2.8. Growth curves and cell cycle analysis

Immortalized and reverted 16-T3 cells were inoculated at a density of 1×10^5 per well in 6-well plates and cultured in ISE-RPMI medium. The cells were regularly detached with trypsin and the number of viable cells counted by trypan blue exclusion test. The cell cycle status of both 16-T3 and reverted cells was analyzed by flow cytometry, as previously reported [19].

2.9. Animal experiments

All procedures performed on animals were within the guidelines for humane care of laboratory animals and approved by the Okayama University Institutional Animal Care and Use Committee. Porcine hepatocytes were prepared from Landrace pigs (JA West of Okayama, Okayama, Japan), weighing 10 kg, by a four-step retrograde perfusion method using collagenase and dispase, as previously described [20]. The isolated hepatocytes with cell viability of >85% were used for transplant experiments. 16-T3 cells were expanded in a roller bottle (Falcon BD) using a cell preparation roller apparatus (Bellco Co., Tokyo, Japan), and used for the transplantation experiments. Landrace male pigs, weighing approximately 6 kg, were used as a recipient for transplantation experiment. An atom multipurpose tube (6 Fr.) (Atom Medical Co., Tokyo, Japan) was surgically inserted into a left external jugular vein for D-galactosamine (D-Gal) injection under general anesthesia. At the same time, the spleen was exposed after abdominal incision. An atom tube of 6 Fr. was inserted from a branch of the splenic vein and the tip of the tube was placed in the main portal vein via for cell transplantation. After closing the abdomen, intravenous administration of 0.5 g/kg of D-Gal (Sigma, Saint Louis, MI) was conducted via the jugular route 24 h prior to cell transplantation. Both of the tubes were flushed with 1000 U of heparin (Aventis, Tokyo, Japan) to prevent blood clotting, capped, and then subcutaneously embedded for the following transplantation experiment, as previously reported [20]. Recipient pigs were randomly divided in the following groups: G1, single intraportal injection of reverted 16-T3 cells (1×10^9 cells) ($n = 5$); G2, single intraportal injection of primary isolated pig hepatocytes (1×10^9 cells, which were compatible to 5% mass of the whole of the pig liver) as a positive control ($n = 3$); and G3, intraportal injection of Ringer's Lactate solution as a negative control ($n = 5$). After the transplantation procedure, pigs had free access to food and water. A daily intramuscular injection of 0.5 mg/kg of FK506 was conducted in the pigs from 1 day before cell transplantation to 7 days after transplantation. Blood samples were regularly taken from the pigs for analyzing liver functions and for measuring human specific albumin to evaluate engraftment of the reverted 16-T3 cells in pigs using a Human ALBUWELL II ELISA kit (Exocell Inc.).

2.10. Histopathological examinations

Autopsies were performed in all pigs at the time of spontaneous death or sacrifice. The liver and spleen specimens obtained from animals subjected to cell transplant as well as D-Gal-treated animals that received medium and only Ringer's Lactate were fixed with 20% formalin embedded in paraffin and processed for staining with hematoxylin and eosin (HE). The liver biopsy specimens obtained from pigs transplanted with reverted 16-T3 cells were also cryopreserved with OCT-Tissue TECH compound (R&D Systems, Minneapolis, MN). Five μm sections were prepared for immunofluorescent staining for human specific albumin expression by means of a mouse monoclonal anti-human albumin antibody (Santa Cruz Biotech, Santa Cruz, CA) and followed by FITC-conjugated goat anti-mouse IgG (Sigma, Saint Louis, MI). Localization of albumin was visualized by an Axiophot FL fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2.11. Statistical analysis

Mean values are presented with standard deviations. A two-tailed Student's t test was used to calculate the significance of difference in mean values. The Kaplan–Meier method was used to calculate survival data and the significance of differences determined by a Logrank test. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Establishment of a reversibly immortalized human hepatocyte cell line 16-T3 with Tamoxifen-mediated self-recombination

