and 24 markers reported in major histocompatibility complex (MHC) associated reports of humans (Martin et al. 1998; Foissac et al. 2000). The primers were selected from the NCBI database (http://www.ncbi.nlm.nih.gov/). PCR was performed using the Peltier Model PTC-200 Thermal Cycler (MJ Research, Tokyo, Japan). Ten microliters of reaction mixture contained 50 ng genomic DNA, 250 μmol L^{-1} each of dATP, dCTP, dGTP, and dTTP, 0.4 units of ExTaq, 0.33 μmol L^{-1} forward and reverse primers, and manufacturer's PCR buffer (all purchased from Takara Biosystems, Ohtsu, Japan). The forward primers were labeled with one of three fluorescent dyes, 6-FAM, HEX, or NED. Initial denaturation was performed at 94°C for 5 min, and 30 cycles of amplification were performed at 94°C (30 s), 55°C (30 s), and 72°C (30 s), followed by an additional extension at 72°C for 10 min. The same PCR conditions were applied to all markers.

Amplified PCR products were visualized by electrophoresis in 3.0% agarose gels stained with 0.5 mg mL^{-1} ethidium bromide under ultraviolet transillumination. To analyze microsatellite loci, PCR samples were mixed with a gel-loading cocktail containing deionized formamide and labeled size standards (GeneScan-ROX400; Applied Biosystems, CA, USA). Samples were run on an ABI Prism 3100-Avant automated by use of Genescan software (Applied Biosystems).

DNA sequencing was performed with an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems). Heterozygosity values (H) for alleles were calculated by use of the formula: $H = 1 - \sum p_i^2$ (Nei et al. 1974).

Results and discussion

Among the 148 microsatellite markers tested, at least 106 (72%) gave PCR products approximately the same size as those from human and rhesus monkey DNA (Table 1). Polymorphism across the 20 unrelated individuals tested was observed for 66 of these markers (62%). The remaining 40 primers amplified monomorphic PCR products in these 20 monkeys. The average number of alleles in 66 polymorphic loci was 5.86 (range 2–10), and the average heterozygosity was 0.63 (range 0.10–0.88, Table 1). These results are almost the same as those from an amplification experiment with rhesus monkeys based on the human genome database (Hadfield et al. 2001). Markers with heterozygosity >0.75 are regarded as highly polymorphic (Rogers et al. 2006). Among 66 polymorphic markers, 29 had heterozygosity >0.75 in this study. Six markers (D8S1100, D8S1119, D11S1975, D18S851, D18S861, and Na/HLA F2) reported to be monomorphic in rhesus monkeys were also monomorphic in cynomolgus monkeys. Among markers reported to be polymorphic in rhesus monkeys, 14 microsatellite markers were monomorphic in cynomolgus monkeys. Average heterozygosity for these markers in rhesus monkeys was 0.58 (range 0.11–0.89), and heterozygosity for six markers was >0.75 (D4S1575, D4S1592, D4S243, D4S405, D4S413, and D6S1610).

Three families consisting of 20 animals were analyzed to confirm the inheritance of the polymorphic markers. Single-gene inheritance was confirmed for all 66 polymorphic markers listed in Table 1.

Knowledge of the position of microsatellite markers on chromosomes is necessary for genome-wide screening of cynomolgus monkeys. In humans, for example, 400 markers locations have been determined one to cover all areas of the human genome with an interval of 10 cM. A greater number of polymorphic markers and their map position on the genome of cynomolgus monkeys is still required.

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Large-scale analysis of Macaca fascicularis transcripts and inference of genetic divergence between M. fascicularis and M. mulatta.

Large-scale analysis of *Macaca fascicularis* transcripts and inference of the genetic divergence between *M. fascicularis* and *M. mulatta*

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Abstract

BACKGROUND

Cynomolgus macaques (*Macaca fascicularis*) are widely used experimental animals in biomedical research and are closely related to other laboratory macaques, such as rhesus macaques (*M. mulatta*). We isolated 85,721 clones and determined 9407 full-insert sequences derived from cynomolgus monkey brain, testis, and liver. These sequences were annotated based on homology to human genes and stored in a database, QFbase [http://genebank.nibio.go.jp/qfbase/].

RESULTS

We found that 1024 transcripts did not represent any public human cDNA sequence and examined their expression using the *M. fascicularis* oligonucleotide microarrays. Significant expression was detected for 544 (51%) of the unidentified transcripts. We also identified 226 genes that contained exon-alterations in the untranslated regions of macaque transcripts, despite the highly conserved structure of coding regions. Considering the polymorphism in the common ancestors of cynomolgus and rhesus macaques and the rate of PCR errors, the divergence time of the two species was estimated to be around 0.9 Mya.

CONCLUSIONS

Transcript data of Old World monkeys provide us a method to infer not only the evolutionary difference between human and non-human primates but also to unveil hidden transcripts in the human genome. Increasing the genomic resources and information of macaque monkeys will greatly contribute to the development of evolutionary biology and biomedical sciences.

