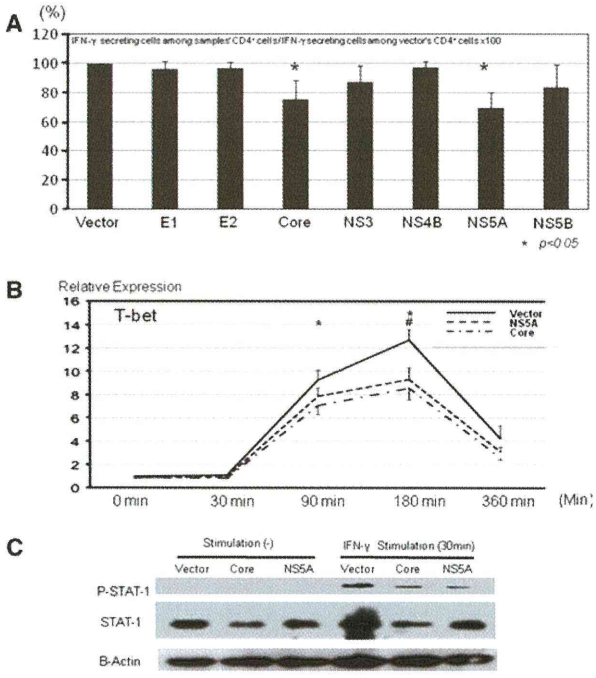

Fig. 4 HCV-Core and NS5A proteins are the proteins that contribute to the suppression of IFN-γ secretion. **a** HCV E1, E2, Core, NS3, NS4B, NS5A, and NS5B expression plasmids were used to transfect into primary $CD4^+$ lymphocytes by Nucleofector. The frequencies of IFN-γ-secreting cells among the samples' $CD4^+$ cells/the frequencies of IFN-γ-secreting cells among the vector's $CD4^+$ cells $\times 100$ are shown in this *bar graph*. **b** HCV core and NS5A transfected primary $CD4^+$ lymphocytes were stimulated with IFN-γ (500 ng/ml). The relative expression of T-bet-mRNA was sequentially analyzed by real-time polymerase chain reaction (PCR). The relative amount of target mRNA was obtained by using a comparative the threshold cycle (CT) method. The expression level of mRNAs of the nonstimulation sample of vector transfected-primary $CD4^+$ cells is represented as 1.0 and the relative amount of target mRNA in a stimulated sample was calculated. Three independent experiments were carried out. *Error bars* indicate the standard deviation. **c** Immunoblotting assay was carried out to detect the protein of signal transducer and activator of transcription-1 (*STAT-1*), phospho-STAT-1 (*p-STAT-1*), and actin in the HCV-core, NS5A, and vector-plasmid transfected human primary $CD4^+$ cells with or without IFN-γ stimulation (30 min)

SB-HCV infection could induce apoptosis of naïve $CD4^+$ cells

Annexin V and PI double staining were carried out to detect early apoptotic cells. The frequency of Annexin-V-positive PI-negative early apoptotic cells in SB-HCV-infected naïve T cells was significantly higher than those in the control groups ($p < 0.01$) (Fig. 3a, b). UV-irradiated SB-HCV did not enhance the induction of apoptosis in naïve T cells with CD3CD28 stimulation. During T-cell activation, apoptosis is easily induced in order to maintain an appropriate immune response. In line with this feature, 3.04% of early apoptotic cells were detected in naïve T cells with CD3CD28 beads stimulation and Mock serum. These data indicate that SB-HCV replication could induce apoptosis, as seen in Molt-4 cells [14].

HCV core and NS5A proteins could suppress IFN-γ secretion from primary $CD4^+$ cells

We investigated the HCV proteins responsible for the suppression of IFN-γ secretion. HCV E1, E2, Core, NS3, NS4B, NS5A, and NS5B expression plasmids were used to transfect into primary $CD4^+$ lymphocytes by Nucleofector. The intracellular staining of these proteins was carried out and the transfection efficiency was about 35–55% (Suppl. Fig. 3). Among these proteins, HCV core and NS5A could significantly suppress the IFN-γ secretion ($p < 0.05$) (Fig. 4). HCV core and NS5A transfected primary $CD4^+$ lymphocytes were stimulated with IFN-γ. The relative expression of T-bet-mRNA was sequentially analyzed by real-time PCR. T-bet-mRNA expression in HCV core or NS5A transfected primary $CD4^+$ T lymphocytes was significantly suppressed at 90 and 180 min post-transfection in comparison to vector-transfected primary $CD4^+$ T lymphocytes. Moreover, the amount of STAT-1 protein in HCV-Core-expressing $CD4^+$ cells was remarkably lower than the amounts in vector and HCV-E2 transfected $CD4^+$ cells

Table 1 Cytokine conditions for various kinds of lymphoid cell culture

Cells	Cytokine condition	Other stimulant	Cell viability (%)
PBMC	IL2 (50 ng/ml) + IL6 (20 ng/ml) + CSF (250 ng/ml)	None	80
PBMC-CD8	IL2 (50 ng/ml) + IL6 (20 ng/ml) + CSF (250 ng/ml)	None	80
CD3	IL2 (50 ng/ml)	CD3CD28 coated beads	70
CD4	IL2 (50 ng/ml)	CD3CD28 coated beads	70
CD8	IL2 (50 ng/ml)	CD3CD28 coated beads	70
CD14	CSF (250 ng/ml)	None	60
CD19	IL-6 (20 ng/ml)	None	70

The conditions of the cell culture are shown. Peripheral blood mononuclear cell (PBMC)-CD8 indicates CD8 cell-depleted PBMCs

IL interleukin, *CSF* colony stimulating factor

Table 2 Strand-specific hepatitis C virus (HCV)-RNA detection in various kinds of lymphoid cells

Subset	PBMC	PBMC-CD8	CD3	CD4	CD8	CD14	CD19
Positive strand							
2 days	+	+	−	+	−	−	+
7 days	++	++	+	++	−	++	+++
7 days UV-irradiated	−	−	−	−	−	−	−
Negative strand							
2 days	−	−	−	−	−	−	−
7 days	−	+	+/−	+	−	+	++
7 days UV-irradiated	−	−	−	−	−	−	−

Subset	Whole $CD4^+$	$CD4^+CD45RA^+RO^-$	$CD4^+CD45RA^-RO^+$
Positive strand			
2 days	+	+	+
7 days	++	+++	+
7 days UV-irradiated	−	−	−
Negative strand			
2 days	−	−	−
7 days	+	++	+/−
7 days UV-irradiated	−	−	−

Positive- and negative-strand-specific HCV-RNA was detected by semiquantitative nested polymerase chain reaction (PCR) methods

−, negative detection; +, positive detection without dilution; ++, positive detection with 4 times dilution; +++, positive detection with 16 times dilution; ±, only one detection in three independent experiments. Three independent experiments were carried out. Similar results were obtained three times

(Fig. 4c). The amount of phosphorylated STAT-1 (p-STAT-1) after IFN-γ stimulation was also analyzed. The amount of p-STAT-1 in HCV-Core and NS5A expressing $CD4^+$ cells was remarkably lower than that in the vector control.

Discussion

There are many reports about the existence of extrahepatic HCV replication that might contribute to immune dysfunction [13, 14, 23–25]. We have reported that a specific SB-HCV strain could replicate in B- and T-cell lines and affect various immune systems [13, 14, 25]. However, the results of these studies were not definitely conclusive, since the cell lines were inappropriate to investigate the development and commitment of the lymphocytes. In the present study, we demonstrated that the SB-HCV strain could replicate in primary $CD19^+$ B cells, $CD4^+$ T cells, and $CD14^+$ monocytes with cytokine stimulation. Among the $CD4^+$ T cells, $CD4^+CD45RA^+RO^-$ naïve $CD4^+$ cells were the most susceptible to SB-HCV infection. One of the speculated reasons to explain why naïve $CD4^+$ cells with stimulation were most susceptible to SB-HCV infection is that T cells might temporarily express various kinds of molecules which may contribute to the HCV infection during T-cell development. The infectivity of naïve $CD4^+$ T cells was not as high as that of Molt-4 cells. However, significant suppression of cell development and IFN-γ secretion were seen in SB-HCV-infected naïve T cells with CD3, CD28, and IL2 stimulation. UV-irradiated-HCV that could not replicate in the cells suppressed the IFN-γ secretion slightly. These data indicate that not only the effect of HCV replication but also the direct binding effects of HCV structured proteins might contribute to the suppression of IFN-γ secretion. One report indicated that HCV-core protein could interact with the complement receptor gC1qR and upregulate suppressor of cytokine signaling-1 (SOCS-1), accompanied by downregulation of signal transducer and activator of transcription-1 (STAT-1) phosphorylation in T cells [7]. Another possible explanation of the discrepancies between HCV infectivity and suppression of proliferation and IFN-γ secretion might be the low sensitivity of HCV antigen-immunostaining, since lower sensitivity of immunostaining in comparison to the nested PCR method was found in our previous study [13].

HCV-Core and -NS5A proteins were the proteins responsible for the suppression of IFN-γ secretion from T cells. Lin et al. [26] have documented that HCV-core protein causes the degradation of STAT-1 protein and suppresses the Jak-STAT pathway in hepatocytes. In our previous study, reduction of STAT-1 protein was detected in HCV-core transfected primary naïve T cells and HCV-replicating Molt-4 cells [13]. Moreover, inhibition of intrahepatic gamma interferon production by HCV-NS5A

in transgenic mice was recently reported [27]. Recently, detection of HCV replicative intermediate RNA in perihepatic lymph nodes was reported [28]. The disturbance of Th1 commitment might influence the development of HCV-specific CTL in perihepatic lymph nodes. The selective infection of certain T cells by HCV in vivo may explain why there is only relative HCV-specific T-cell suppression without general immune suppression.

Suppression of proliferation activity was seen in HCV-infected naïve T cells as well as HCV-infected Molt-4 cells [14]. The expression level of CD45RA, which is a surface marker of T-cell development, gradually declined along with cell proliferation. However, HCV-infected naïve T cells expressed significantly higher levels of CD45RA than the control groups. We previously reported that HCV replication could suppress Ras/MEK/ERK signaling of Molt-4 [14]. During T-cell development, T cells showed strong proliferation activity that might facilitate HCV replication in T cells. However, extensive proliferation of HCV in T cells might interfere with the proper development of T cells.

