotoxic T lymphocytes (CTLs) in specific antitumor

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immune responses.[21,22] In addition, we reported previously that IFN-α transduction of poorly immunogenic tumor cells reduces tumorigenicity and leads to a long-lasting tumor immunity,[23] and that IFN-α-expressing tumor cells promote the survival of tumor-specific CTLs by preventing apoptosis.[24]

IFN-α has been reported to induce the maturation of DCs. Santini *et al*.[25] demonstrated that IFN-α upregulated the expression of costimulatory molecules (CD80, 86) and MHC class II (HLA-DR) and induced CD83 expression, which is considered to be a marker of mature and activated human DCs. Then, they showed that DCs gained a greater capability to stimulate the proliferation of allogeneic lymphocytes in the presence of IFN-α.[25] Others have also reported the effects of IFN-α on enhancement of DC maturation.[26,27] However, it has also been reported that IFN-α fails to induce DC maturation, and that the presence of IFN-α prior to or during the differentiation of DCs from the monocyte precursors alters their response to maturation stimuli in the human system.[28]

In our previous studies, we demonstrated that IFN-α gene therapy in combination with CD80 transduction reduces tumorigenicity and the growth of established tumors in poorly immunogenic tumor models,[23] and that IFN-α has additive effects on suppressing tumor growth in cooperation with interleukin (IL)-12.[29] Costimulatory molecules such as CD80 are highly expressed on the surface of DCs, and IL-12 is produced mainly by DCs. Therefore, the combined use of DC-based immunotherapy with IFN-α gene therapy is considered reasonable. Recently, Tsugawa *et al*.[30] reported that combined use of DC with adenoviral vector encoding IFN-α elicits antitumor response in a murine intracranial gliomas model. In this study, as a preliminary investigation of the combined therapy, we investigated the effects of bone marrow-derived DCs and IFN-α-expressing colorectal cancer cells on the proliferation of allogeneic splenocytes. Then, we examined whether DCs and IFN-α-expressing tumor cells display synergistic effects on the induction of antitumor immunity in a therapeutic model to evaluate the possibility of applying this combined therapy to clinical trial.

Results

IFN-α gene transduction does not affect growth of tumor cells in vitro

Each of the tumor cells (wild type (WT), neomycin-resistance gene-transduced MC38 (MC38-Neo)-, murine IFN-α-overexpressing MC38 cells (MC38-IFN-α)) with or without γ-irradiation were seeded at 5×10^5 cells/well in six-well plates, and enumerated every day in duplicate to compare the growth *in vitro* of the genetically modified MC38 cells. The growth rates did not differ significantly between MC38-WT, MC38-Neo, and MC38-IFN-α cells within 72 h incubation (data not shown). Cell counts of each tumor cell were decreased to approximately one-third 48 h after 100 Gy γ-irradiation.

When nonirradiated tumor cells were incubated with DCs, cell growth of each tumor cell line was almost the same with that of MC38-WT without DCs (data not shown). DCs did not affect the cell growth of tumor cell lines.

With the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling assay (TUNEL), we found approximately 50% of the γ-irradiated (100 Gy) MC38 cells to be apoptotic 48 h after irradiation (data not shown). The proportion of apoptotic cells after γ-irradiation did not differ between the genetically modified MC38 cell lines.

Coincubation with MC38-IFN-α cells does not enhance cytokine production by DCs and phenotypic maturation of DCs

The production of murine IFN-α by modified tumor cells or DCs was confirmed by enzyme-linked immunosorbent assay (ELISA). Nonirradiated MC38-IFN-α cells produced large amounts of IFN-α as shown in Table 1. On the other hand, 100 Gy γ-irradiated MC38-IFN-α cells produced about half as much IFN-α as nonirradiated MC38-IFN-α. There was no difference in IFN-α production between the irradiated MC38-IFN-α cells alone and DCs coincubated with irradiated MC38-IFN-α. IFN-α was not detected in supernatants of MC38-WT cells, MC38-Neo cells, DCs, splenocytes, and DCs with MC38-Neo cells.