Figure 1

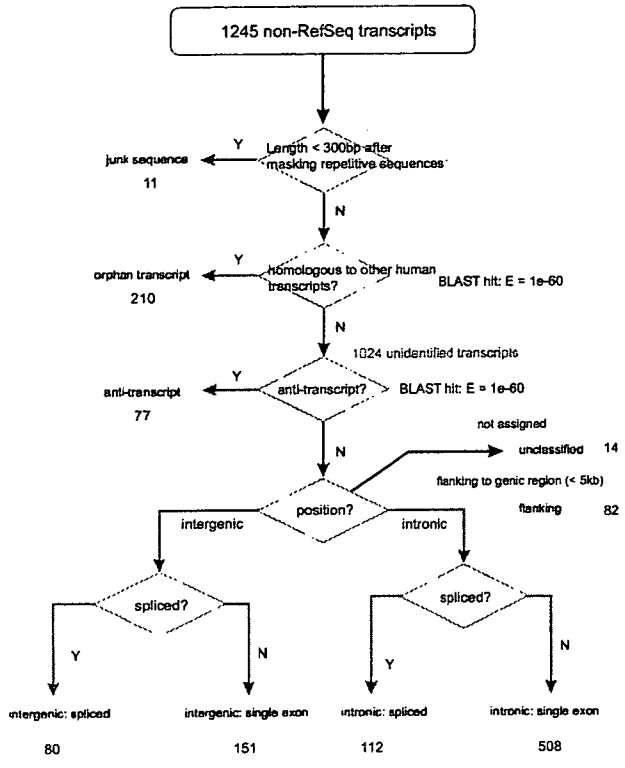


Figure 2

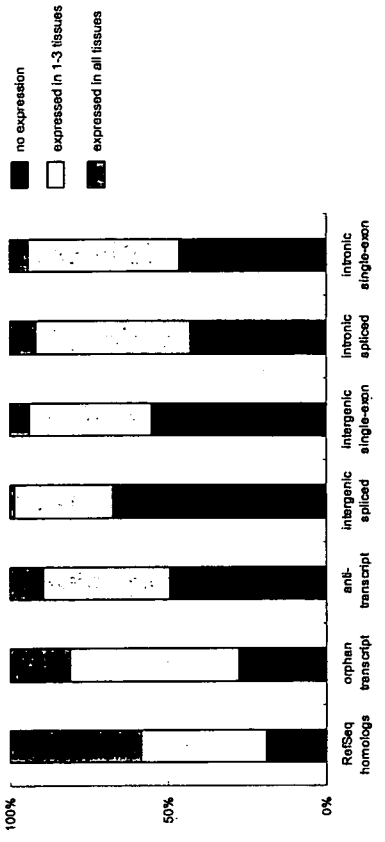


Figure 3

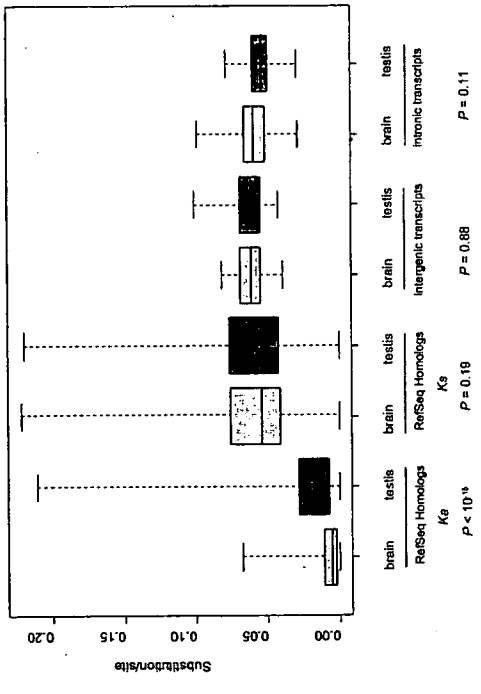


Figure 4

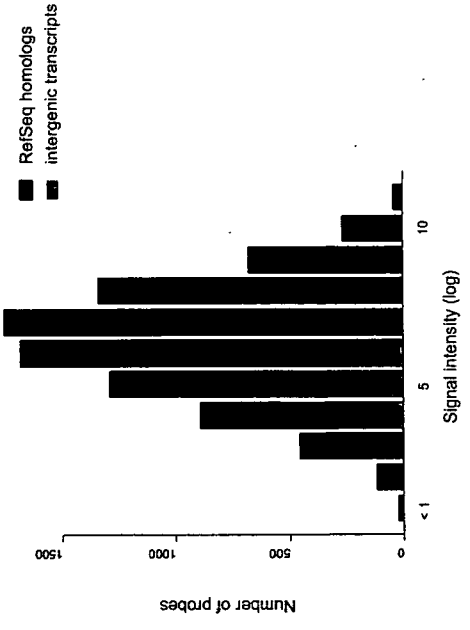


Figure 7

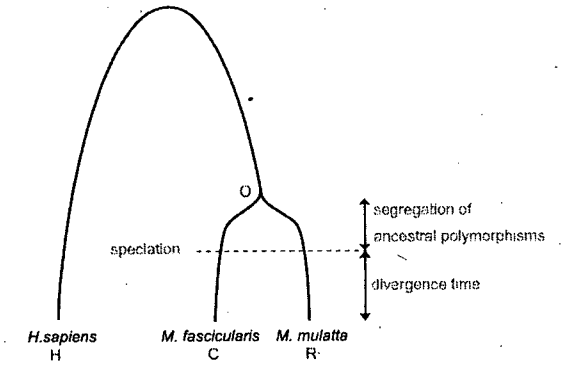


Figure 5

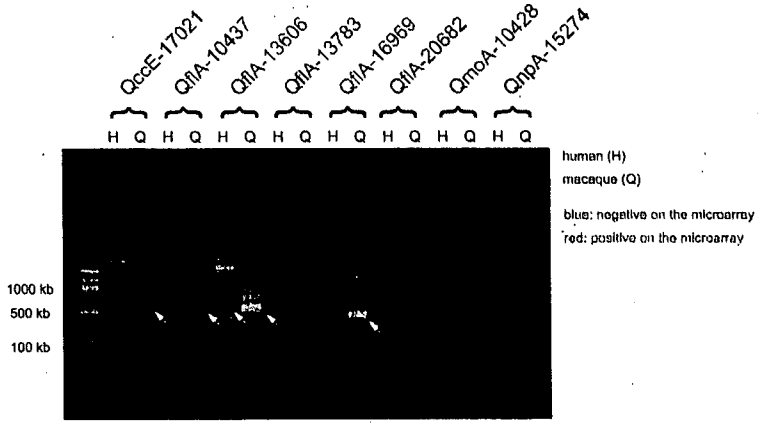
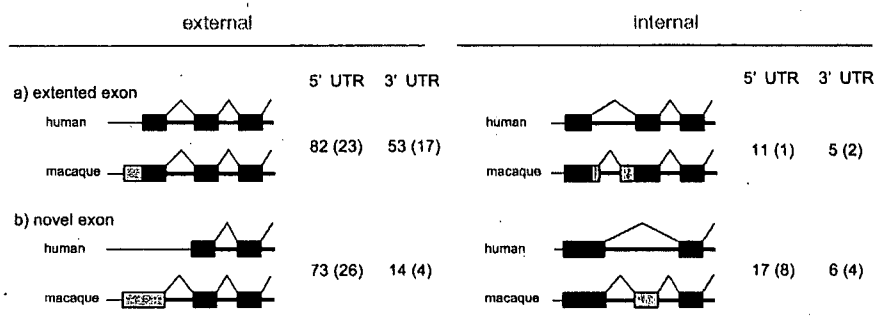


Figure 6



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Original article

GBV-B as a pleiotropic virus: distribution of GBV-B in extrahepatic tissues *in vivo*

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Abstract

GB virus B (GBV-B) infection of New World monkeys is considered to be a useful surrogate model for hepatitis C virus (HCV) infection. GBV-B replicates in the liver and induces acute resolving hepatitis but little is known whether the other organs could be permissive for the virus. We investigated the viral tropism of GBV-B in tamarins in the acute stage of viral infection and found that the viral genomic RNA could be detected in a variety of tissues. Notably, a GBV-B-infected tamarin with marked acute viremia scarcely showed a sign of hepatitis, due to preferential infection in lymphoid tissues such as lymph nodes and spleen. These results indicate that GBV-B as well as HCV is a pleiotropic virus *in vivo*.

Keywords: GB virus B; Hepatitis C virus; Tamarin; Surrogate model

1. Introduction

Over 100 million people worldwide are carriers of hepatitis C virus (HCV) and the viral infection is a significant cause of human morbidity and mortality; chronic HCV infection in many cases will lead to liver cirrhosis and hepatocellular carcinoma. Furthermore, HCV infection manifests a variety of extrahepatic, at least in part due to the extrahepatic tropisms of HCV, particularly lymphotropism diseases (for review see [1]).

Other than humans, only chimpanzees that are endangered as species can be productively infected by HCV. Together with ethical issues regarding animal experiments, it has becomes increasingly difficult to access chimpanzees for experimental studies. Tamarins (*Saguinus* species), one of the new world monkeys, develop acute, self-limited hepatitis upon infection with the GB virus B (GBV-B), which is most closely related to HCV [2–4]. Although the acute nature of GBV-B infection in tamarins has been distinguished this hepatitis from HCV infection in humans, recent studies demonstrated that tamarins could be persistently infected by GBV-B and developed chronic hepatitis [5,6]. Therefore, the GBV-B infection of tamarins is proposed as a good surrogate model for hepatitis C. While GBV-B appeared to infect liver, comprehensive documentation of the *in vivo* tropism of GBV-B has not been

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reported yet. A previous report that GBV-B RNA was observed in peripheral blood mononuclear cells (PBMCs) from a GBV-B-infected marmoset [7] suggests that GBV-B may be lymphotropic as well as HCV. Considering the close similarity between HCV and GBV-B, we examined the viral distribution and tropism in tamarins in the acute phase of the viral infection.

2. Materials and methods

2.1. Animals

Adult white-lipped and Red-handed tamarins (*Saguinus labiatus* and *Saguinus midas*, respectively) were housed in individual cages at the Tsukuba Primate Research Center. All animal studies were conducted in accordance with the protocols of experimental procedures that were approved by the Animal Welfare and Animal Care Committees of the National Institute of Biomedical Innovation and National Institute of Infectious Diseases. The details of tamarins used in this study were summarized in Table 1.

2.2. GBV-B infection in tamarins

GBV-B RNA was transcribed *in vitro* with T7 RNA polymerase (Promega, Madison, WI) from 10 μg of *Xho*I-digested pGBB [2] that harbors infectious cDNA for GBV-B (kind gift of Dr. J. Bukh, National Institutes of Health, USA). The integrity of the RNA was checked by electrophoresis through an agarose gel stained with ethidium bromide. Each transcription mixture (400 μg of GBV-B RNA) was diluted with 400 μl of ice-cold water and then immediately frozen on dry ice and stored at −80 °C. Transcription mixtures were injected into each tamarin intrahepatically. For transmission of GBV-B, animals were infected intrahepatically with 100 μl of GBV-B infectious plasma containing 8×10^8 genome equivalents (GE) of the viral RNA. Blood samples were periodically collected from the monkeys from femoral vein under anesthetization and were tested for plasma ALT level.

2.3. Quantification of GBV-B genomic RNA

GBV-B-infected tamarins were euthanized and perfused with saline thoroughly before the collection of specimens including plasma, PBMCs and a variety of tissues (esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas, submandibular gland, trachea, lung, bone marrow, thymus, spleen, submandibular lymph nodes, axillary lymph nodes, intestinal lymph nodes, mesenteric lymph nodes, inguinal lymph nodes, tonsil, heart, kidney, adrenal gland, bladder, brain, spinal cord, testis, uterus and ovary). GBV-B RNA from these specimens was quantified by a real-time, 5′ exonuclease PCR (TaqMan) assay using a primer-probe combination that recognized a portion of the GBV-B capsid gene. The primers 558F [5′AACGAGCAAAGCGCAAAGTC] and 626R [5′CATCATGGATACCAGCAATTTTGT] and probe 579P [5′6FAM-AGCGCGATGCTCGGCCTCGTATAMRA] [8] were obtained from PE Biosystems. The primers were used at 15 pmol/50 μl reaction, and the probe was used at 10 pmol/50 μl reaction. Synthesized GBV-B RNA was used as a reference standard of GBV-positive plasma. PBMCs were isolated from whole blood by density-gradient centrifugation. Approximately 10 mg of tissues were removed under sterile conditions and immediately homogenized in 1 ml of TRIzol (Invitrogen, Carlsbad, CA) to extract RNA. We set our lowest detection cutoff at 10^2 GE per ml. All the specimens were evaluated in duplicates and the averages were shown.