The induction of apoptosis was seen in SB-HCV-infected naive T lymphocytes with CD3CD28 and IL2 stimulation. It is known that, during T-cell activation from naïve to effector cells, T cells have to survive activation-induced cell death (AICD), which may contribute to the maintenance of an appropriate level of the immune response [29, 30]. However, some groups reported that HCV replication could inhibit apoptosis in hepatoma cell lines [31, 32]. The developmental stages and characteristics of naïve T cells might explain these contradictory results. During T-cell activation, apoptosis is easily induced in order to maintain an appropriate immune response.

In conclusion, HCV replication in human naïve T cells might affect their proliferation activity and Th1 development, as was shown in the cell lines used in a previous study. The results suggest that the infectivity of HCV in human naïve T lymphocytes is low, although the biological effect of this infection might be significant because of its bystander effects.

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Four-year study of lamivudine and adefovir combination therapy in lamivudine-resistant hepatitis B patients: influence of hepatitis B virus genotype and resistance mutation pattern

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SUMMARY. To investigate the efficacy of long-term lamivudine (3TC) and adefovir dipivoxil (ADV) combination therapy in 3TC-resistant chronic hepatitis B virus (HBV) infected patients, we analysed 28 3TC-resistant patients treated with the combination therapy during 47 months (range, 9–75). At 12, 24, 36, and 48 months, the rates of virological response with undetectable HBV DNA (≤2.6 log copies/mL) were 56, 80, 86, and 92%, respectively. Among 17 hepatitis B e antigen (HBeAg)-positive patients, HBeAg disappeared in 24% at 12 months, 25% at 24 months, 62% at 36 months, and 88% at 48 months. When HBV genotypes were compared, patients with genotype B achieved virological response significantly more rapidly than those with genotype C ($P = 0.0496$). One patient developed virological breakthrough after 54 months, and sequence analysis of HBV obtained from the patient was performed. An rtA200V mutation was present in the majority of HBV clones, in addition to the 3TC-resistant mutations of rtL180M+M204V. The rtN236T ADV-resistant mutation was observed in only 25% clones. *In vitro* analysis showed that the rtA200V mutation recovered the impaired replication capacity of the clone with the rtL180M+M204V mutations and induced resistance to ADV. Moreover, rtT184S and rtS202C, which are known entecavir-resistant mutations, emerged in some rtL180M+M204V clones without rtA200V or rtN236T. In conclusion, 3TC+ADV combination therapy was effective for most 3TC-resistant patients, especially with genotype B HBV, but the risk of emergence of multiple drug-resistant strains with long-term therapy should be considered. The mutation rtA200V with rtL180M+M204V may be sufficient for failure of 3TC+ADV therapy.

Keywords: chronic hepatitis B, drug resistance, HBV, rtA200V.

INTRODUCTION

Hepatitis B virus (HBV) causes acute and chronic infection, and chronic hepatitis often leads to liver cirrhosis and hepatocellular carcinoma (HCC) [1]. HBV contains a small (3.2 kb), circular, partially double-stranded DNA genome, and nucleoside or nucleotide analogues inhibit HBV replication by interfering with reverse transcriptase/DNA polymerase of the virus [2]. Although therapy with these drugs results in virological, biochemical, and histological improvement in most patients [3], the effect is often transient because of the emergence of drug-resistant HBV mutants [4].

Lamivudine (3TC), a nucleoside analogue of L-deoxycytidine, is associated with highly frequent emergence of drug-resistant mutants: the cumulative rate is about 20% per year [5,6]. Mutations that result in the replacement of methionine at amino acid 204 to valine or isoleucine (rtM204V/I) within the tyrosine-methionine-aspartate-aspartate (YMDD) motif in the reverse transcriptase (RT) region of HBV polymerase are found in most of the 3TC-resistant isolates [7]. Compensatory mutations rtV173L and rtL180M, which restore the replication capacity of the YMDD mutant *in vitro*, are observed frequently together with the YMDD mutation [8,9]. Adefovir dipivoxil (ADV) is a phosphonate nucleotide analogue of adenosine monophosphate, and ADV-resistance rates are lower than those of 3TC [10]. Two mutations, rtA181V/T and rtN236T, are associated with resistance to ADV [11–14], and the cumulative 5-year occurrence of genotypic resistance is reported to be 29% [15]. *In vitro* studies showed that these mutations confer a weaker

Abbreviations: ADV, adefovir dipivoxil; ALT, alanine aminotransferase; eGFR, estimated glomerular filtration rate; ETV, entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; RT, reverse transcriptase; TDF, tenofovir disoproxil fumarate.

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decrease in the susceptibility to ADV, in comparison with the greater decrease in 3TC susceptibility because of the YMDD mutant [11,16]. This finding may explain the lower rate of the emergence of ADV resistance.

Although the number of approved drugs has increased in recent years, the treatment of chronic HBV infection remains a clinical challenge. Especially, how to manage drug-resistant patients including 3TC-resistant patients is a major problem. Continuation of 3TC monotherapy or retreatment with 3TC after its temporary discontinuation is ineffective options for 3TC-resistant patients [17]: the lack of any further benefit and the possibility of rapid re-emergence of resistant HBV have been reported [18]. Against 3TC-resistant HBV, ADV and entecavir (ETV) have a suppressive effect *in vivo* and *in vitro* [19–21]. Combination therapy of ADV and 3TC is effective for 3TC-refractory patients and has a low frequency of viral breakthrough [22]: the 3-year cumulative rate of *de novo* resistant mutants was 4% with no development of viral breakthrough in 3TC-resistant patients. However, further longer-term efficacy of the combination therapy remains unknown. ETV is a potent drug with infrequent development of resistance for treatment-naïve patients [23]. ETV monotherapy was shown to be effective during the first year of therapy in 3TC-resistant patients [20], but pre-existing 3TC-resistant mutants are favourable for the emergence of ETV resistance [21], and a comparatively high rate of the emergence of ETV-resistant strains has been reported in long-term studies [23]. Therefore, ETV monotherapy seems to be a less attractive option for the long-term treatment of 3TC-resistant patients.

Several previous reports have described the differences in the responses to antiviral therapy between HBV genotypes. A case–control study of 3TC treatment for genotypes B and C showed that the responses were not different, but the emergence of the YMDD mutation was more frequent in genotype C [24]. It was also reported that the YMDD mutation and breakthrough hepatitis developed more often in patients with genotype A than in patients with genotype B or C [25]. However, the impact of the genotype on the efficacy to ADV is uncertain.

Here, we studied the long-term efficacy of 28 3TC-resistant patients treated with the combination of 3TC and ADV and compared the response between HBV genotypes. Sequence analysis of HBV from a patient with resistance to the combination therapy was performed, and *in vitro* drug susceptibility of the mutant HBV clones was assessed to clarify the mechanism of the emergence of resistance.

MATERIALS AND METHODS

Patients

A total of 28 consecutive Japanese patients with chronic HBV infection who were treated with 3TC+ADV at Tohoku University Hospital from June 2003 to August 2009 for more than 6 months were enrolled in this study. All patients developed virological breakthrough during 3TC monotherapy, and ADV was added in. Virological breakthrough was defined as an increase in the serum HBV DNA level of ≥1 log copies/mL, which was determined using the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan), at two or more consecutive examinations in comparison with the lowest level after treatment. To evaluate renal function, the estimated glomerular filtration rate (eGFR) level using the Cockcroft-Gault formula [(140 - age) × (weight in kilograms) × (0.85 if female)/(72 × serum creatinine)] [26] was calculated. No patients were infected with HCV, nor had a history of other liver diseases. The patients were evaluated for the rate of virological response (undetectable HBV DNA: <2.6 log copies/mL), biochemical response [alanine aminotransferase (ALT) normalization: ≤35 IU/L], hepatitis B e antigen (HBeAg) loss, and virological breakthrough.

Antiviral treatment

Adefovir dipivoxil was administered at a dosage of 10 mg/day in all but one patient in addition to 3TC at a dosage of 100 mg/day. One patient received 10 mg of ADV on alternate days and 50 mg/day of 3TC daily because of reduced eGFR at the start of treatment. This occurred when the eGFR level dropped to <50 mL/min.

Determination of HBV genotype

The HBV genotype was determined as described previously [27] with minor modifications. Briefly, total DNA was extracted from 50 μL of serum sample by QIAamp Blood Mini kit (QIAGEN GmbH, Hilden, Germany) and subjected to nested polymerase chain reaction (PCR) with high fidelity polymerase (PrimeSTAR HS DNA polymerase; TaKaRa Bio Inc., Shiga, Japan), to amplify a 396-nt sequence in the S gene. The amplification products were sequenced on both strands directly using the BigDye Terminator v3.1 Cycle Sequencing kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using Genetyx-Mac (Version 12.2.7; Genetyx Corp., Tokyo, Japan). The genotype of HBV was determined by phylogenetic analysis with HBV isolates whose genotype was known.

Sequencing analysis of HBV reverse transcriptase region

Total DNA extracted from 50 μL of serum sample was subjected to nested PCR to amplify the 1148-nt sequence [nt 52 to 1199, the nucleotide numbers are in accordance with a genotype C HBV isolate of 3,215 nt (AB033550)] including the RT region of HBV polymerase. The first-round PCR was carried out with primers B026 [5′-TCA TCC WCA GGC CAT GCA GTG GA-3′ (W = A or T)] and B025 (5′-CTA GGA GTT CCG CAG TAT GGA TCG-3′), and the second round with

primers B011 [5′-YTT YCC TGC TGG TGG CTC CAG TTC-3′ (Y = C or T)] and B024 (5′-GGG GTT GCG TCA GCA AAC ACT TG-3′). The amplification products were sequenced on both strands directly or after cloning into pUC118. Sequencing analysis after cloning was performed at nt 497-1161.