IL-1β, IL-12, and TNF-α production by DCs was also confirmed by ELISA. Enhancement of these cytokine production was not observed when DCs were coincubated with parental or genetically modified tumor cells (data not shown). Expression of CD80, CD86, and I-A^b molecules on DCs was compared by flow cytometry. Coincubation with genetically modified tumor cells did not enhance the expression of those molecules (data not shown). From these data, coincubation with parental or genetically modified tumor cells does not enhance the cytokine production by DCs as well as the phenotypic maturation of DCs in this system.

Proliferation of allogeneic splenocytes is markedly enhanced by coincubation with MC38-IFN-α cells

To investigate proliferative effects of DCs and MC38-IFN-α cells on allogeneic splenocytes, we performed cell proliferation assays. As shown in Figure 1, stimulation by both DCs and MC38-IFN-α cells markedly enhanced the proliferation of allogeneic splenocytes compared with stimulation by DCs alone or by DCs and MC38-Neo ($P = 0.007$ or 0.020, respectively). When exogenous IFN-α was added to the culture of DCs and MC38-Neo cells, the proliferation of allogeneic splenocytes was

Table 1 Production of IFN-α by genetically modified MC38 tumor cells and DCs

Cell	*IFN-α production (ng/48 h)*
DC	Not detected
MC38-Neo	Not detected
MC38-IFN-α	20.8±0.5
MC38-IFN-α (irradiated)	10.8±0.3
DC+MC38-IFN-α (irradiated)	11.0±1.1
Splenocyte+MC38-IFN-α (irradiated)	7.2±0.6

A total of 1×10^6 DCs or splenocytes were incubated with or without 1×10^5 γ-irradiated (100 Gy) MC38-IFN-α cells in 5 ml of CM in six-well plates. After 48 h incubation, concentration of IFN-α in the culture supernatant was confirmed by ELISA according to the manufacture's protocol.

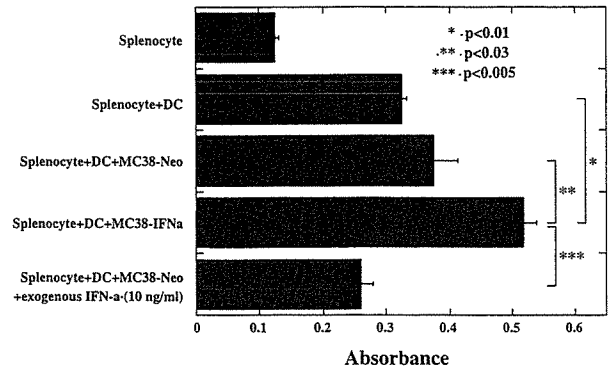

Figure 1 Proliferation of allogeneic splenocytes is markedly enhanced by coincubation with MC38-IFN-α cells. Proliferation of allogeneic splenocytes is markedly enhanced by coincubation with MC38-IFN-α cells. Purified DCs were incubated with γ-irradiated (100 Gy) MC38-Neo or MC38-IFN-α cells at a DC to tumor ratio of 10 for 2 days. The cells were γ-irradiated (30 Gy) for the purpose of using as stimulator cells. In some wells, exogenous murine IFN-α was added at a concentration of 10 ng/ml. After allogeneic splenocytes (5×10^5) were incubated with the stimulator cells (5×10^4) for 3 days, cell proliferation assay was performed. This experiment was performed twice with similar results.

significantly suppressed compared with the stimulation by DCs and MC38-IFN-α cells ($P = 0.005$). The results suggested that MC38-IFN-α cells and DCs stimulate allogeneic splenocytes more effectively than control gene-transduced MC38 cells, and that continuous secretion of IFN-α may be responsible for the proliferative effect on allogeneic splenocytes in this system.

Inoculation of DCs and MC38-IFN-α cells prevents development of parental MC38 tumors in vivo

We investigated the preventive effects of DCs and MC38-IFN-α cells on the development of parental MC38 tumors. At 1 week after the second intraperitoneal inoculation of DCs (or splenocytes) and the modified MC38 cells, MC38-WT cells were inoculated subcutaneously (s.c.). 12 of 17 mice inoculated with DCs+MC38-IFN-α cells did not develop parental tumors, although all mice injected with only DCs or splenocytes had growing parental tumors on day 28 as shown in Figure 2 and Table 2. All these 12 tumor-free mice rejected the subsequent parental MC38 cell challenge. DCs+MC38-Neo and splenocytes+MC38-IFN-α cells also had preventive effects on the development of parental tumors compared with splenocytes alone, although the preventive effects were less than those of DCs+MC38-IFN-α.