Table 1
Summary of the results of GBV-B RNA levels in the tissues of the virus-infected tamarins

		Tm3	Tm4	Tm5	Tm6
Animals		*S. labiatus*	*S. midas*	*S. labiatus*	*S. midas*
Sex		Female	Female	Male	Female
GBV-B inoculum		Plasma	Plasma	RNA	RNA
Weeks at necropsy		4	4	3	ND[a]
ALT		321	522	38	554
Viral loads in:					
Blood	Plasma	3.8×10^8	5.9×10^8	1.3×10^{10}	2.8×10^9
	PBMC	270	1630	35650	ND
Spleen		(−)[b]	540	5980	ND
Lymph nodes	Inguinal	(−)	(−)	3090	ND
	Intestinal	(−)	(−)	640	ND
Liver		70080	33480	16080	ND
Kidney		(−)	(−)	380	ND
Testis				600	ND
Ovary		1290	150		ND
Bone marrow		120	(−)	750	ND

Viral loads in each tissues were presented as GE/mg except for plasma (GE/ml) and PBMC (GE/10^6 cells). Data for Tm6 were obtained at week 4.
[a] ND: not done.
[b] (−): undetectable.

2.4. Detection of anti-GBV-B core and NS3 antibodies by ELISA

The TrpE-core (aa 1 to 132) fusion protein and TrpE-NS3 (aa 1135 to 1378) fusion protein, representing a portion of NS3 identified as being immunogenic in infected animals [9], was expressed in *Escherichia coli* [10] to serve as an antigen to generate polyclonal rabbit antisera. Tamarin sera were tested for the presence of antibodies to GBV-B core and NS3 by ELISA as described previously [8].

2.5. Cloning of entire GBV-B genome from plasma, liver and PBMCs of infected tamarins

GBV-B RNA was isolated from plasma, liver and PBMCs as described above. GBV-B cDNA was synthesized using SuperScript reverse transcriptase II (Invitrogen) with GB-5145R primer (5′-GCG AGT GCG GCT GTC CCA GAA GTA TTG ACT-3′) or GB-9051R primer (5′-AAT TTG GGG GTT CAG CTG ATG GCT AAT CCA-3′). After RNase H (Invitrogen) treatment at 42 °C, a cDNA mixture was subjected to PCR with LA-taq DNA polymerase (TaKaRa), GB-5145R primer and GB-35S primer (5′-ACC ACA AAC ACT CCA GTT TGT TAC ACT CCG CTA GG-3′) or GB-9051R primer and GB-3999S primer (5′-CGT ACG GCG TGA ATC CAA ATT GCT ATT TTA-3′) for 30 cycles of denaturation at 94 °C for 20 s and extension at 68 °C for 5 min. PCR products were purified from the gel using a QIA-quick gel kit (Qiagen), and then cloned into pGEM-T Easy vector (Promega). Four clones of each fragment were determined using a CEQ-2000XL analysis system with a DTCS quick start kit and GBV-B specific primers according to the manufacturer's instructions. Sequence data were analyzed on Macintosh computers with the Sequencer (Gene Code Corp.) and MacVector (Accelrys) software packages.

2.6. Synthesis of positive and negative standard RNAs for RT–PCR controls

Recombinant positive and negative strand RNAs were generated from pGBB containing 3′ sequences of GBV-B. Positions 8569–9359 were amplified and inserted into pGEM-T easy vector. Clones were selected for sense and antisense orientation of the insert corresponding to positive and negative strands, respectively. Ten micrograms of the selected plasmids were linearized using *Pst*I and positive- and negative-strand RNAs were synthesized by transcription from the upstream T7 RNA polymerase promoter by Ambion MEGAscript T7 kit (Ambion, Austin, TX).

2.7. Detection of strand-specific viral RNA by tagging PCR system

One microgram of total RNA obtained from tissues or cells was subjected to RT–PCR. cDNAs were synthesized using Superscript III first strand synthesis system (Invitrogen). In order to overcome the detection of falsely primed cDNA products and make the PCR system strand-specific, additional nucleotides (TCATGGTGGCGAATAA) were added to the 5′ end of the reverse transcription primer (5′-TCATGGTGGC GAATAATTGGATTAGCCATCAGCTGAACC-3′), forming a "tag" (underlined) [11,12]. This "tag" sequence was neither complementary nor homologous to any part of the GBV-B genome. PCR amplification of a tagged cDNA was performed using only the tag portion of the cDNA primer (5′-TCATGG TGGCGAATAA-3′) as one of the primers and a GBV-B specific oligonucleotide for the opposing primer (5′-CTTGGTAC TACGCTCTGCACA-3′, positions 9339–9359). For the first round of PCR using 2 µl of cDNA in a final volume of 25 µl, the reactions were performed using a TaKaRa PCR kit (TaKaRa) with following conditions; a 20 s and 94 °C denaturation step followed by 20 s and 55 °C annealing and 2 min and 72 °C extension steps. After 30 cycles of first round amplification, 2 µl of reaction samples were subjected to 30 cycles of nested PCR using 5′-TTTTAGGGCAGCGGCAACAG-3′ (positions 9105–9124) and 5′-CACACAGCCAGGACTCCTCA-3′ (positions 9260–9279) as primers.

2.8. Histopathology

Five tamarin livers were used in this study. Of these, three livers were from GBV-B-infected tamarins (Table 1), and two were from uninfected tamarins. Liver samples obtained by necropsy were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4 µm thick-sections. Deparaffinized sections were stained with hematoxylin and eosin (H&E) for histopathological analyses. To investigate apoptotic cells in the livers, we also examined both DNA fragmentation and immunohistochemistry for an active form of caspase-3. To diminish autofluorescence mainly caused by lipofuscin, sections were pre-stained with 1% Sudan black B. DNA fragmentation was evaluated by a TUNEL assay with an ApopTag Direct *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions. Briefly, the specimens were digested with a solution of proteinase K (20 µg/ml) in PBS for 5 min and then incubated with terminal deoxynucleotidyl transferase (TdT) and fluorescein-labeled nucleotides (ApopTag Direct) in a humid atmosphere at 37 °C for 1 h. Specimens were viewed with a BX-FLA fluorescence microscope (Olympus, Tokyo, Japan). To control for nonspecific incorporation of nucleotides and nonspecific binding of TdT, cells were treated with proteinase K as usual, but staining was performed in the absence of active TdT. This served as a negative control. In parallel, immunohistochemistry for an active form of caspase-3 was examined by using an FITC-conjugated monoclonal antibody against the active caspase-3 (C92-605; BD Pharmingen, San Jose, CA) in order to confirm the degree of apoptotic cells detected by TUNEL staining. Sections were deparaffinized followed by autoclaving for 5 min at 121 °C, and then incubated free floating in the primary antibody solution overnight at 4 °C. Following brief washes, sections were then incubated with DAPI (1:800; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. These sections were examined with a Digital Eclipse C1 confocal microscope (Nikon, Japan).

3. Results

3.1. GBV-B infection in tamarins

Firstly, two tamarins were intrahepatically inoculated with RNA transcripts from GBV-B infectious molecular clone pGBB (Fig. 1). Both monkeys showed viremia at 2 weeks post inoculation; peak viral titers in plasma reached up to 10^9 GE/ml and both monkeys developed hepatitis with dramatically elevated plasma ALT levels. The viremia was maintained up to 8 weeks, followed by rapid decline in parallel with the resolution of the ALT abnormalities. Within 6–8 weeks of the inoculation, the development of antibodies reactive with the viral core and NS3 proteins was observed (Fig. 1). Multiple plasma samples collected at later time points contained no detectable viral RNA and showed no ALT abnormalities; however, antibodies against GBV-B core and NS3 proteins were maintained at relatively high levels at least until 28 weeks after inoculation (Fig. 1). These results confirmed that inoculation of GBV-B viral RNA caused acute hepatitis in parallel with typical viremia in tamarins.

Next, in order to examine the tissue tropism of GBV-B *in vivo*, four tamarins were inoculated intrahepatically with week 2 plasma of tamarin Tm1 containing 8×10^8 GE of GBV-B (Tm3 and Tm4) or synthetic GBV-B RNA as described above (Tm5 and Tm6). These tamarins developed a typical acute infection that were marked by high levels of viremia, indicating that inoculation of either viral RNA or plasma of the infected tamarin resulted in comparable outcome (Fig. 2). It is noteworthy that in Tm5 the plasma ALT level was scarcely elevated in contrast with other three tamarins during the acute period of GBV-B infection, although this tamarin developed highest viremia (1.3×10^{10} GE/ml).