Construction of plasmid

A cloned mutant sequence including the RT region from a sample obtained after the development of 3TC and ADV resistance was digested with BlnI (TaKaRa Bio Inc.) and EcoT22I (TaKaRa Bio Inc.). The digested fragment (nt 179-1068) was ligated into the BlnI-EcoT22I site of pBFH2R, which contained a 1.3-fold HBV genome [28]. Quick Change II-E Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to introduce nucleotide substitutions into the plasmid. Each mutation found in the RT region, rtL180M [C to A at nt 667 (C667A)], rtT184S (A679T), rtA200V (C728T), rtS202C (A733T), rtM204V (A739G), and rtN236T (A836C), was converted into the wild type or another mutant nucleotide. To construct plasmids with combined nucleotide substitutions, these converted plasmids were used next as templates. As a result, variant constructs harbouring rtM204I, rtL180M+M204V, rtL180M+T184S+M204V, rtL180M+A200V+M204V, rtL180M+S202C+M204V, rtL180M+M204V+N236T, and rtL180M+A200V+M204V+N236T were composed, and all constructs were sequenced to confirm the nucleotide substitutions.

Cell culture and transfection

Human hepatoma HepG2 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% bovine serum at 37 °C and 5% CO_2. Cells were seeded in 24-well plates at 1.25×10^5 cells/well. On the next day, 375 ng of plasmid DNA were transfected into these cells using TransIT LT-1 Transfection Reagent (Mirus, Madison, WI, USA), and cells were washed twice with phosphate-buffered saline after 4 h. Five hundred microliter of the medium and various amounts of adefovir (Toronto Research Chemicals Inc., Ontario, Canada) were added, and the culture supernatant was collected 4 days later. Experiments were performed at least in triplicate.

Real-time PCR and determination of IC_{50}

HBV DNA in the culture supernatant was quantified by real-time PCR as described previously [28] to determine the 50% inhibitory concentration (IC50) for ADV of each mutant HBV clone. Briefly, to digest the input plasmid DNA in the culture supernatant, 5 μL of the supernatant were treated with 5 units of DNase I (TaKaRa Bio Inc.) at 37 °C for 2 h, and the reaction was stopped with EDTA. Then, total DNA was extracted with a QIAamp DNA Blood Mini kit, and 10 μL of 200 μL DNA solution were subjected to real-time PCR using a LightCycler (Roche Diagnostics). Dose–response curves were plotted to determine the ADV IC50.

Statistical analysis

Statistical analyses were performed using Fisher's exact probability test for comparison of proportions between two groups and Mann–Whitney *U* test for comparison of continuous variables between two groups. The cumulative rate of undetectable HBV DNA or ALT normalization was calculated using the Kaplan–Meier method, and differences between the curves were tested using Log-rank test. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Study profile

The demographic and clinical profiles of the 28 patients [20 men and 8 women, median age 53.5 years (range 18–72)] at commencement of 3TC+ADV therapy are shown in Table 1. One (3.6%), 7 (25.0%), and 19 (67.9%) patients had HBV of genotypes A, B, and C, respectively. Eight (28.6%) patients had cirrhosis, 7 (25.0%) had HCC, and 17 (60.7%) patients were HBeAg positive. The mutations of the YMDD motif were determined by direct sequencing, and the YIDD, YVDD, and YIDD+YVDD mixed pattern were found in 14 (50%), 11 (39%), and 2 (7%)of the patients, respectively. Only one (4%) patient had no mutation in the YMDD motif. There were no significant differences in the profiles between patients with genotype B and those with genotype C.

Response to lamivudine and adefovir dipivoxil combination therapy

The 3TC-resistant patients treated with the combination therapy were followed up for a median of 47 months (range, 9–75). All patients continued to be treated with 3TC and ADV until virological breakthrough. The 6-, 12-, 24-, 36-, and 48-month rates of virological response with HBV DNA ≤ 2.6 log copies/mL were 39, 56, 80, 86, and 92%, respectively (Table 2). The ALT normalization rates were 57% at 6 months, 70% at 12 months, 84% at 24 months, 82% at 36 months, and 77% at 48 months. When compared between genotype B and C, the results of patients with genotype B tended to be favourable for both virological and biochemical response (Figs 1a,b). The cumulative probability of undetectable HBV DNA was significantly higher in genotype B than in genotype C ($P = 0.0496$), whereas there was no significant difference in that of ALT normalization. Notably, patients with genotype B achieved early virological response (HBV DNA < 2.6 log copies/mL at 6 months) significantly more frequently than those with genotype C

Table 1 Demographic and clinical characteristics of the 28 lamivudine-resistant patients at the start of adefovir addition to the treatment

	Overall (n = 28)*	Genotype B (n = 7)	Genotype C (n = 20)
Age (years), median (range)	53.5 (18–72)	51.0 (18–72)	53.5 (35–68)
Male patients, no. (%)	20 (71.4)	5 (71.4)	14 (70.0)
Patients with cirrhosis, no. (%)	8 (28.6)	1 (14.3)	7 (35.0)
Patients with HCC, no. (%)	7 (25.0)	0 (0)	7 (35.0)
HBeAg positive, no. (%)	17 (60.7)	3 (42.9)	13 (65.0)
HBV DNA (log copies/mL), median (range)	7.6 (4.3 to >7.6)	7.2 (5.3 to >7.6)	7.6 (4.3 to >7.6)
Patients with rtM204 mutation (M:I:V:I/V, no.)	1:14:11:2	1:3:2:1	0:11:8:1
ALT (IU/L), median (range)	86.5 (29–1027)	314.0 (47–760)	78.5 (29–1027)
T. Bil (mg/dL), median (range)	1.1 (0.5–4.5)	1.1 (0.5–1.5)	1.1 (0.5–4.5)
Albumin (g/dL), median (range)	4.1 (2.7–4.8)	4.2 (3.8–4.8)	4.0 (2.7–4.6)
Serum creatinine (mg/dL), median (range)	0.7 (0.4–1.2)	0.7 (0.6–1.2)	0.7 (0.4–1.2)
Prior lamivudine therapy (month), median (range)	28.6 (2–76)	36.5 (2–76)	28.6 (5–65)

HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; T. Bil, total bilirubin. *One patient had genotype A HBV.

Table 2 Virological and biochemical response to lamivudine and adefovir combination therapy during a median of 47 months

	Months of treatment						
Response	0 (n = 28)	6 (n = 28)	12 (n = 27)	24 (n = 25)	36 (n = 22)	48 (n = 13)	60 (n = 7)
HBV DNA < 2.6	0 (0)	11 (39.3)	15 (55.6)	20 (80.0)	19 (86.4)	12 (92.3)	6 (85.7)
HBV DNA 2.6 to <5.0	1 (3.6)	15 (53.6)	11 (40.7)	5 (20.0)	3 (13.6)	1 (7.7)	1 (14.3)
HBV DNA ≥ 5.0	27 (96.4)	2 (7.1)	1 (3.7)	0 (0)	0 (0)	0 (0)	0 (0)
ALT normalization*	NA	16 (57.1)	19 (70.4)	21 (84.0)	18 (81.8)	10 (76.9)	6 (85.7)
HBeAg disappearance†	NA	1/17 (5.9)	4/17 (23.5)	4/16 (25.0)	8/13 (61.5)	7/8 (87.5)	4/5 (80.0)
Virological breakthrough	NA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (14.3)

Values are shown as numbers of patients followed by percentages in parentheses. NA, not applicable. *ALT ≤ 35 IU/L. †Values are shown as numbers of patients/total followed by percentages in parentheses.

[5/7 (71%) vs. 5/20 (25%), P = 0.0427]. Although the status of HBeAg at the start of ADV seemed to influence the response, the difference was not significant (Figs 1c,d). Among 17 HBeAg-positive patients, HBeAg disappeared in 6% at 6 months, 24% at 12 months, 25% at 24 months, 62% at 36 months, and 88% at 48 months. There was no patient with hepatitis B surface antigen (HBsAg) loss during follow-up in this study.

Three of 22 patients who were treated for more than 36 months did not achieve virological response. One of them developed virological breakthrough after 54 months of combination therapy. The other patients had 2.8 and 3.5 log copies/mL of serum HBV DNA at 36 months of therapy but did not develop breakthrough. None of the patients experienced biochemical breakthrough. One patient with HCC died of HCC progression at 9 months after ADV. None of the 21 patients without HCC at the start of ADV developed HCC during follow-up.

The renal toxicity with a ≥0.3 mg/dL increase in serum creatinine level was observed in five of the 28 patients. Two of them had a ≥0.5 mg/dL increase: the serum creatinine levels were increased from 0.8 to 1.4 mg/dL after 31 months in a patient, and from 0.9 to 1.7 mg/dL after 34 months in another patient. As their eGFR levels were lowered to 39 and 29 mL/min, the dosage of ADV was reduced to alternate-day administration. After the reduction of ADV, their serum creatinine and eGFR recovered.

Profile of a patient with lamivudine and adefovir dipivoxil resistance

He was a 53-year-old Japanese man with HBeAg-positive liver cirrhosis at the start of 3TC monotherapy in April 2002. The genotype of HBV was found to be genotype C. His clinical course is shown in Fig. 2. He developed breakthrough hepatitis with serum HBV DNA of >7.6 log copies/mL and alanine aminotransferase (ALT) of 236 IU/L in March 2003. ADV was added to the ongoing 3TC therapy in June 2003, and HBV DNA was gradually reduced reaching <2.6 log copies/mL 3 years later. However, virological