Human hepatocytes were immortalized with SSR#197 that constitutively expresses LoxP-flanked hTERT and EGFP (Fig. 1). One of 24 EGFP-positive clones, clone no. 16, was chosen on the basis of results from gene expression profiles assessed by RT-PCR and tumorigenic assays (Table 1). The clone no. 16 was further subjected to transfection with the plasmid pCAGMerCreMer/Puro^R, which expresses a Cre recombinase protein fused to two mutant estrogen-receptor ligand-binding domains (MerCreMer) (Fig. 1). The MerCreMer/Puro^R cDNA was placed under the control of the CAG promoter. After transfection with a plasmid conferring resistance to puromycin and subsequent selection, a resultant clone, 16-T3, was isolated for further studies on the basis of Cre/LoxP recombination efficiency. 16-T3 cells grew steadily in culture with serum-free medium and doubled in cell number in approximately 48 h.

3.2. Cre/LoxP recombination in the presence of 4-hydroxytamoxifen

The EGFP-negative population was recovered by high-speed cell sorting in 16-T3 cells after a week-exposure to 4-hydroxytamoxifen (4-HT). The cells (reverted 16-T3 cells) were uniformly negative for EGFP expression by histogram study and FITC-based phase contrast microscopy (Fig. 2a). The cells lost expression of hTERT (Fig. 2b) analysis and telomerase activity (Fig. 2c), resulting in loss of proliferation (Fig. 2d). No change in the proportion of cells expressing EGFP was observed in a control experiment employing 17β-estradiol instead of 4-HT.

3.3. Reverted 16-T3 cells express differentiated hepatocyte phenotypes

Expression of hepatocyte-specific genes, including albumin (Alb), glutathione-S-transferase π (GST), and human blood coagulation factor X (HBCFX), was further increased in reverted 16-T3 after 4-HT treatment by both RT-PCR (Fig. 3a) and Northern blot analyses (Fig. 3b). The enhanced expression after Cre/LoxP recombination was at least in part explained by increased expression of the transcriptional factors CCAAT/enhancer binding protein (C/EBP)-α and