3.2. Histopathological analyses of GBV-B infection

Histopathological analyses in Tm3 and Tm4 livers showed inflammatory responses including inflammatory cell invasions around central and/or portal veins and hemorrhages, hepatocytic degenerations, and disruptions of sinusoids (Fig. 3A,B,E,F). Although there were only minimal pathological changes, hepatocytic degenerations and dilation of sinusoids were also found in the Tm5 liver (Fig. 3C and G) in contrast to uninfected tamarins (Fig. 3D and H, data not shown). To further evaluate the levels of apoptotic hepatocytes in these monkeys, we employed two different methods, detecting fragmented DNA (TUNEL assay) and an active form of caspase-3 as previously described [13]. It was found that substantial numbers of fragmented DNA-positive cells were observed in the Tm3 and Tm4 livers while much less in the Tm5 liver (Fig. 3I–K). Consistent results were obtained when the active form of caspase-3 was stained (Fig. 3M–O). On the other hand, we found neither DNA fragmentation nor caspase-3 activation in uninfected tamarin livers (Fig. 3L and P, data not shown). The minimal levels of pathological changes in the Tm5 liver were well correlated with a lower level of plasma ALT in Tm5 (Fig. 2, Table 1).

3.3. Tissue distribution of GBV-B

The results described above suggested the possibility that the substantial levels of viral replication occurred in other tissues rather than in the liver of Tm5. To ascertain the possibility, we euthanized three tamarins (Tm3, Tm4 and Tm5) and the viral levels in a variety of tissues were compared. Table 1 summarizes the data obtained in this experiment. It is reasonable to consider that GBV-B replicated in the liver accounts for majority of the viral load *in vivo*. However,

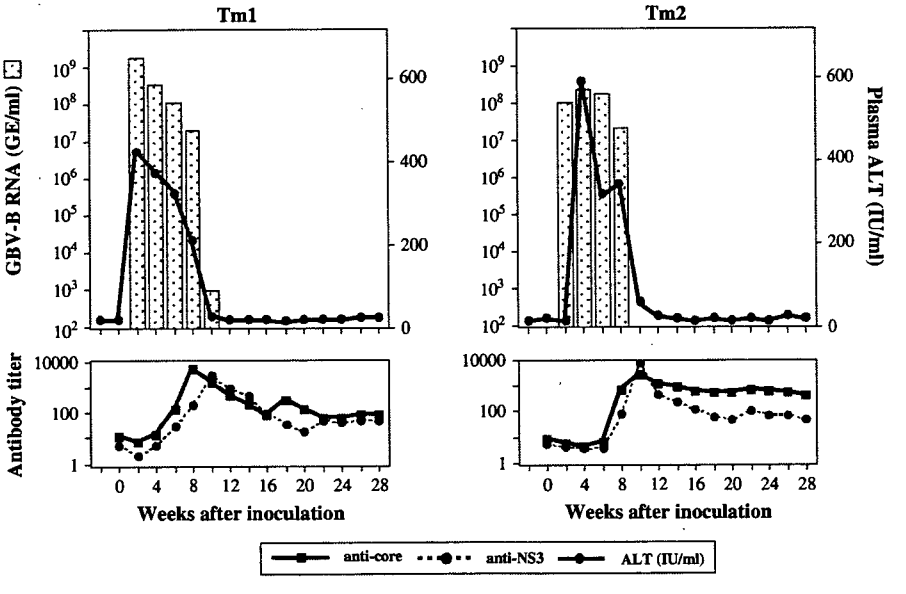

Fig. 1. Course of GBV-B infection in tamarins Tm1 and Tm2. Synthesized infectious RNA transcript of GBV-B from a pGBB molecular clone was inoculated into each tamarin intrahepatically. Plasma samples were collected from each tamarin at 2-week intervals post inoculation. The viral RNA copies, ALT levels, and titers of anti-viral antibodies (anti-core and anti-NS3) in the plasma samples until 28 weeks after inoculation were shown.

substantial levels of GBV-B RNA were detected not only in the liver but also in a variety of extrahepatic tissues such as hematolymphoid and genital tissues, suggesting that GBV-B may infect and replicate in these organs. Notably, the viral RNA levels of Tm5 were much greater in the lymphoid tissues but lower in the liver as compared with those of other two tamarins, indicating that the highest plasma viral load in Tm5 derived from extrahepatic tissues, mainly hematolymphoid tissues. We could not detect GBV-B RNA from other tissues tested (data not shown). From these results, we concluded that the preferential distribution of GBV-B in the extrahepatic tissues rather than in the liver of Tm5 may attribute to the highest plasma viral load in spite of the mild disorder and the lower viral load in the liver.

In addition, the unique viral distribution implied that the GBV-B disseminated in Tm5 might acquire novel tissue tropism as a result of genomic mutation. To ascertain the possibility, we amplified the entire viral genomes by RT–PCR from the liver, PBMCs and plasma collected from Tm5 at euthanasia and compared with the original nucleotide sequence. The sequences determined were completely identical to the original sequence of GBV-B (data not shown), indicating that the sequence heterogeneity of GBV-B was not responsible for the different tropism observed in Tm5 and thus GBV-B intrinsically exhibits pleiotropism in a host-dependent manner.

3.4. Detection of strand-specific viral RNA in the tamarin tissues

To confirm that the virus was actually replicated in the tissues other than the liver, we sought to deferentially determine negative-strand viral RNA which is shown to be a viral replication intermediate in case of HCV. We thus newly developed an assay system for detecting replication intermediate of GBV-B.

To determine the sensitivity of this method, synthetic positive- and negative-strand GBV-B transcripts (ranging from 10^8 to 10^0 copies of GBV-B) in 100-fold serial dilutions were subjected to RT–PCR. As shown in Fig. 4A, at least 100 copies of GBV-B negative-strand RNA could be detected by this method. When the primer for cDNA synthesis was omitted, no PCR products were obtained (Fig. 4A, negative control), indicating that the PCR signals were derived specifically from the GBV-B negative-strand RNA. In the presence of 10^8 copies of positive-strand HCV RNA, false positive PCR signals appeared (Fig. 4A). We then analyzed the samples from liver, spleen, pancreas, stomach and PBMCs from Tm5 using the GBV-B strand-specific PCR assay and found that the negative-strand viral RNAs were detected in the liver, spleen and PBMC samples (Fig. 4B). No negative-strand or replicating forms of the virus were detected from RNA extracted from pancreas, stomach and HeLa cells.

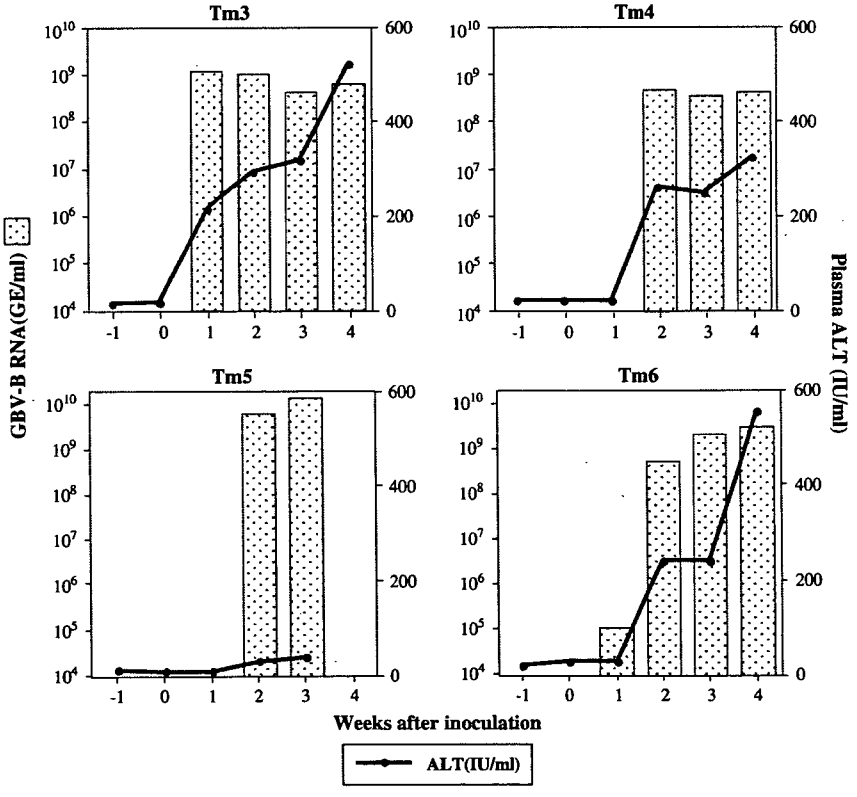

Fig. 2. Acute course of GBV-B infection in tamarins (Tm3 and Tm4) by *in vivo* passage of plasma (7.9×10^8 GE/head) obtained from the GBV-B RNA-inoculated Tm1 in comparison with GBV-B RNA transcript-inoculated tamarins (Tm5 and Tm6). The viral RNA copies and ALT levels in the plasma samples collected from each tamarin were indicated.