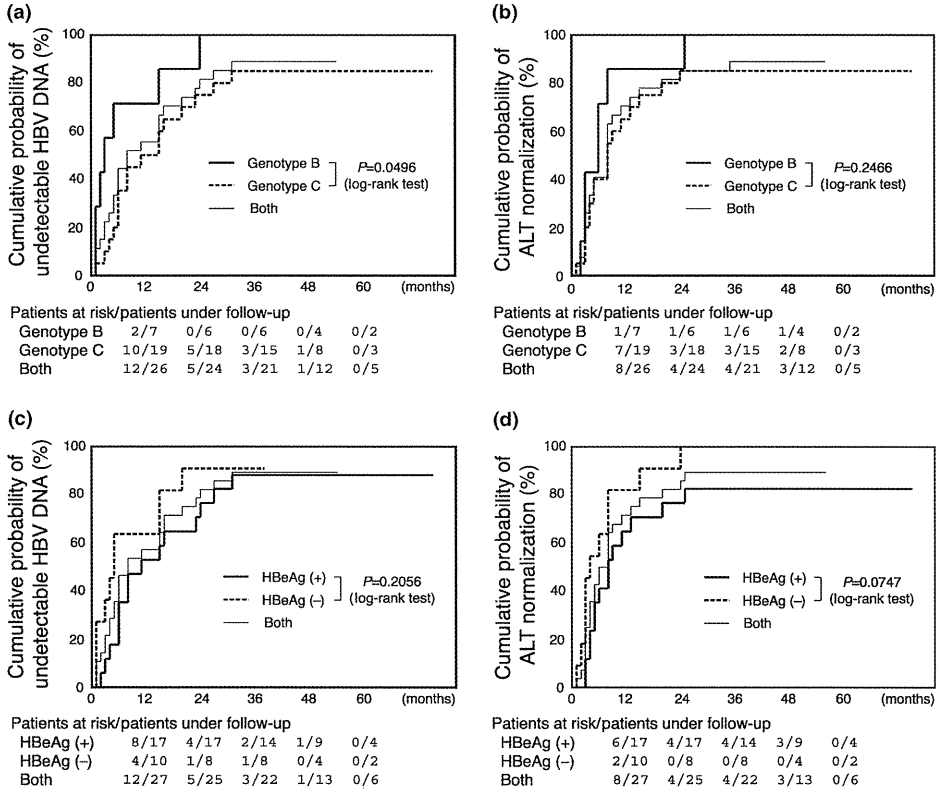


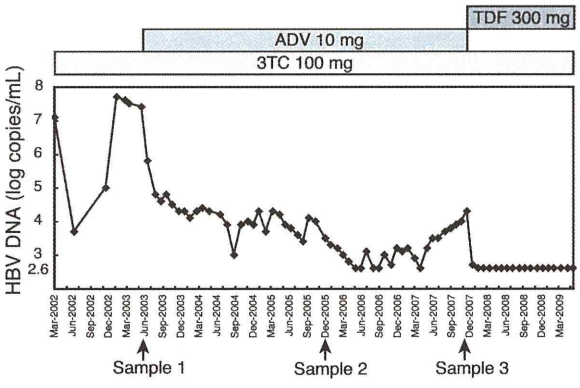
Fig. 1 Cumulative probability of virological or biochemical response during lamivudine (3TC) and adefovir dipivoxil (ADV) combination therapy. (a) Cumulative probability of undetectable HBV DNA (<2.6 log copies/mL) in patients with genotype B and those with genotype C. (b) Cumulative probability of ALT normalization (≤35 IU/L) in patients with genotype B and those with genotype C. (c) Cumulative probability of undetectable HBV DNA in HBeAg-positive patients and HBeAg-negative patients. (d) Cumulative probability of ALT normalization in HBeAg-positive patients and HBeAg-negative patients.

breakthrough was observed at 4 years after starting ADV, and his HBV DNA reached 4.3 log copies/mL in December 2007. Because his liver was cirrhotic and the hepatic functional reserve was impaired, combination therapy of tenofovir disoproxil fumarate (TDF) and 3TC was started before ALT flair. Two months later, his HBV DNA was suppressed to <2.6 log copies/mL, and viral breakthrough has not been observed to date (20 months later).

Mutations found in the HBV reverse transcriptase region of the lamivudine and adefovir dipivoxil-resistant patient

To investigate the mutations responsible for the viral breakthrough during the 3TC and ADV combination therapy, nucleotide sequences of the HBV RT region of the patient were compared between 3 time points: at the beginning of ADV treatment, at 30 months after ADV therapy, and at the time of viral breakthrough (54 months after ADV therapy). Direct sequencing analysis showed 10 amino acid changes during the clinical course (Fig. 2). The 3TC-resistant mutation of rtM204I changed to rtM204V after ADV treatment. Along with the change, the mixed mutation of rtL180L/M changed to rtL180M, which was reported to emerge with rtM204V during 3TC therapy [9]. The rtN236T mutation, which is a known ADV-resistance mutation [11], emerged as a mixed mutation with wild type (rtN236N/T) after viral breakthrough. Notably, rtA200V, which has never been reported as an ADV-resistant mutation, emerged also after viral breakthrough as a mixed mutation (rtA200V/A). Meanwhile, no specific mutation was found in the 2 patients without virological breakthrough who did not achieve virological response after 3 years of the combination therapy.

Clonal analysis was performed to examine the significance of these mutations of the RT region (Table S1). Several minor mutations were found during the 3TC and ADV therapy. After viral breakthrough, rtA200V was found in 63% of the clones, while rtN236T was found in only 25% of the clones. Therefore, rtA200V seemed to be responsible for the treatment failure of ADV. Moreover, rtT184S and S202C, which were reported as ETV resistance–associated mutations [29], were found as a minor population.



RT mutation	L80	P109	D134	R153	L180	A200	M204	V214	L229	N236
Sample 1	I/L	P/Q	G/D	R/Q	L/M	–	I	–	L/W	–
Sample 2	–	–	–	–	M	–	V	A	–	–
Sample 3	–	–	G/D	–	M	V/A	V	–	–	N/T

Fig. 2 Clinical course of a lamivudine (3TC)-resistant patient who developed virological breakthrough during 3TC and adefovir dipivoxil (ADV) combination therapy, and changes of amino acids in the reverse transcriptase (RT) region detected by direct sequencing analysis. After breakthrough, therapy was switched to 3TC plus tenofovir disoproxil fumarate (TDF) combination. The arrows indicate the time point when serum samples were obtained for sequencing analysis. Sample 1, 2, and 3 were obtained at the start of ADV, 30 months after ADV, and 54 months after ADV, respectively.

To investigate further the mutant populations, the combinations of these mutations and 3TC-resistant mutations were analysed (Fig. 3). At 30 months after ADV therapy, 100% of clones had mutations rtL180M+M204V. Subsequently, the mutations of rtT184S, A200V, S202C, and N236T emerged in the rtL180M+M204V clones after viral breakthrough. Of note, rtN236T was not found in clones without rtA200V.

Replication capacity and drug susceptibility of HBV mutants

We analysed the replication capacity of HBV clones with combined mutations as shown in Fig. 3. A clone with rtL180M+M204V+N236T mutations, which was not found in the patient, was also included for comparison. Consistent with a previous report [30], 3TC-resistant mutations of rtM204I or rtL180M+M204V lowered the replication capacity significantly in comparison with the wild-type clone (Table 3). From additional mutations to rtL180M+M204V found in the patient, only rtA200V restored the impaired replication capacity significantly. The ETV-resistant mutation of rtT184S and rtS202C did not seem to have such an effect. The ADV-resistant mutation, rtN236T, lowered the replication capacity further, and rtA200V did not restore the lowered capacity caused by rtN236T.

The 7 HBV clones with mutations in the RT region were analysed for their susceptibility to ADV. The IC_{50} of each clone is shown in Table 3. The clones with the 3TC-resistant mutations of rtM204I or rtL180M+M204V showed moderate resistance to ADV. In comparison with the clone with rtL180M+M204V, clones with additional mutations of rtT184S, A200V, or S202C showed significantly higher resistance to ADV. An additional mutation of rtN236T led to much greater resistance to ADV. Taking into account the results from the clonal analysis of serum samples and the replication capacity of each clone, rtA200V may be responsible for the treatment failure of 3TC+ADV therapy when it presents with 3TC-resistant mutations such as rtL180M+M204V. The mutations of rtT184S or S202C with rtL180M+M204V also confer ADV resistance, but the clones

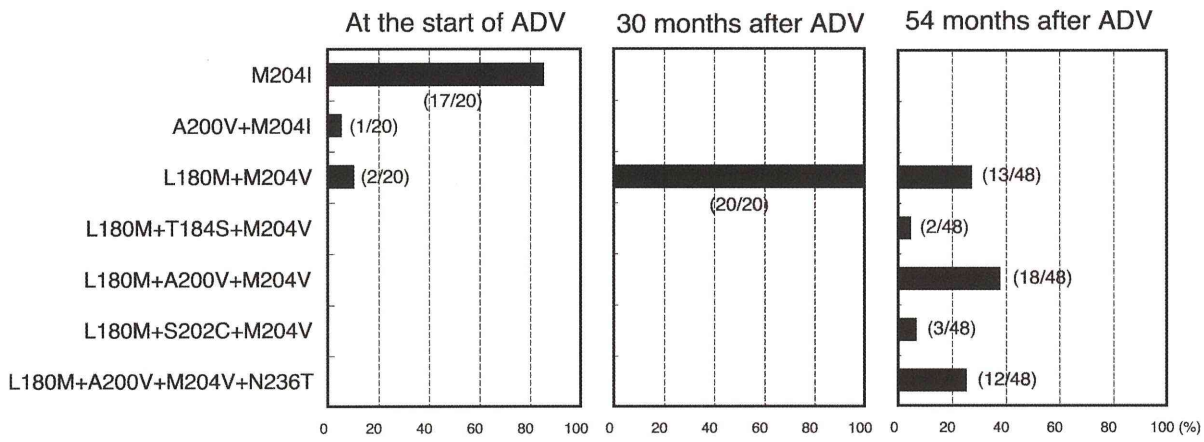


Fig. 3 Clonal analysis of HBV obtained from the patient with 3TC and ADV resistance. The serum samples were collected at the time points indicated in Fig. 2. The percentages (no. of clones/total in parentheses) of the clones with the combined mutations in the RT region are shown.

Table 3 Replication capacity and susceptibility to adefovir of the HBV mutants

HBV mutants	HBV DNA ($\times 10^7$ log copies/mL)*	Fold replication†	IC_{50} (μM)*	Fold resistance†
Wild type	13.60 ± 3.50	1	0.42 ± 0.06	1
M204I	2.17 ± 0.38	0.16	0.87 ± 0.2	2.07
L180M+M204V	4.38 ± 0.77	0.32	0.73 ± 0.06	1.74
L180M+T184S+M204V	5.98 ± 0.80	0.44	0.91 ± 0.04	2.17‡
L180M+A200V+M204V	8.90 ± 0.56	0.65‡	1.09 ± 0.12	2.60‡
L180M+S202C+M204V	4.86 ± 0.19	0.36	2.19 ± 0.63	5.21‡
L180M+M204V+N236T	0.88 ± 0.68	0.07‡	>10	>25
L180M+A200V+M204V+N236T	0.54 ± 0.38	0.04‡	>10	>25

*Values are expressed as means ± SD of experiments performed in triplicate. †(Mean value of the mutant)/(mean value of the wild type). ‡$P < 0.05$ in comparison with the clone with rtL180M+M204V.

with these mutations were not major, because they had no effect in enhancing the replication capacity of HBV.