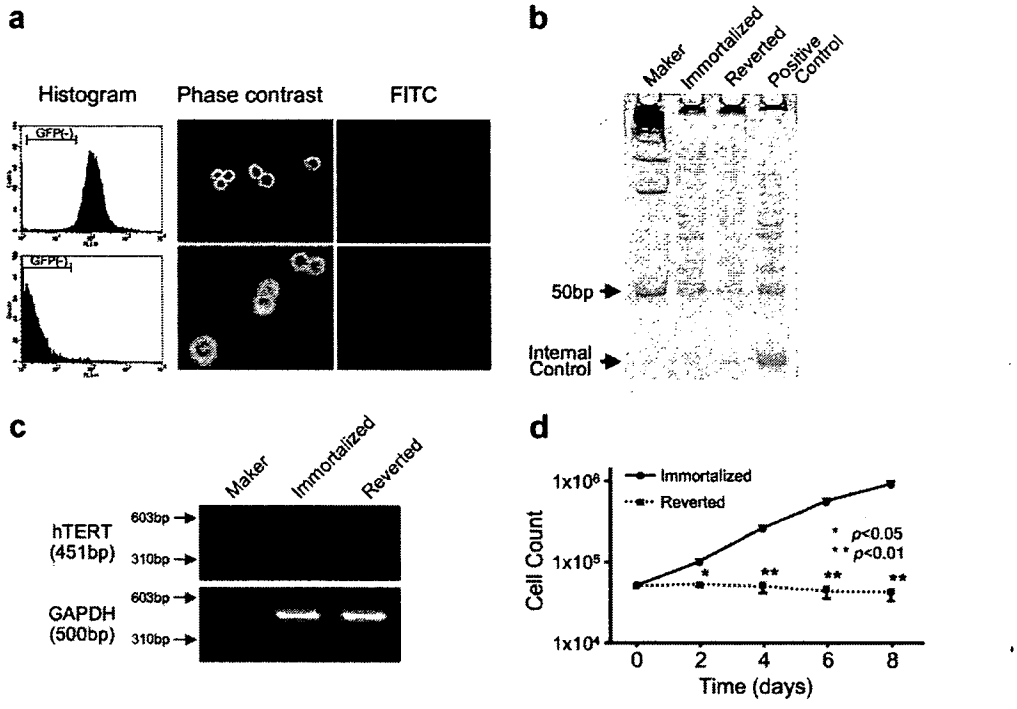

Fig. 2. EGFP expression, telomerase activity, and cell growth of reversibly immortalized human hepatocyte 16-T3 cells. (a) GFP expression. Reversion in 16-T3 cells was conducted by 500 nM Tamoxifen (4-HT) treatment followed by cell sorting with MoFlo. Reverted 16-T3 cell population was negative for GFP expression by the histogram study and FITC-based phase contrast microscopic examination. (b) RT-PCR analysis for hTERT expression. hTERT mRNA was detected in immortalized 16-T3 cells but not in the reverted cell population. (c) Telomerase assay with the TRAPEZE telomerase detection kit. Reverted 16-T3 cells lost telomerase activity. The positive control was provided by the kit. (d) Comparative growth curves of 16-T3 cells, before and after recombination. Reverted cells stopped proliferation. Data are representative of 3 independent experiments.

hepatocyte nuclear factor (HNF)-4α (Fig. 3b). Increased production of albumin was also observed in reverted 16-T3 by immunofluorescent study (Figs. 3c and d) and by measuring secreted albumin into the culture medium by ELISA (Fig. 3g). Reverted cells progressively stopped proliferating in the G0/G1 phase of the cell cycle (Figs. 3e and f). Lidocaine metabolic activity was significantly increased in reverted cells (Fig. 3h). Compared to normal human hepatocytes, albumin production and lidocaine-metabolizing activities of reverted 16-T3 cells were 0.32 and 0.50-fold, respectively.

3.4. Transplantation of reverted cells via the portal vein ensures long-term survival of pigs with ALF

We evaluated the therapeutic effect of the reverted 16-T3 cells in a pig model of ALF induced by intravenous injection of 0.5 g/kg of D-Gal. Animals were injected with the immunosuppressant FK 506 to delay rejection of the injected cells in this xenotransplantation model until spontaneous liver regeneration occurred. While blood levels of total bilirubin (Fig. 4a), AST (aspartate aminotransferase) (Fig. 4b), ammonia (NH3) (Fig. 4c) and prothrombin time (PT) (Fig. 4d) increased up to 72 h after D-Gal injection in all animals, they gradually recovered to normal levels in the pigs with intraportal transplantation of either reverted 16-T3 cells or porcine hepatocytes. In contrast, control pigs injected with Ringer's Lactate solution showed rapid increase in these parameters and eventually died of liver failure with hemorrhage and jaundice within 4 days after D-Gal injection. Blood levels of tumor necrosis factor (TNF)-α were considerably higher in control pigs, whereas pigs transplanted with either reverted 16-T3 or porcine hepatocytes had almost normal values of TNF-α (Fig. 4e). However, G1 and G2 pigs transiently showed the higher blood level of ALP (alkaline phosphatase) for a couple of days after cell transplantation than control pigs due to cell injection in the portal vein (Fig. 4f).

3.5. Survival and histological findings

The one-month survival rate was 100% for pigs transplanted with porcine hepatocytes and 80% for pigs with reverted 16-T3 transplant, respectively, whereas none of the vehicle-injected pigs survived (Fig. 5a). The status of engraftment of transplanted reverted 16-T3 cells was evaluated by measuring the blood level of human specific albumin in the pigs. Under FK506 administration, reverted 16-T3 cells survived in the liver of pigs for at least 4 days that were a crucial period of time to support the damaged liver to regenerate (Fig. 5b). These data suggest that the transplanted reverted 16-T3 cells degraded inflammatory cytokines, such as TNF-α, effec-