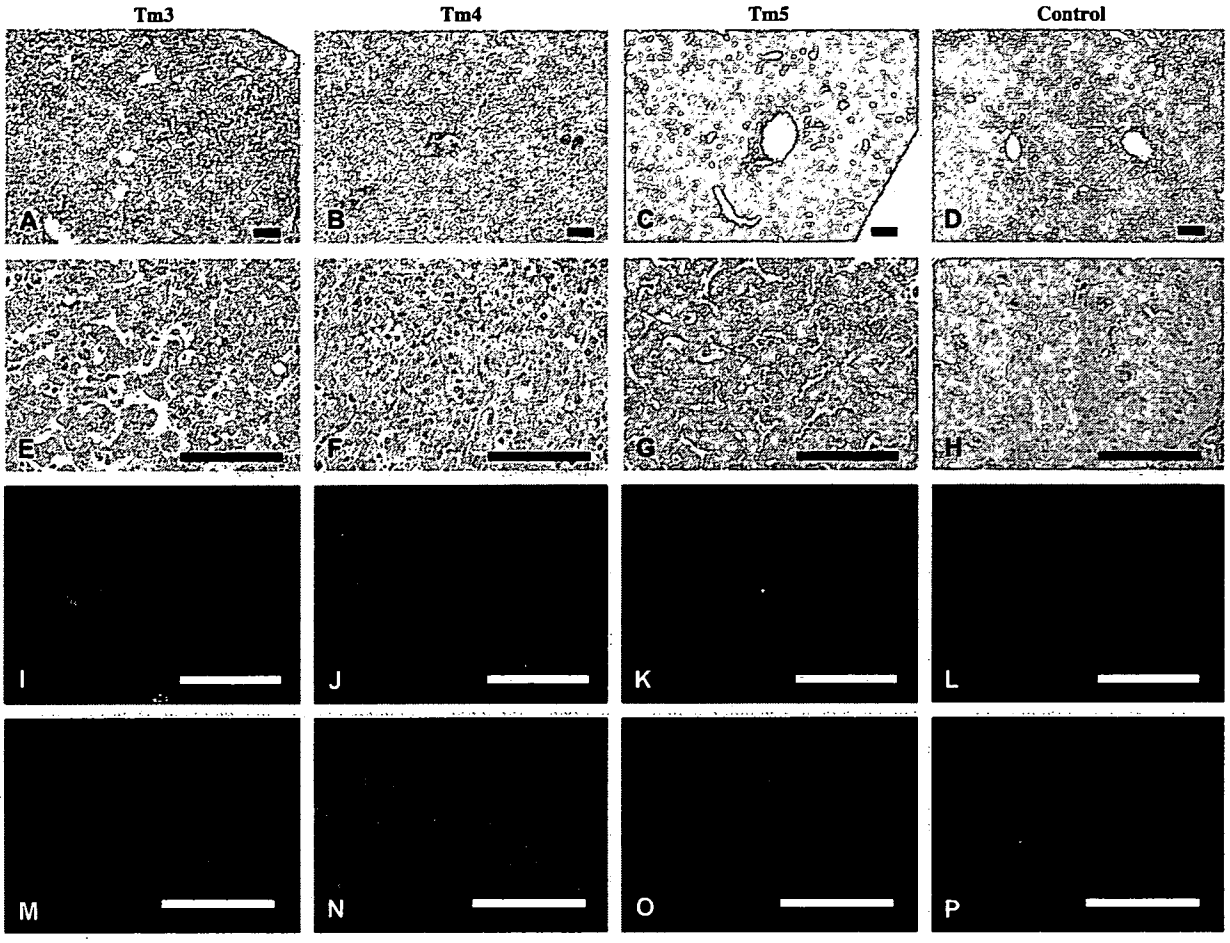

Fig. 3. Photomicrographs of liver sections from Tm3 (A, E, I, M), Tm4 (B, F, J, N), Tm5 (C, G, K, O), and an uninfected tamarin (D, H, L, P). A–H show sections with H&E staining, while I–L and M–P indicate sections with a TUNEL assay and immunohistochemistry for an active form of caspase-3, respectively. Sections immunostained for an active form of caspase-3 (green fluorescent) were counterstained with DAPI (blue fluorescent). Scale bars: 100 μm.

4. Discussion

GBV-B is most closely related to HCV and induces acute resolving hepatitis in tamarins. It is therefore reasonable that GBV-B has been considered to be a hepatotropic virus; in this study, however, we show for the first time that GBV-B is a pleiotropic virus and can disseminate to not only liver but also a variety of extrahepatic tissues such as hematolymphoid and genital tissues. Of note, there is ample evidence that persistent HCV infection manifests a variety of extrahepatic diseases, at least in part due to the extrahepatic tropisms of HCV (for review see [1]). This also suggests that extrahepatic tissues may serve as alternative reservoirs for HCV, while further analyses should still be required to understand the viral dynamics *in vivo*. Considering the similar pleiotropism of HCV and GBV-B, our results support and extend the usefulness of New World primates infected with GBV-B as a surrogate model for the study of pathogenesis and tropism of HCV infection.

Tamarins infected with GBV-B generally develop semi-acute viremia, of which peak levels regularly ranged from 10^7 to 10^9 GE/ml on the basis of previous reports [2,5,6, 14,15]. From this point of view, the peal viremia (1.3×10^{10} GE/ml) in Tm5 euthanized at the acute phase of the viral infection appeared to be much greater than other cases. It seems likely that in Tm5 the lymphoid tissues but not liver were responsible for the highly efficient viral production, because (i)

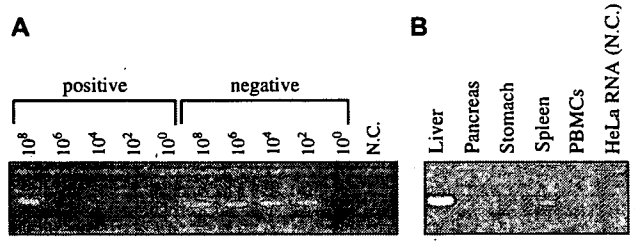

Fig. 4. (A) Titration of synthetic GBV-B RNA transcripts. Synthetic RNA transcripts corresponding to the positive- and negative-strands of part of the GBV-B were serially diluted and each transcript was subjected to amplification using strand-specific RT–PCR to determine the specificity and sensitivity of the assays. (B) Detection of negative-strand GBV-B RNA from various tissues. One microgram of total RNA obtained from tissues or cells was subjected to RT–PCR.

viral titer in the liver was lowest among three monkeys, which was consistent with minimal plasma ALT level and liver damage; (ii) yet, Tm5 exhibited highest viremia levels, (iii) the viral RNA levels in PBMCs, spleen and inguinal and intestinal lymph nodes of Tm5 were much greater than others, and (iv) we could detect negative-strand GBV-B RNA from not only liver but also spleen and PBMCs. Supposing that the entire virus in Tm3 plasma (3.8×10^8 GE/ml) was produced in the liver of which RNA titer was highest among tamarins, most of the virus in Tm5 plasma (1.3×10^{10} GE/ml) should be derived from extrahepatic tissues. Taken together, our data demonstrate preferential dissemination of GBV-B in extrahepatic tissues. In order to further define the cell type(s) in which GBV-B replicates efficiently, *in situ* histological analysis should be needed as indicated in the case of HCV [16].

It was possible that differential lymphotropism among GBV-B-infected tamarins could be due to adaptive mutation in the viral genome. From this point of view, we cloned the viral RNA obtained from plasma and liver; however, we did not find any sequence heterogeneity in the viral genome (data not shown). Furthermore, challenge of Tm5 plasma to naïve tamarins developed typical semi-acute hepatitis with regular viremia and did not reproduce the preferential lymphotropism (data not shown). These results indicate that GBV-B intrinsically has pleiotropism in a host-dependent manner. It is possible that multiple surface molecules in the host cells, which act as alternative receptors, would determine the pleiotropism of GBV-B. It remains to be investigated whether host molecules which are used as receptors for HCV [17] would also be used by GBV-B.

Histopathological studies showed the inflammatory responses in Tm3 and Tm4 livers; especially, the Tm4 liver developed strong degenerative changes, which was consistent with high ALT levels (Fig. 2G,H). Furthermore, the livers of Tm3 and Tm4 showed substantial proportions of apoptotic cells as revealed by greater signals of DNA fragmentation and caspase-3 activation, both of which were popular markers of apoptosis, than those in Tm5 (Fig. 3). It needs to be clarified whether such cytopathic effects could be directly induced by GBV-B infection into hepatocytes or whether effector cytotoxic T lymphocytes would be responsible for the cytopathicity.