DISCUSSION

As clinical and histological improvement accompanies reductions in HBV replication, therapies that reduce HBV replication are expected to limit the progression of liver disease and improve the natural history of chronic HBV infection [10]. Currently, the management of hepatitis B patients with drug resistance is one of the major problems in clinical practice for hepatitis B. A substantial part of 3TC-treated patients has mutant HBV with the YMDD mutation, and several clinical trials to treat 3TC-resistant hepatitis B have been performed. It has been reported first that with ADV alone and in combination with 3TC, the viral and biochemical responses were the same for 3TC-resistant patients in a 1 year study [31]. However, several studies of longer term treatment have shown that adding ADV was superior to switching to ADV monotherapy for patients with 3TC resistance [32–34]. In this study, we demonstrated that the add-on ADV therapy for 3TC-resistant hepatitis B patients effectively suppressed serum HBV DNA for a median of 47 months. Moreover, the biochemical response of ALT normalization was achieved in 77% patients and HBeAg loss in 88% of the HBeAg-positive patients at 48 months. The undetectability of HBV DNA was assessed by the Amplicor HBV monitor test, but recently, this can be assessed by a more sensitive real-time assay such as the Cobas TaqMan HBV test (Roche Diagnostics). The treatment duration to achieve HBV DNA undetectability might be longer if a more sensitive assay was used.

The influence of HBV genotype on the response or resistance to ADV has not been clarified, whereas the efficacy to 3TC was reported to be different between HBV genotypes [24,25]. This study showed that the virological response to 3TC+ADV was significantly earlier in genotype B than in C. However, there were several limitations of the results: the patients with genotype B were fewer, and no multivariate analysis was performed. In addition, all patients with HCC were genotype C, and ALT levels of genotype B tended to be higher, although there were no significant differences. The effect of genotype on the response to 3TC±ADV should be confirmed in larger studies. The baseline HBeAg status in 3TC+ADV combination therapy in 3TC-resistant patients was reported to influence the viral response: HBeAg-negative patients showed better virological and biological response [35]. In this study, the same tendency was observed, but the difference was not significant.

Initial virological suppression by ADV monotherapy was reported to be a good prognostic factor for the treatment of both naïve patients [36] and 3TC-resistant patients [37]. Taking into account the results of this study and previous reports, it is suggested that patients with genotype B HBV might develop resistance to 3TC+ADV less frequently than those with genotype C. In fact, the 3TC+ADV-resistant patient in this study was infected with genotype C HBV. Because the development of resistance to 3TC+ADV combination therapy is rare [22,35], it is difficult to evaluate whether the early virological response or genotype B is associated with the lower frequency of resistance to 3TC+ADV combination therapy. Further long-term study is needed to clarify this issue.

Although the emergence of resistance in this study was rare during the combination therapy as previously reported [22,35], one patient developed virological breakthrough after 4.5 years. We identified a characteristic mutation pattern of HBV in this patient. The mutation of rtA200V rescued the *in vitro* replication capacity that was impaired by rtL180M+M204V and reduced the susceptibility to ADV. In previous reports, rtA200V emerged as an additional mutation with the 3TC-resistant mutation in patients under 3TC monotherapy [38,39]. The effect of this mutation is not as strong as the effect of rtM204I/V±L180M on 3TC susceptibility *in vitro*, which showed >1000-fold resistance [40]. However, the clinical dose of ADV is comparatively low

because of renal toxicity [41], and the weakly resistant profile *in vitro* can explain the great clinical impact. Villet *et al.* reported that rtA200V was observed in a patient with 3TC monotherapy, and it was no longer detected after the combination therapy with ADV and 3TC [39]. The difference of results between the previous study and our study may be because of the emergence of mutations with a potent effect on ADV resistance, such as rtV173L and rtA181V, in the previous study. Because these mutations may have a greater effect on ADV resistance than rtA200V, the HBV clones with rtA200V seemed to disappear in the previous study case.

The known ADV-resistant mutation of rtN236T was found in only 25% clones, exclusively with rtA200V. This may indicate that rtN236T appeared after the emergence of rtA200V. In the active replication of the clones with rtA200V, which restored the replication capacity and enhanced ADV resistance, other mutations including rtN236T might occur more readily.

The rtA200V mutation is the result of nucleotide substitution C728T. This change in the overlapping S region results in an amino acid substitution affecting HBsAg: Leu to Phe at aa192 (sL192F). There is a possibility that sL192F may affect the replication capacity of HBV, but the actual mechanism is unknown.

Interestingly, the ETV-resistant mutations of rtT184S and rtS202C were also detected during 3TC+ADV combination therapy by clonal analysis. These mutations confer ETV resistance in the presence of the 3TC-resistant mutations of rtM204I/V±L180M [21]. This study showed that these mutations also have an ADV-resistance profile. These mutations may not cause viral breakthrough, because the population of these mutants in the patient was minor (4% and 6%, respectively), and their replication capacity was lower than that with rtA200V *in vitro*. The emergence of these mutations suggested that long-term 3TC+ADV therapy has the possibility of leading to multiple drug resistance including ETV resistance.

The combination therapy of 3TC and ADV is very effective with little frequency of viral breakthrough for 3TC-refractory patients. However, some patients do not achieve complete viral suppression of serum HBV DNA to under 2.6 log copies/mL. It was considered that the incomplete suppression of viral replication might favour further selection of drug-resistant mutants [42]. Although there have been a few reports of cases that showed resistance to 3TC+ADV therapy to date, the number of resistant cases will increase along with the increase in cases with long-term therapy. The 3TC- and ADV-resistant patient in this study was treated with 3TC and TDF after the virological breakthrough, and HBV DNA was promptly suppressed. Although TDF was reported to show cross-resistance with ADV *in vitro* [16,40,43], there are several reports that showed the effectiveness of TDF for ADV-refractory patients [44–46]. It is thought that the potency of TDF might result from its higher clinical dose compared to that of ADV [47].

In conclusion, this study showed that the combination therapy of 3TC and ADV effectively suppressed HBV replication in 3TC-resistant patients with chronic HBV infection for 4 years. Especially, patients with genotype B achieved earlier virological response than those with genotype C. However, one of the 28 patients developed virological breakthrough during the combination therapy over 4 years, and the HBV mutation of rtA200V, in addition to 3TC-resistant mutations, was demonstrated to contribute to the ADV resistance. Moreover, ETV-resistant mutations emerged coincidentally in minor HBV clones. The risk of emergence of multiple drug-resistant mutant should be considered in cases with long-term therapy with nucleos(t)ide analogues, especially when serum HBV DNA cannot be suppressed completely. Potent antiviral agents should be administered in such cases to prevent the emergence of multiple drug-resistant HBV mutants that are difficult to treat.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Clonal analysis of HBV RT region of samples from the patient with lamivudine and adefovir resistance.

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PLoS one

Plasma L-Cystine/L-Glutamate Imbalance Increases Tumor Necrosis Factor-Alpha from CD14+ Circulating Monocytes in Patients with Advanced Cirrhosis

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Abstract

Background and Aims: The innate immune cells can not normally respond to the pathogen in patients with decompensated cirrhosis. Previous studies reported that antigen-presenting cells take up L-Cystine (L-Cys) and secrete substantial amounts of L-Glutamate (L-Glu) via the transport system Xc- (4F2hc+xCT), and that this exchange influences the immune responses. The aim of this study is to investigate the influence of the plasma L-Cys/L-Glu imbalance observed in patients with advanced cirrhosis on the function of circulating monocytes.

Methods: We used a serum-free culture medium consistent with the average concentrations of plasma amino acids from patients with advanced cirrhosis (ACM), and examined the function of CD14+ monocytes or THP-1 under ACM that contained 0–300 nmol/mL L-Cys with LPS. In patients with advanced cirrhosis, we actually determined the TNF-alpha and xCT mRNA of monocytes, and evaluated the correlation between the plasma L-Cys/L-Glu ratio and TNF-alpha.

Results: The addition of L-Cys significantly increased the production of TNF alpha from monocytes under ACM. Monocytes with LPS and THP-1 expressed xCT and a high level of extracellular L-Cys enhanced L-Cys/L-Glu antiport, and the intracellular GSH/GSSG ratio was decreased. The L-Cys transport was inhibited by excess L-Glu. In patients with advanced cirrhosis (n = 19), the TNF-alpha and xCT mRNA of monocytes were increased according to the Child-Pugh grade. The TNF-alpha mRNA of monocytes was significantly higher in the high L-Cys/L-Glu ratio group than in the low ratio group, and the plasma TNF-alpha was significantly correlated with the L-Cys/L-Glu ratio.

Conclusions: A plasma L-Cys/L-Glu imbalance, which appears in patients with advanced cirrhosis, increased the TNF-alpha from circulating monocytes via increasing the intracellular oxidative stress. These results may reflect the immune abnormality that appears in patients with decompensated cirrhosis.

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Introduction

Circulating levels of proinflammatory cytokines such as TNF-alpha, IL-1 beta and IL-6 are increased in patients with cirrhosis [1,2,3]. Endotoxemia has been assumed to be responsible for the increased of such cytokines in patients with cirrhosis [4], because the activation of monocytes, macrophages and dendritc cells (DCs) by lipopolysaccharide (LPS) plays a key role in the pathogenesis of cytokine overproduction. This overproduction of proinflammatory cytokines leads to various complications, such as spontaneous bacterial peritonitis (SBP) and hepatorenal syndrome (HRS) in patients with advanced cirrhosis [5,6].