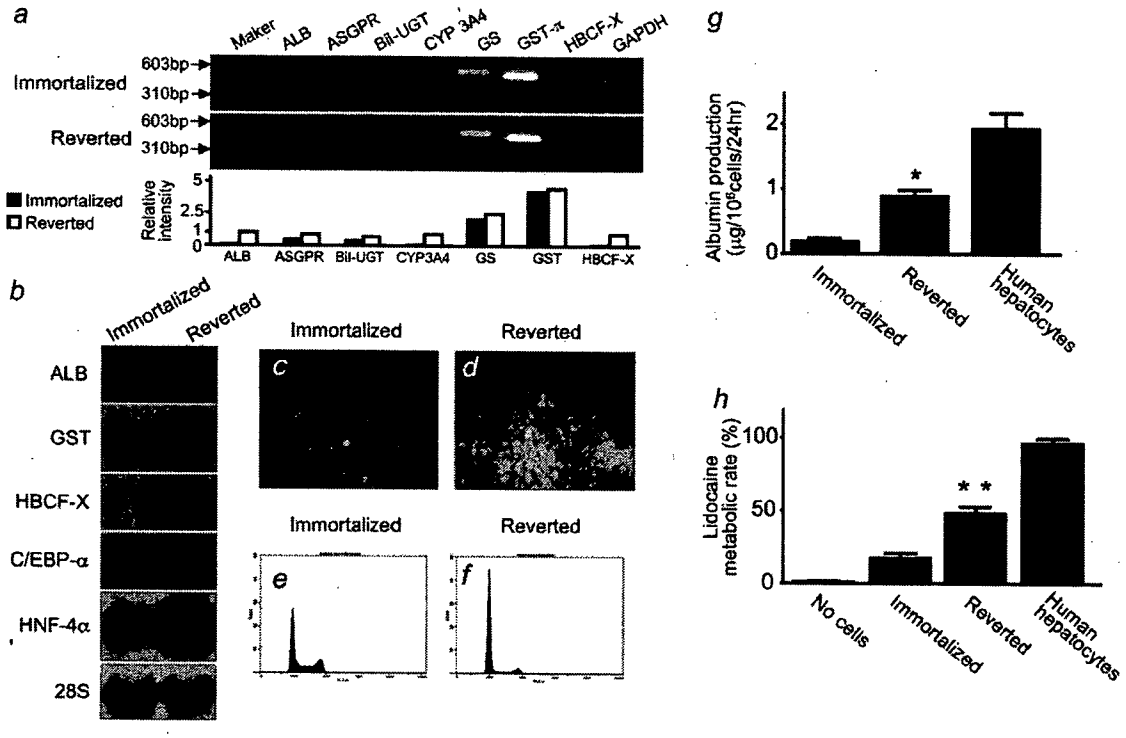

Fig. 3. In vitro biological characteristics of 16-T3 cells. (a and b) PR-PCR and Northern blot analyses for RNA expression of several hepatocyte-specific markers, showing higher overall expression in reverted 16-T3 cells than in the immortalized population before recombination. The intensity of the bands of each of the markers in RT-PCR was assessed quantitatively (a). Specific pairs of primers were utilized in Northern blot, and equal loading was verified by detection of 28S ribosomal RNA (b). (c and d) Immunofluorescence staining with a labeled anti-human albumin antibody showed that reverted 16-T3 cells produce more albumin (red) than immortalized cells before recombination. Actin filaments and cell nuclei were revealed with Oregon green Phalloidin and Hoechst staining, respectively (magnification × 100). (e and f) In the cell cycle analysis, reverted 16-T3 cells progressively stopped proliferating in the G0/G1 phase of the cell cycle. (g) Albumin production as assayed by ELISA was increased by 4.5-fold after reversion of 16-T3 cells. (h) Reverted 16-T3 cells significantly favorably metabolized lidocaine (50% of loaded dose) compared to immortalized cells (15% of loaded dose). Data are representative of 3 independent experiments.

tively and supported hepatic functions until pigs recovered from ALF. Liver biopsy samples obtained on day 3 post-transplantation with reverted 16-T3 cells showed clusters of human cells that were positive for human specific albumin expression in the portal areas, accompanied with severe necrosis of the liver (Figs. 5c and d). Liver specimens taken 15 days after D-Gal injection in the 16-T3 cell transplanted group revealed almost normal hepatic trabecular architecture with moderate mononuclear infiltrate, indicating complete recovery from ALF and host rejection of the transplanted cells (Figs. 5e and f). At the time of sacrifice 3 months post-transplantation, there was no evidence of tumor development in liver, lung, bone marrow and other organs of the pigs injected with the reverted 16-T3 cells.