Acknowledgments

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Efficient regulation of viral replication by siRNA in a non-human primate surrogate model for hepatitis C

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Abstract

RNA interference (RNAi) represents a new technology which could offer potential applications for the therapeutics of human diseases. RNAi-mediated therapy has recently been shown to be effective toward infectious diseases in *in vitro* and rodent models, however, it remains unclear whether RNAi therapy with systemic application could be effective in primates. In this study, we examined if RNAi therapy could be effective toward infectious diseases by using a non-human primate surrogate model for hepatitis C. Administration into marmosets of cationic liposome-encapsulated siRNA (CL-siRNA) for GB virus B (GBV-B), which is most closely related to hepatitis C virus, repressed GBV-B replication in a dose-dependent manner. Especially, 5 mg/kg of the CL-siRNA completely inhibited the viral replication. Since the serum interferons (IFNs) were induced by CL-siRNA *in vivo*, inhibition of viral regulation by anti-GBV-B CL-siRNA may include an antiviral effect of IFN. However, contribution of induced IFN may be partial, since the control CL-siRNA which induced a stronger IFN response than GBV-B CL-siRNA could only delay the viral replication. Our results suggest the feasibility of systemic administration of CL-siRNA as an antiviral strategy.

Keywords: siRNA; Hepatitis C; Marmoset; Interferon; GB virus B

RNA interference is a powerful tool for silencing gene expression and has spurred considerable interest in its experimental and therapeutic potential. RNAi has been characterized as a cellular process of post-transcriptional gene silencing. An RNaseIII-like enzyme, called Dicer, cleaves double stranded RNA (dsRNA) in to 21–23 nucleotide RNA duplex, termed small interfering RNAs (siRNAs). siRNAs are unwound in the RNA-induced-silencing-complex (RISC), and single-stranded siRNAs then act as a guide to substrate selection, leading to the cleavage of a homologous target RNA molecule [1].

Hepatitis C virus (HCV) infection contributes significantly to human morbidity and mortality worldwide. It is estimated that 40–60% of infected individuals progress to chronic liver disease, and many of these patients develop liver cirrhosis and hepatocellular carcinoma [2]. Currently,

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the only treatment available for patients with chronic HCV infections is combinational therapy with interferon (IFN) and ribavirin. The standard therapy is only effective for approximately 50% of patients with chronic HCV hepatitis [3]. Therefore, there is a great need for less complicated and more generally efficient therapeutics for HCV infection.

We and others reported that the synthetic siRNA and the siRNA-expressing adenovirus targeting 5′-UTR of HCV genome efficiently and specifically inhibited the HCV replication *in vitro* [4–6]. Other than humans, only chimpanzees can be productively infected by HCV. Together with ethical issues it has becomes increasingly difficult to access chimpanzees for experimental studies. The new world monkeys, tamarins and marmosets, undergo hepatitis upon infection with the GBV-B, which is most closely related to HCV. The significant similarity between HCV and GBV-B at the genomic and biochemical levels led to the proposal of the GBV-B/monkey system as a good surrogate model for hepatitis C [7,8]. Taking advantage of this non-human primate surrogate model, we investigated the feasibility of siRNA-mediated therapy against infectious diseases caused by pathogenic viruses.

Materials and methods

Preparation of siRNA. The sequence of siRNA for GBV-B was cucguagaccguagcacau dTdT in the sense strand and augugcuacggucuacgagdTdT in the antisense strand which was designed to target the GBV-B RNA (Fig. 1). The sequence of control siRNA for experiments of Fig. 2A and B was uua ugc cga ucg cgu cac a dTdT in the sense strand and ugu gac gcg auc ggc aua a dTdT in the antisense strand which was designed to target beta-galactosidase RNA, and that for experiments of Figs. 3 and 4 was gct atg aaa cga tat ggg c dTdT in the sense strand and g ccc aua ucg uuu cau ugc dTdT in the antisense strand which was designed to target *firefly*-luciferase RNA. siRNA oligonucleotides were chemically synthesized and purified by reverse-phase high-performance liquid chromatography, while the unconjugated RNA oligonucleotides were purified by anion-exchange high-performance liquid chromatography. The sense and antisense strands were annealed at 95 °C for 1 min followed by slow cooling in RNAse free water. Positively charged liposomes containing cationic lipid analogue were synthesized at Nippon Shinyaku Co., as described previously [9]. To prepare CL-siRNA,

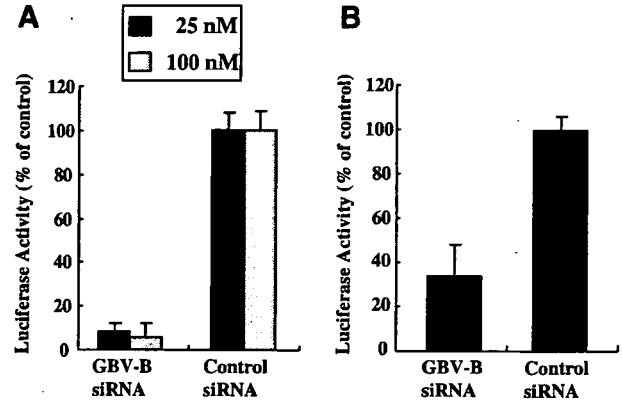
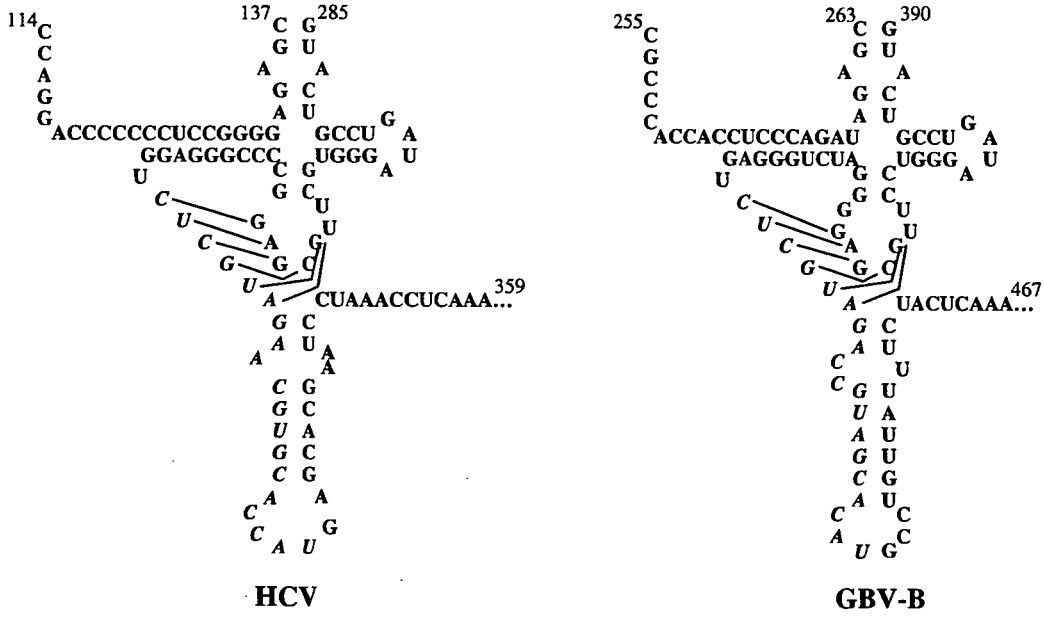


Fig. 2. Effects of the siRNA oligonucleotides on expression of GBV-B-reporter gene in culture cells. (A) and liver of mice (B).



Sense sequence of siRNA

HCV : CUCGUAGA*A*CGU(*A*)GCACAU

GBV-B : CUCGUAGA*C*CGU *A* GCACAU

Fig. 1. Predicted secondary structure of the 5′-untranslated region around the target site (italic) in the HCV and GBV-B genome (nucleotide 114–137 and 285–359 of HCV, and 263–255 and 390–467 of GBV-B), and the sense sequences of siRNA.

annealed siRNA was added to the same volume of liposome solution with sonication. The ratio of oligonucleotide to LIC-101 was 1:16 (w/w).

Cells culture and transfection. The human embryonic kidney cell line, 293 T, was maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, Missouri) supplemented with 10% fetal calf serum at 37 °C under 5% CO_2. Transfections of the siRNA oligonucleotides and the plasmids were performed in 24-well plates using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. GBV-B-RNA-reporter gene vector, pGBV-B-Rluc, was used as a target, which expressed mRNA consisting of GBV-B 5′-untranslated region and upstream part of the core region (nucleotide 1–377) connected with upstream of *renilla* luciferase (RLuc) gene. Fifty nanograms of the pGBV-B-Rluc and 2 and 25 nM of siRNA were transiently transfected with 20 ng of *firefly* luciferase (FLuc)-expressing plasmid (pRL-RSV, Promega). The RLuc activity was adjusted by the FLuc activity, to normalize the transfection efficiency.