On the other hand, various types of amino acid imbalance appear in the plasma of patients with decompensated cirrhosis, since the liver plays a major role in metabolism involving glucose, lipids, vitamins,minerals and amino acids. An imbalance of plasma amino acids, with decreased levels of branched-chain amino acids (BCAAs) and increased levels of aromatic amino acids (AAAs), is commonly seen in patients with advanced cirrhosis [7]. Previously, we reported that extracellular branched-chain amino acids (BCAAs) regulate the maturation and function of monocyte derived dendritic cells [8], and that the addition of branched chain amino acids enhances the maturation and function of myeloid dendritic cells ex vivo in patients with advanced cirrhosis [9]. However, it is not clear whether the imbalance of amino acids other than BCAAs influence the immune responses in patients with advanced cirrhosis. A previous study showed that the concentration of plasma L- Cystine (L-Cys) is higher in patients with cirrhosis and shows a wide range of variation [10]. Increased levels of L-Glutamine (L-Gln) and decreasing levels of

L-Glutamate (L-Glu) are seen in patients with advanced cirrhosis, because the L-Glu-L-Gln exchange regulates the high levels of toxic ammonia in such patients [11]. Furthermore, previous studies demonstrated that antigen-presenting cells take up L-Cys via the Na-independent anionic amino acid transport system Xc^-(4F2hc+xCT) and secrete substantial amounts of L-Glu, influencing the immune-responses through this exchange [12,13,14]. This transporter is composed of two protein components, xCT and 4F2hc (CD98), and the transport activity is thought to be mediated by xCT [15,16]. The aim of this study is to investigate the influence of the extracellular L-Cys/L-Glu imbalance observed in patients with advanced cirrhosis on the function of peripheral monocytes using a serum-free culture medium with the average concentration of plasma amino acids from patients with advanced cirrhosis [9], thereby approximating the actual environment of the living body.

Materials and Methods

Ethics Statement

Written informed consent was obtained from each individual and the study protocol was approved by the Ethics Committee of Tohoku University School of Medicine (2009-209, 2009-535).

Monocyte count and isolation

In patients with cirrhosis, the monocyte and lymphocyte counts were measured by a Beckman Coulter LH 750 Analyzer (Fullerton, CA, USA). PBMCs were separated from the peripheral blood of healthy volunteers or patients with cirrhosis by centrifugation on a density gradient. The CD14-positive monocytes were isolated from PBMCs using magnetic microbeads (Miltenyi Biotec, Bergish Gladbach).

The serum free culture media used in this study

A serum free culture medium with the average concentration of plasma amino acids from healthy volunteers (HCM), that from patients with advanced cirrhosis (ACM) and complete culture media (CCM) were described previously [9]. Other components except amino acids were identical among media. Various concentrations of L-Cys were added to L-Cys free ACM , and the final concentration was adjusted to 0–300 nmol/mL (Table S1). We cultured CD14+monocytes, THP-1, Jurkat and Molt-4 under the these media with stimulant and measured the amino acid concentrations of these media. The viability of monocytes and PBMCs was determined using Annexin V^{FITC}, with dead cells identified by propidium iodide (PI) staining (Annexin V^{-FITC} Apoptosis Detection Kit, BioVision, Mountain View, CA), according to the manufacturer's instructions. We confirmed the viability of PBMCs cultured in ACM and ACM plus L-Cys to be equal to that of complete culture medium (CCM) and X-VIVO 10 (Cambrex Bio Science Walkersville, Inc. Walkersville, MD USA).

Patients and Healthy volunteers

The concentrations of the plasma amino acids from fasting patients with chronic hepatitis (n = 17), and patients with cirrhosis (n = 130) were measured by high-performance liquid

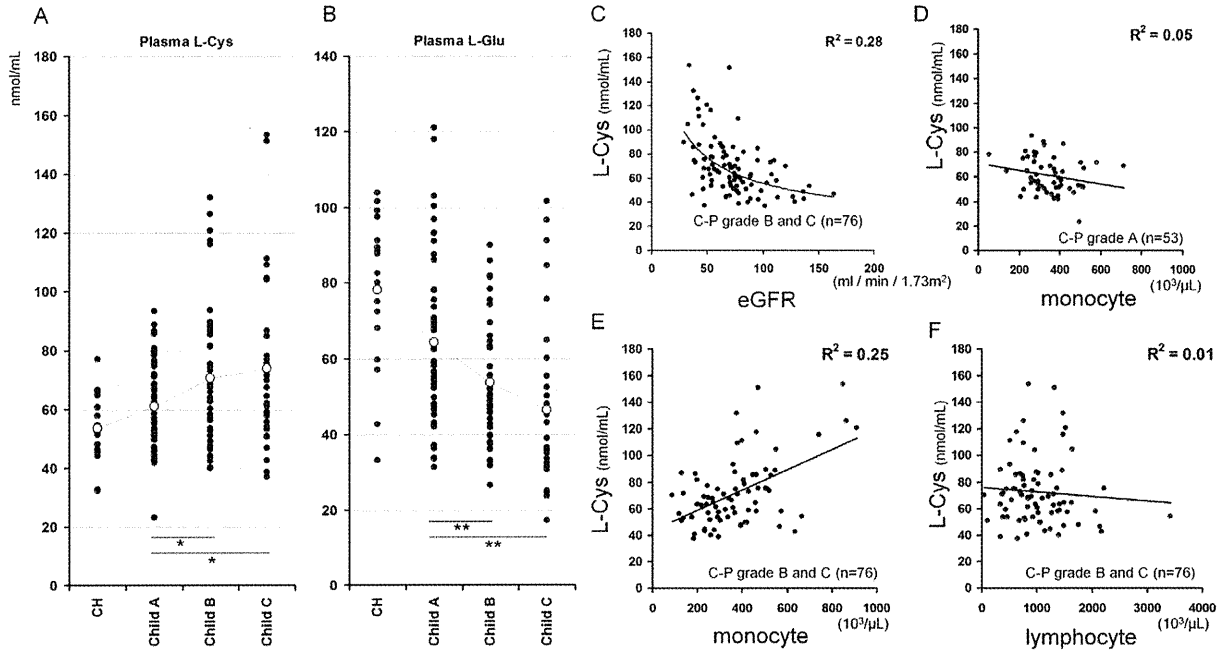

Figure 1. The counts of peripheral monocyte were increased in association with plasma L-Cystine in patients with advanced cirrhosis. A, B, The concentrations of plasma L-Cys in patients with cirrhosis was increased and that of plasma L-Glu was decreased acccording to Child-Pugh grade. The levels of plasma L-Cys and L-Glu in patients with cirrrhosis (n = 130) were measured using HPLC and classfied by the Child-Pugh classfication. C, Nonliner regression model was used to model variation in plasma L-Cys and eGFR. D, E,F, Linear regression model was used to model variation in plasma L-Cys and monocyte and lymphocyte counts. Individual correlations between plasma L-Cys levels and monocyte counts in patients with early cirrhosis (D), that in patients with advanced cirrhosis (E), and lymphocyte counts in patients with advanced cirrhosis (F). A,B, **, $p<0.01$, _*, $p<0.05$ vs Child-Pugh grade A. Statistical significance was determined by one-way ANOVA and Dunnett's post-hoc procedure. C, D, E, F, R^2 represents coefficient of determination.
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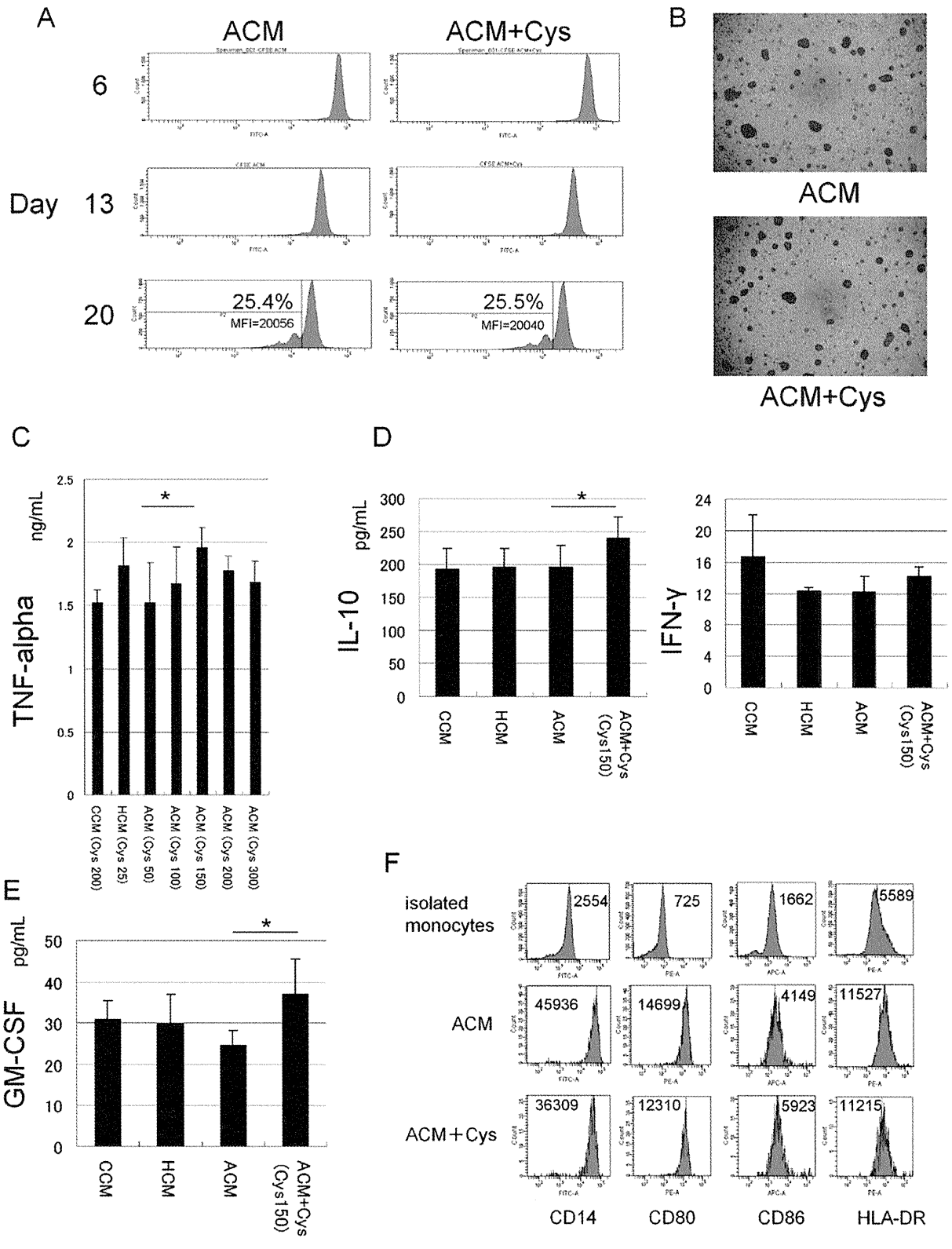
A
ACM
ACM+Cys
6
Day 13
20
25.4%
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B
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TNF-alpha
ng/mL
CCM (Cys 200)
HCM (Cys 25)
ACM (Cys 50)
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D
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IFN-γ
CCM
HCM
ACM
ACM+Cys (Cys150)
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GM-CSF
pg/mL
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isolated monocytes
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2554
725
1662
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45936
14699
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11527
36309
12310
5923
11215
CD14
CD80
CD86
HLA-DR