4. Discussion

In many cases of ALF, the damaged liver would spontaneously regenerate if bridging life-support could be provided by transient transplantation of functional hepatocytes [4]. In an effort to provide a practical source of cells for this therapy despite the shortage in liver donors, we previously developed a reversible cell immortalization procedure by retroviral transfer of a viral oncogene of SV40 that could be subsequently excised by Adenovirus-mediated Cre/LoxP site-specific recombination [10,11]. However, three major hurdles remained to obtain a sufficient number of reverted cells for clinical applications. First, SV40 T-mediated cell expansion was unable to avoid replicative cell senescence at the time of excision of the oncogene, because telomere attrition was not prevented, thus limiting the number of viable cells that could be obtained. Second, oncogenic concerns remained with the use of the viral oncogene SV40 T. Lastly, adenovirus-associated cytotoxicity induced considerable cell loss, and possible lingering contamination by adenoviral structural proteins could not be ruled out.

In this study, we have use two prolonged approaches to surmount these hurdles. First, hTERT was used as the immortalizing protein, thus preserving telomere length and avoiding the use of a viral oncogene [21]. Second, we pre-integrated in the immortalized hepatocyte clone a DNA construct that expresses a Tamoxifen-dependent Cre recombinase fusion protein, thereby

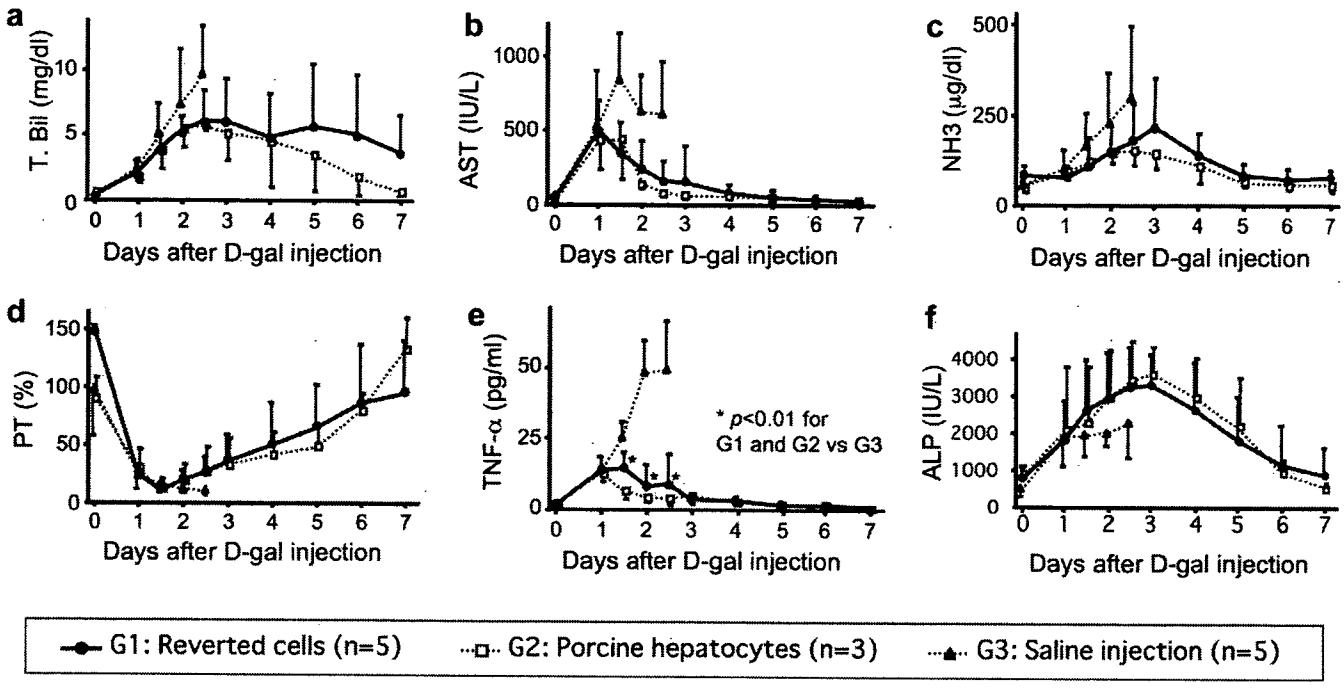

Fig. 4. Intraportal injection of reverted 16-T3 cells into pigs in ALF. After induction of ALF by injection of D-Gal, pigs were randomly divided into 3 groups: G1, single injection of reverted 16-T3 cells (1×10^9 cells) into the liver via the portal vein ($n = 5$); G2, single intraportal injection of primary isolated pig hepatocytes (1×10^9) as a positive control ($n = 3$); and G3, intraportal injection of 100 ml Ringer's Lactate solution as a negative control ($n = 5$). Control G3 pigs experienced a rapid increase in blood total bilirubin levels (T. Bil) (a) and ammonia (NH3) (c) with marked decrease in prothrombin time (PT) (d). In contrast, these parameters as well as AST (b) and ALP (f) returned to normal levels 72 h after D-Gal injection in G1 pigs and G2 pigs. Pigs with cell transplant (G1 and G2) showed the higher ALP level due to intraportal cell infusion (f). Blood levels of TNF-α were considerably elevated in control G3 pigs, whereas G1 and G2 pigs demonstrated almost normal levels of TNF-α after D-Gal administration (e). Data are averages ± SD of repeated samples from individual animals. 16-T3 cells were used for the transplantation experiments.

making secondary virus-mediated transfer of the recombinase gene unnecessary.