Luciferase assays. Luciferase activities were quantified with a luminometer (Lumat LB9501, Promega) using the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and the results expressed as means ± SD as percentages of controls.

Animals. Male BALB/c or ICR mice, 9 weeks of age, were obtained from CLEA Japan and subject to a 2-week quarantine and acclimation period before use. Male juvenile common marmosets (*Callithrix jacchus*) were housed in individual cages at the Tsukuba Primate Medical Center. All animal studies were conducted in accordance with the protocols of experimental procedures which were approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation and Tokyo Medical and Dental University.

In vivo efficacy experiments in mice. For the *in vivo* delivery of the siRNA to the liver of mice and monkeys, we used a novel cationic liposome that was synthesized by Nippon Shinyaku Co., Ltd. This cationic liposome was reported to be a good vehicle for the delivery of nucleic acid polymers and siRNAs to the liver when it was administered intravenously [9,10] or to the bladder by intravesical administration [11]. For the delivery of plasmid DNA to the liver of mice we used the hydrodynamic injection method in which a large volume of nucleotides solution was rapidly injected from tail vein [12]. *Three mice for each group were examined.* 5.0 mg/kg GBV-B or control CL-siRNA was administered as a regular intravenous injection from the tail vein in 0.2 ml 10% maltose over a period of 1–3 s. Three minutes later, the 50 μg of the pGBV-B-Rluc and 20 μg of pRL-RSV plasmids in a volume equivalent to 5% of the body weight were rapidly injected in 3–5 s into the mouse tail vein according to the hydrodynamic injection method. Phosphate buffer saline (PBS) was used as a carrier solution for injection. Successful injection was monitored when the conjunctiva of mouse became transiently anemic and confirmed by the luciferase activity in the liver.

In vivo efficacy experiments in monkeys. Negative control (n = 2; with or without control siRNA) and treatment group (n = 3; 1.0, 2.5 and 5.0 mg/kg of anti-GBV-B siRNA) were employed in this study. GBV-B-infectious serum obtained from a tamarin [8] was intrahepatically inoculated with the GBV-B RNA. The siRNA to GBV-B and control siRNA formulated by the cationic liposome, or just 10% maltose (sham) was administered by standard intravenous injection via the saphenous vein of the marmosets for three days. On the second day, the GBV-B infectious serum (1.3×10^9 viral RNA copies/inoculum) was directly injected to the liver of five marmosets. Blood samples were periodically collected from the femoral vein of the monkeys under anesthetization. GBV-B RNA in plasma from the monkeys was quantified by a real-time, 5′ exonuclease PCR (TaqMan) assay using a primer-probe combination that recognized a portion of the GBV-B capsid gene as previously described [8]. The Platelet cell counts were performed at FALCO Biosystems, Co., Ltd.

Measurement of IFNs in mice and monkeys. The siRNA/cationic liposome was injected from tail vein of ICR mice or saphenous vein of the marmosets. Bood samples were taken 3 h after the injection. Mouse IFN-α levels were quantified by using sandwich ELISA kits for mouse IFNs (PBL Biomedical Laboratories, Biosource). Marmoset IFN-α and -γ levels were by using sandwich ELISA kits for human and rhesus macaque IFN, respectively (U-CyTech bioscience) according to the manufacture's instructions. Assays were performed in duplicate and the results expressed as means ± SD as percentages of controls.

Results

We selected the siRNA-targeting site to the GBV-B genome from its 5′-UTR, the most conservative portion in both GBV-B and HCV genomes [13], to protect the siRNA from escape mutations of the virus [4]. The secondary structures of virus genome RNAs of HCV and GBV-B around the target site are very similar to each other, and the designed siRNA was different from the corresponding sequence of HCV by only two nucleotides (Fig. 1).

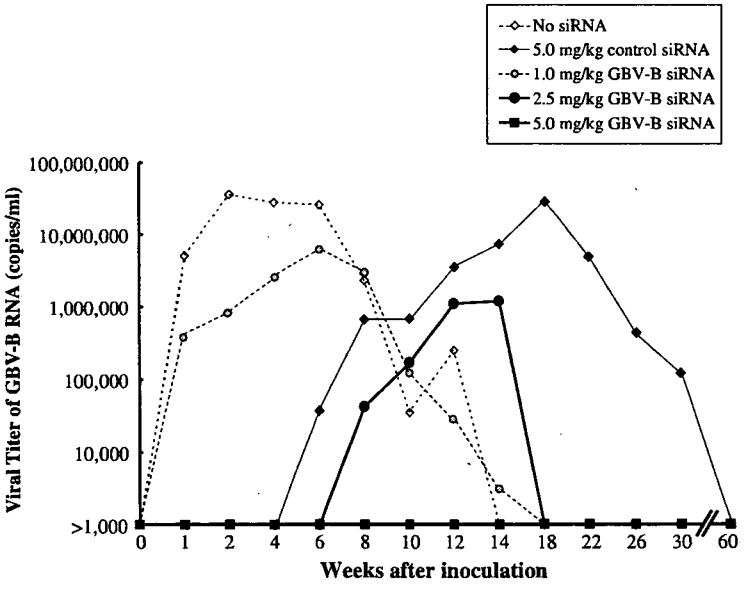


Fig. 3. Effect of the GBV-B siRNA/cationic liposome complex on replication of GBV-B in marmosets.