Figure 2. L-Cystine dose-dependently increased TNF-alpha from CD14+ monocytes with LPS under the amino acid environment of patients with advanced cirrhosis. A, Isolated CD14+ monocytes (purity >90%) were cultured at a density of 2.5×10^5 cells/well in 96-well plates containing in ACM and ACM plus L-Cys (L-Cys : 150 nmol/mL) with 1,000 U/mL M-CSF. One half the amount of culture fluid was exchanged every one day. Cells were maintained for 20 days and the proliferation rate of the cells was measured using CFSE staining. B, Influence of L-Cys on microscopic appearance of monocyte proliferation under serum-free conditions. Day 20, cells in firmly adherent clusters in both ACM and ACM+Cys. C, Monocytes were cultured under CCM, HCM, ACM and ACM plus L-Cys (100–300 nmol/mL). Cells were pre-incubated at a density of 2.5×10^5 cells/well in 96-well flat-bottom plates for 2 hours in each of the media, and 100 ng/mL LPS was added. The supernatants were collected after 24 hours and immediately TNF-alpha was determined by specific cytokine ELISA kits. D,E, Similarly as in Fig. 2C, IL-10, IFN gamma from monocytes and GM-CSF from PBMCs under CCM, HCM, ACM, ACM+Cys (L-Cys 150 nmol/mL) were measured with ELISA. F, Cells were harvested after 24 hours, stained with different mAbs, and analyzed using flow cytometry. Cells were stained with FITC-labeled anti-CD14, -CD80, -CD86, and -HLA-DR. A, B and F results are representative of four experiments from three different donors. C, D and E, Mean ± SEM values from five different donors are shown. C,D,E *, $p<0.05$ vs ACM (paired Student's t test, two-tailed).
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chromatography (HPLC) in the early morning. Briefly, sulfosalicylic acid was added to the plasma to a final concentration of 5%. The samples were then placed on ice for 15 minutes followed by centrifugation to remove precipitated proteins. The extracts were then analyzed for the amino acid content with a JLC-500/V (Japan Electron Optics Laboratories, Tokyo, Japan). Also, the patients with cirrhosis were classified according to the Child-Pugh classification. We defined as Child-Pugh grade B or C the patients with advanced cirrhosis. The estimated glomerular filtration rate (eGFR) was calculated using the new Japanese equation [17].

We selected nineteen patients with cirrhosis for in vitro or ex vivo studies (Table S2). All of these patients were inpatients. The MELD score [18] was calculated by an on-line worksheet available on the internet at www.mayoclinic.org/meld/mayomodel5.html.

Monocyte proliferation assay

Monocytes were cultured at a density of 2.5×10^5 cells/well in 96-well plates containing each media with 1,000 U/mL M-CSF (PEPROTECH EC, London, UK). One half the amount of culture fluid was exchanged every one day. Cells were maintained for 20 days and the proliferation rate of the cells was measured using Carboxyfluorescein Succinimidyl Ester (CFSE) staining; CellTrace CFSE Cell Proliferation Kit (Molecular Probes, Oregon). The staining methods followed the manufacturer's protocol.

Cytokine analysis

PBMCs or monocytes were preincubated at a density of 2.5×10^5 cells/well in 96-well flat-bottom plates (CORNING, NY) for 2 hours in each of the media, and 100 ng/mL LPS (Escherichia coli 026:B6 (SIGMA) were added. The supernatants were collected after 24 hours and immediately TNF-alpha, IFN-gamma, IL-10, GM-CSF were determined by specific cytokine ELISA kits (Bender MedSystems) according to the manufacturer's instructions.

Surface marker analysis

Monocytes were harvested and labeled with FITC-, PE- and APC-labeled monoclonal antibodies (mAbs) (anti-human CD14, CD80, CD86, CD98, HLA-DR, or the relevant isotype controls: BD PharMingen, San Diego, CA), according to the manufacturer's instructions. On xCT expression, indirect staining was performed; primary antibody (xCT (H-121) scc-98552: Santa Cruz) secondary antibody (goat anti-rabbit IgG-FITC sc-2012: Santa Cruz) Using a FACS Canto II (BD Immunocytometry Systems, San Diego, CA) flow cytometer, surface marker expressions were analyzed using the BD FACSDiva (BD Immunocytometry Systems) program.

Intra-extracellular amino acid quantification

The THP-1, Jurkat and Molt4 cells were pre-incubated for 2 hr in ACM, then 1.0×10^7 cells were re-suspended with LPS (100 ng/mL) or IL-2 (1000 IU/mL) in 1 mL of ACM, L-Cys-free ACM or ACM with L-Cys. After 2 hr incubation, the supernatants were measured by HPLC for the extracellular amino acid quantification. The concentration of the intracellular amino acids was determined as described in ref [19]. Briefly, cells were washed two times by PBS and resuspended in 500 µL PBS sonicated with four 10-s pulses. Cell debris was removed by centrifugation, and sulfosalicylic acid was added to the supernatant to a final concentration of 2%. The samples were then placed on ice for 30 min followed by centrifugation to remove precipitated proteins. The extracts were then analyzed for amino acid content with an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo).

Measurement of reduced glutathione (GSH) and oxidized glutathione (GSSG)

CD14+ monocytes were pre incubated at a density of 2.0×10^5 cells/well in 96-well plates containing HCM for 2 h and then cultured in HCM, ACM or ACM plus L-Cys for an additional 2 h. The culture medium was carefully removed from the wells. 100 µl of prepared GSH-Glo™ Reagent were added to each well of a 96-well plate, mixed briefly on a plate shaker, and incubated at room temperature for 30 minutes. 100 µl of reconstituted Luciferin Detection Reagent were added to each well of a 96-well plate, mixed briefly on a plate shaker, and incubated for 15 minutes. luminescence was read by a Lumino Skan Ascent (Thermo BioAnalysis, Helsinki, Finland).

Real-time PCR

THP-1, Jurkat, Molt-4 and CD14+ monocytes were collected. After the extraction of total RNA and the RT procedure, real-time PCR using aTaqMan Chemistry System () was carried out. The ready-made sets of primers and probes for the amplification of xCT (Assay ID : Hs00921937_m1), TNF-alpha (Assay ID : Hs99999043_m1) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, Assay ID : Hs02758991_g1) were purchased from Perkin-Elmer/Applied Biosystems. The relative amount of target mRNA was obtained by using a comparative threshold cycle (CT) method. The expression level of mRNAs of the Molt-4 was represented as 1.0 and the relative amounts of target mRNA in THP-1 and Jurkat were calculated according to the manufacturer's protocol. For CD14+ monocytes, The expression level of monocyte mRNA from a healthy volunteer was represented as 1.0 and the relative amounts of target mRNA in monocytes from patients were calculated.

Statistical Analysis

The data were analyzed with ANOVA, and multiple comparisons were performed with Dunnett's post-hoc procedure for the plasma aminogram. When 2 groups were analyzed, the differences between media were analyzed by the Wilcoxon t test, and the