CD8⁺ cells and asialo-GM-1⁺ cells contribute to the antitumor effects induced by DCs and IFN-α-expressing tumor cells

We depleted immune cells using anti-CD4, anti-CD8, and anti-asialo-GM-1 antibodies to explore the mechanism of the antitumor effects induced by DCs and IFN-α-expressing tumor cells. We depleted these immune cells *in vivo* after inoculation with DCs and IFN-α-expressing tumor cells. Then, MC38-WT cells were injected and the WT tumor development was measured. When $CD8^+$ cells or asialo-GM-1^+ cells were depleted, we observed obvious growing WT tumors in those mice, whereas

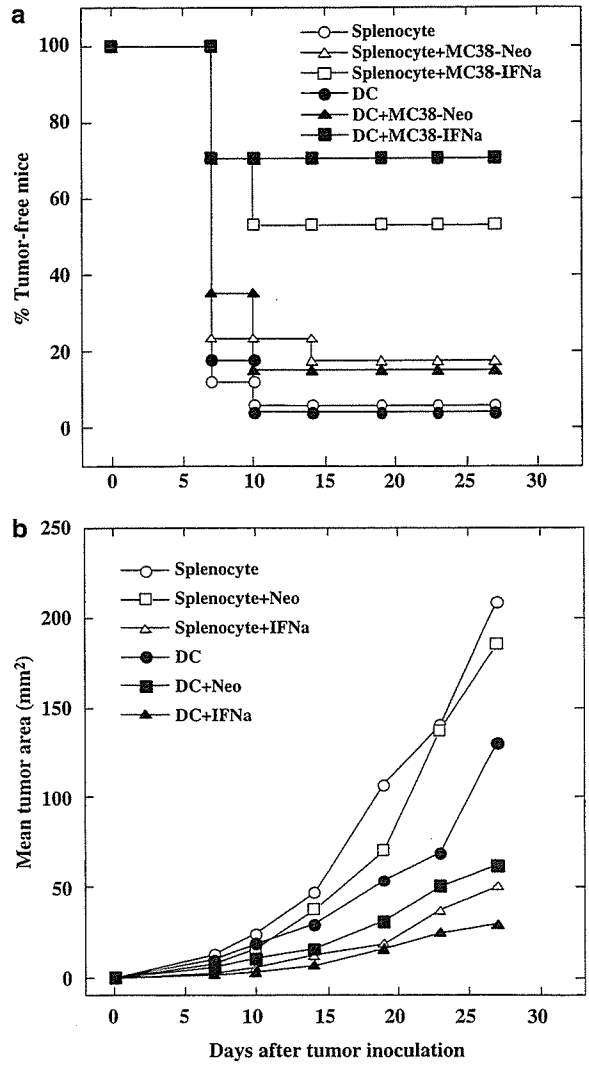

Figure 2 Inoculation of DCs and MC38-IFN-α cells prevents development of parental MC38 tumors *in vivo*. Inoculation of both MC38-IFN-α cells and DCs prevents development of parental MC38 tumor. Mice were injected i.p. with 1×10^6 DCs or splenocytes and 1×10^5 the genetically modified tumor cells twice at a 7-day interval (days –14 and –7). At 1 week after the final vaccination (day 0), the mice were injected s.c. with 1×10^5 MC38-WT cells in the right flank. Each experiment involved five or six mice per group. Results are reported as (**a**) percent of tumor-free mice and (**b**) mean tumor area (mm^2). This experiment was performed three times with similar results.

we detected no tumor in mice not depleted of any immune cells ($P = 0.015$ or 0.023, respectively, Figure 3). On the other hand, in $CD4^+$ cell-depleted mice, the antitumor effects induced by DCs and IFN-α-expressing tumor cells were diminished marginally. Thus, DCs and IFN-α therapy seemed to stimulate $CD8^+$ cells and asialo-GM-1^+ cells mainly *in vivo*.

Therapeutic inoculation of DCs and MC38-IFN-α cells suppresses outgrowth of established parental MC38 tumors

We evaluated the therapeutic effects of DCs and MC38-IFN-α on established parental MC38 tumors. As shown