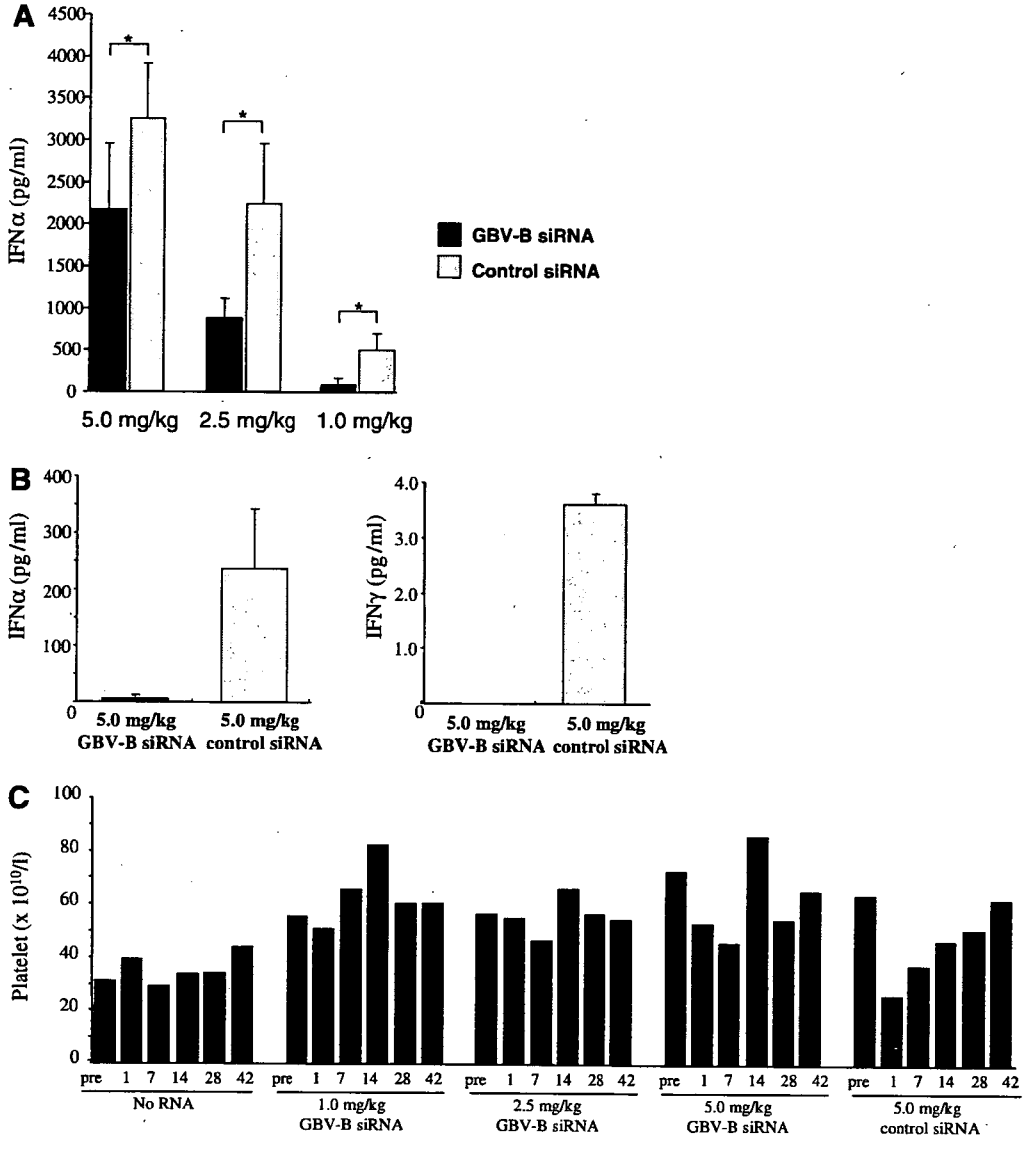


Fig. 4. Side effects of siRNA/cationic liposome complex. (A) Induction of IFN-α was evaluated by measuring mouse serum 3 h after intravenous injection of 1–5 mg/kg GVB-B or control siRNA/cationic liposome complex ($n = 3$). *<0.05 (Student t test). (B) Induction of IFN-α and γ was evaluated by measuring marmoset serum 3 h after intravenous injection of 5 mg/kg GVB-B or control siRNA/cationic liposome complex), respectively ($n = 3$). (C) Peripheral blood platelet was counted in the five marmosets examined in the same experiment shown in Fig. 3.

Effect of siRNA in vitro and in mice

First, we confirmed the efficient cleavage of GBV-B RNA by the siRNA in 293 T cells. The cells were harvested at 24 h of transfection with pGBV-B-*R*luc, pRL-RSV and siRNA oligonucleotides, and internal luciferase activities were measured and ratio of RLuc versus FLuc value was calculated. More than 90% the *R*Luc activities were inhibited by expressing co-transfected siRNA (Fig. 2A). This result clearly indicated that GBV-B siRNA efficiently inhibited the expression of GBV-B RNA in culture cells.

Next, we investigated the *in vivo* effect of siRNA formulated in the cationic liposome on silencing the viral gene expression in the liver of mice. BALB/c mice were injected intravenously from the tail vein with GVB-B CL-siRNA followed by hydrodynamically injection of pGBV-B-*R*luc and pRL-RSV. We found that intravenously administered GBV-B CL-siRNA efficiently suppressed the expression of GBV-B genome in the liver of mice (Fig. 2B).

Effect of siRNA on GBV-B replication in marmosets

The 1.0, 2.5 and 5.0 mg/kg/day of siRNA to GBV-B, 5.0 mg/kg/day of control siRNA formulated by the cationic liposome, or just 10% maltose (sham) were administered by standard intravenous injections via the saphenous vein of the marmosets for three consecutive days. On the second day, GBV-B infectious serum

(1.3×10^9 viral RNA copies/inoculum) was directly injected to the liver. Before and after the inoculation, GBV-B RNA in the serum was quantified by a real-time, 5' exonuclease PCR. In a sham-administered marmoset, the viral RNA was transiently increased in plasma after infection and the viral load reached to the peak level (3.6×10^7 copies/ml) (Fig. 3). It has been reported that GBV-B infection in marmosets as well as tamarins causes semi-acute viremia which generally ceases within 10–12 weeks post-infection [8,14,15]. This viral kinetics is consistent with the cases of HCV-infected human or chimpanzee, thus it appears to be in vivo characteristics of genus hepativirus where HCV and GBV-B belong to. Virological or immunological implication for the transient viremia is not fully addressed.

In contrast to sham-administered marmoset, we could find that the administration of CL-siRNA significantly delayed or suppressed the replication of GBV-B in a dose-dependent manner; the 5.0 mg/kg CL-siRNA completely suppressed the replication of GBV-B for more than 6 months after the infection (Fig. 3), even though the siRNA was administered only for the initial 3 days. Unexpectedly, the 5.0 mg/kg of control CL-siRNA was also able to delay the virus replication, while the peak level was comparable with that of the untreated monkey (Fig. 3).

Induction of interferons

We evaluated the induction of serum IFN-α by intravenous administration of the siRNA with cationic liposome in mice. IFN-α was induced by CL-siRNA but not by the cationic liposome nor siRNA oligonucleotide alone (data not shown). Induced IFN levels in the sera were dose-dependent and were significantly higher in mice with the *control CL-siRNA* than those with the *GBV-B CL-siRNA* (Fig. 4A).

An independent experiment using marmosets showed that single injection of 5.0 mg/kg control CL-siRNA substantially induced the serum interferon (IFN)-α and -γ, whereas the same dose of CL-siRNA induced a minimal level of IFN-α and no detectable level of IFN-γ (Fig. 4B).

In addition, a transient and mild decrease in peripheral blood platelets was more clearly observed in the marmoset treated with 5.0 mg/kg of control than 5.0 mg/kg of GBV-B CL-siRNA (Fig. 4C). There was no other remarkable abnormality related to siRNA administration in biochemical parameters indicating liver dysfunction which include alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase and albumin.

Discussion

Many viruses produce some dsRNA as a byproduct of their replication [16], and RNAi serves as an important defense against viruses in plants [17]. Therefore, mammalian viruses have been expected to be a good therapeutic target of RNAi, and indeed, several animal viruses have been successfully inhibited to replicate *in vitro* [18]. Locally delivered siRNA have proven effective in abrogating infection from respiratory [19–22] and vaginal [23] viruses. Recently, systemically-delivered siRNA in mice has been successfully suppressed the expression of endogeous gene of the liver [24–26]. However, it remains to be ascertained if the RNAi-mediated gene therapy with systemically-delivered siRNA would be applicable to hepatitis virus in non-human primates. In this study, we examined if RNAi therapy could be effective toward infectious diseases by using a non-human primate surrogate model for hepatitis C. Administration into marmosets of CL-siRNA for GBV-B, which is most closely related to hepatitis C virus, repressed GBV-B replication in a dose-dependent manner. Our results suggest the feasibility of systemic administration of CL-siRNA as an antiviral strategy.

The 5.0 mg/kg GBV-B CL-siRNA dramatically inhibited the replication of GBV-B. However, control CL-siRNA also delayed the virus replication. Intravenous injection of siRNA formulated with liposomes was reported to stimulate mammalian immune system [26,27]. In relation to antiviral effect of IFNs, we therefore measured the serum IFN levels. Since the GBV-B siRNA/cationic liposome had less effect in IFN induction than the control but better antiviral effect than the control, it is possible that inhibition of the viral replication by the GBV-B siRNA/cationic liposome complex was at least in part caused by RNA interference. On the other side, it is also likely that IFN locally induced in the marmoset liver contributed the suppression of the viral replication. Because the induced level of mice serum IFN-α by GBV-B CL-siRNA was significant, although it was less than that by control CL-siRNA. Moreover, estimated IFNs level in marmoset serum was minimal but their actual levels might have been more, because the standard IFN in the ELISA was human or rhesus macaque IFN. Therefore, we considered that the antiviral effect of CL-siRNA was made by both RNA interference and induced IFNs.

In therapeutic application of siRNA to humans, general safety is a most important problem. The side effect of CL-siRNA to the liver is thought to consist of direct liver toxicity which is probably caused by its hydrophobic nature and its immuno-stimulatory effect [26–28]. Recently, Zimmermann et al. has reported that siRNA delivered systemically in a cationic liposome, stable nucleic acid lipid particles (SNALP), inhibited endogenous gene expression in the liver of the cynomolgus monkeys, which supports our notion concerning the therapeutic potential of systemically injected siRNA in primates. Although they made excellent chemical modifications to siRNA oligonucleotides to reduce IFN induction, their siRNA complex produced a considerable liver damage with a marked increase of transaminases at the dose (2.5 mg/kg) of maximal suppression effect. This indicated that the therapeutic window of their siRNA complex is overlapped with its toxic window. In contrast, our CL-siRNA induced much less liver damage, since even the 5.0 mg/kg of our CL-siRNA did not show a marked liver damage, but induced a sub-

stantial immune responses. A number of recent studies revealed that siRNA/cationic liposome complex has an immunological effects of siRNAs including the induction of proinflammatory cytokines and type I IFNs (IFN-α and IFN-β) through activation of RNA-sensing immunoreceptors including three members of the Toll-like receptor (TLR) family (TLR3, TLR7 and TLR8) [29]. Detection of siRNA molecules could trigger antiviral innate defense mechanisms including the induction of type I IFNs. In fact, double strand RNA molecule, poly I/C, was reported to eliminate the virus in GBV-B-infected tamarin hepatocytes by activating TLR3 [30,31]. These knowledges lead us to postulate that it is one of sophisticated strategy for siRNA to inhibit hepatitis virus to use this immuno-stimulatory side effect as an antiviral innate defense, only if the systemic side effects are tolerable.

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