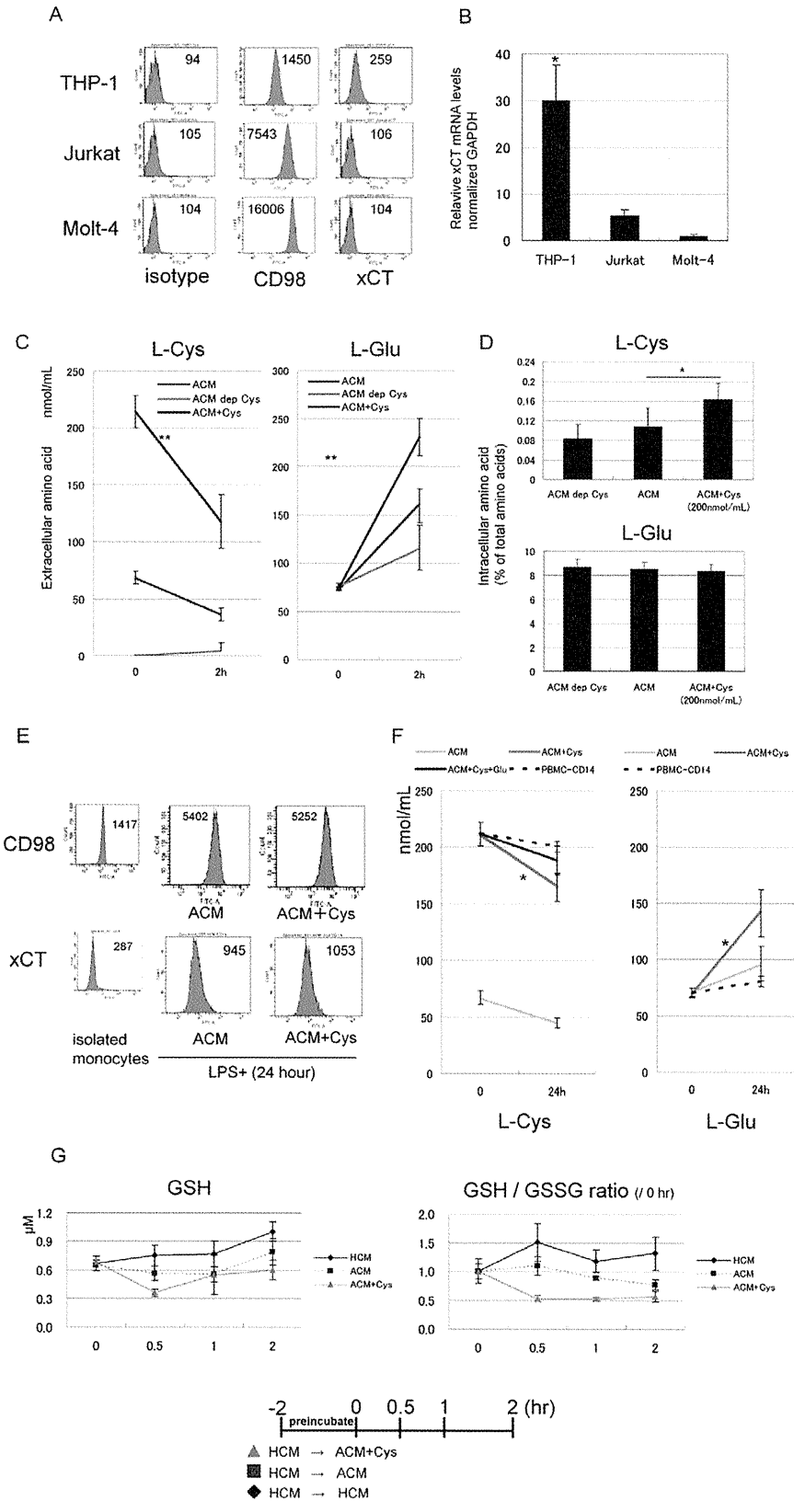
A
THP-1
Jurkat
Molt-4
94
1450
259
105
7543
106
104
16006
104
isotype
CD98
xCT
B
Relavive xCT mRNA levels normalized GAPDH
THP-1
Jurkat
Molt-4
C
L-Cys
L-Glu
nmol/mL
Extracellular amino acid
ACM
ACM dep Cys
ACM+Cys
D
L-Cys
L-Glu
Intracellular amino acid (% of total amino acids)
ACM dep Cys
ACM
ACM+Cys (200nmol/mL)
E
CD98
xCT
1417
5402
5252
287
945
1053
isolated monocytes
ACM
ACM+Cys
LPS+ (24 hour)
F
ACM
ACM+Cys
ACM+Cys+Glu
PBMC-CD14
nmol/mL
24h
L-Cys
L-Glu
G
GSH
GSH / GSSG ratio (/ 0 hr)
μM
HCM
ACM
ACM+Cys
-2
0
0.5
1
2 (hr)
preincubate
HCM → ACM+Cys
HCM → ACM
HCM → HCM

Figure 3. High levels of extracellular L-Cystine promoted the L-Cystine-L-Glutamate antiport via xCT and decreased the intracellular GSH/GSSG ratio in monocyte under the amino acid environment of patients with advanced cirrhosis. A, THP-1, Jurkat and Molt-4 cultured under CCM were harvested and labeled with antibodies (CD98, xCT or the relevant isotype controls). Using flow cytometry, surface marker expressions were analyzed. The figure expresses the mean fluorescence intensity. Data shown are representative of three independent experiments with cells. B, xCT relative mRNA levels of these cell lines were determined by real time PCR: delta-delta CT method. All mRNA expression levels were normalized to GAPDH. C, D, The THP-1 cells were pre-incubated for 2 hours in ACM, then resusupended with LPS (100 ng/mL) in 1 mL of ACM, L-Cys-free ACM and ACM plus L-Cys. The concentration of extracellular (C) and intracellular (D) amino acid was determined as described in material and methods. E, The CD14+ monocytes, cultured under ACM and ACM plus L-Cys for 24 hours, were harvested and labeled with antibodies (CD98, xCT or the relevant isotype controls). Using flow cytometry, surface marker expressions were analyzed. The figure expresses the mean fluorescence Intensity. Data shown are representative of three independent experiments with cells. F, Similarly as in Fig. 4C, the monocytes were cultured for 24 hours in ACM, L-Cys-free ACM and ACM plus L-Cys. The supernatants were measured by HPLC as extracellular amino acids quantification. G, Monocytes were pre incubated at a density of 2.0×10^5 cells/well in 96-well flat-bottom plates for 2 hours in HCM, and then cultured in HCM, ACM and ACM plus L-Cys (200 nmol/mL) for an additional 2 hours. These intracellular glutathione levels were measured by GSH-Glo™ at the time point indicated. C and D, Mean ± SD values from five independent experiments are shown. B *, $p<0.05$ vs Molt-4 (the Mann-Whitney U-test.). C,F **, $p<0.01$ *,$P<0.05$ (mean change vs ACM) D *, $p<0.05$ (paired Student's t test, two-tailed).
doi:10.1371/journal.pone.0023402.g003

differences between healthy controls and patients were analyzed by the Mann-Whitney U-test. All statistical analyses were performed with standard statistical software (SPSS 13.0 for Windows, Chicago, IL).

Results

The counts of peripheral monocytes were increased in association with the plasma L-Cystine in patients with advanced cirrhosis

Firstly, we confirmed that, in patients with advanced cirrhosis (Child-Pugh grade B or C), the plasma concentrations of L-Cys were significantly higher than in those with early cirrhosis (Figure 1A), and there was a wide range of variation. On the other hand, the plasma concentrations of L-Glu were significantly decreased along with the Child-Pugh grade (Figure 1B). In patients with advanced cirrhosis, the wide range of variation of L-Cys was attributed to the eGFR ($R^2=0.28/P=0.0000008$) (Figure 1C). These data mean that plasma L-Cys increases in decompensated cirrhosis. Secondly, we investigate whether the concentration of plasma L-Cys influenced the peripheral monocyte counts. In patients with early cirrhosis, L-Cys was not correlated with the monocyte counts ($R^2=0.05/P=0.119$) (Figure 1D), but in patients with advanced cirrhosis, it was significantly and positively correlated with the monocyte counts ($R^2=0.25/P=0.0000017$) (Figure 1E). On the other hand, the lymphocyte counts were not correlated with the concentration of plasma L-Cys ($R^2=0.01/P=0.523$) (Figure 1F). Interestingly, among all twenty kinds of free amino acids, only L-Cys was significantly correlated with the monocyte counts in patients with advanced cirrhosis (Figure S1). These data mean that, in patients with advanced cirrhosis, plasma L-Cys is increased according to renal dysfunction and influences the counts of monocytes.

Extracellular L-Cystine dose-dependently increased pro-inflammatory cytokines from CD14+ monocytes under the amino acid condition of advanced cirrhosis

Based on the result that the peripheral monocyte counts were positively correlated with the concentration of L-Cys, we hypothesized that the concentration of extracellular L-Cys could influence the proliferation of monocytes. To investigate this hypothesis, we cultured monocytes for 20 days with M-CSF under ACM or ACM plus L-Cys in vitro, and determined the proliferation of monocytes by CFSE assay. An elevated concentration of L-Cys did not influence the proliferation of monocytes (Figure 2A) on microscopic appearance, and also did not affect the morphological appearance and behavior of the cells in culture (Figure 2B). There was also no difference in the proliferation of the monocyte cell line, THP-1 between these media (data not shown). Next, to investigate whether the extracellular L-Cys level influenced the production of inflammatory cytokines from monocytes, we cultured monocytes under ACM that contained 50–300 nmol/mL L-Cys and measured the producton of TNF alpha from monocytes. The addition of L-Cys increased the production of TNF alpha from monocytes in a dose-dependent manner (Figure 2C), and the values were maximum under 150 nmol/mL L-Cys. Interestingly, this range was in remarkable agreement with the range in patients with advanced cirrhosis (Fig. 1A). The IL-10 level from monocytes was also significantly higher under ACM plus L-Cys than that under ACM (Figure 2D), and there was no difference the interferon gamma (IFN γ) level from monocytes between these media. The GM-CSF from PBMCs was also significantly higher under ACM plus L-Cys than that under ACM (Figure 2E). Regarding monocyte phenotypes, there was no difference between ACM and ACM plus L-Cys (Figure 2F). These data mean that high levels of extracellular L-Cys increased pro-inflammatory cytokines from CD14+ monocytes under the amino acid environment of patients with advanced cirrhosis.

High levels of extracellular L-Cystine promoted the L-Cystine-L-Glutamate antiport and decreased the intracellular GSH/GSSG ratio in monocyte under the amino acid environment of patients with advanced cirrhosis

We investigated whether high levels of extracellular L-Cys influence the L-Cys/L-Glu transport under the amino acid environment of patients with advanced cirrhosis.

Firstly, we determined the expression of 4F2hc (CD98) and xCT in THP-1, Jurkat and Molt-4. All cell lines expressed CD98, but only THP-1 expressed xCT at the protein level (Fig. 3A) and mRNA level (Fig. 3B). Secondly, we measured the intra-extracellular L-Cys and L-Glu concentration of THP-1 under ACM at various L-Cys levels. After 2 hours culture, ACM plus L-Cys significantly decreased the extracellular L-Cys (mean change, ACM+Cys, -96.8 ± 15.8; ACM, -32.3 ± 0.6 and ACM dep L-Cys, 4.8 ± 6.6 nmol/mL) (Fig. 3C) and significantly increased intracellular L-Cys (Fig. 3D) and extracellular L-Glu (mean change, ACM+Cys, 157.5 ± 19; ACM, 86.1 ± 17.7 and ACM dep L-Cys, 40.4 ± 24.9 nmol/mL) (Fig. 3C) more than that by ACM or ACM deprived of L-Cys. For intracellular L-Glu, there was no difference among these media. Such L-Cys/L-Glu changes were not seen for Jurkat and Molt-4 (data not shown). These data indicate that high levels of extracellular L-Cys enhances L-Cys/L-Glu antiport in the monocyte cell line THP-1. Similarly, CD14+ monocytes expressed CD98 and xCT after adding LPS (Fig. 3E) and extracellular L-Cys/L-Glu changes were seen (L-Cys mean change, ACM+Cys, -46.9 ± 5.0; ACM, -22.4 ± 6.0; ACM+Cys+Glu, -22.9 ± 6.4 and