After removal of hTERT in immortalized 16-T3 cells by adding Tamoxifen, the reverted 16-T3 cells progressively arrested in the G0/G1 phase of the cell cycle. Loss of proliferating capacity in immortalized cells may allow the cells to use the energy for expressing the more differentiated phenotypes. In fact, the reverted 16-T3 cells showed the increased expression of hepatic markers in association with enhanced levels of transcriptional factors C/EBP-α and HNF-4α, resulting in higher albumin production and lidocaine metabolism (Figs. 3a and b). Furthermore, transplantation of reverted 16-T3 cells successfully prevented the elevation of the blood level of inflammatory cytokine TNF-α in the pigs with ALF induced by D-Gal, leading to the significant prolongation of the survival. In our current bioartificial liver (BAL) experiments, D-Gal-induced ALF pigs showed decrease in the blood TNF-α level after BAL therapy and survived. In contrast, control pigs revealed the higher level of TNF-α and died within 4 days of D-Gal injection (data not shown).

Complementary measures to enable the rapid clinical isolation of reverted cells while avoiding the need for EGFP-mediated cell sorting could be further developed, such as by means of a membrane marker recognized by biodegradable, magnetic immunolabeled nanoparticles for rapid, single-batch cell purification [22]. Other means of triggering excision of the immortalizing gene without the need for gene transfer could also be explored, such as the use of a TAT-Cre recombinase fusion protein capable of penetrating cell membranes [23].

Results from the porcine xenotransplantation model suggest that non-histocompatible allogenic transplantation in the presence of FK506 would be effective to bridge patients with ALF until their liver spontaneously regenerates and transplanted cells are ultimately rejected. Repeated intraportal administration of reverted hepatocytes is available by means of a PORT-A CATH catheter inserted from the middle portion of the splenic vein with the tip positioned inside the portal vein and the site of cell infusion subcutaneously embedded into the groin region [24]. In this setting, the use of non-histocompatible cells is a powerful added security against any potential tumorigenicity.

This approach may also be extended to the long-term cell therapy of terminal, chronic liver diseases and liver inborn errors of metabolism. HLA-matched liver cells could be obtained by limited liver biopsy of related individuals and submitted to the procedure of reversible immortalization. In the case of terminal, chronic liver disease, the donor cells could be further modified by gene correction by additional gene transfer. For instance, expression of a dominant negative mutant or

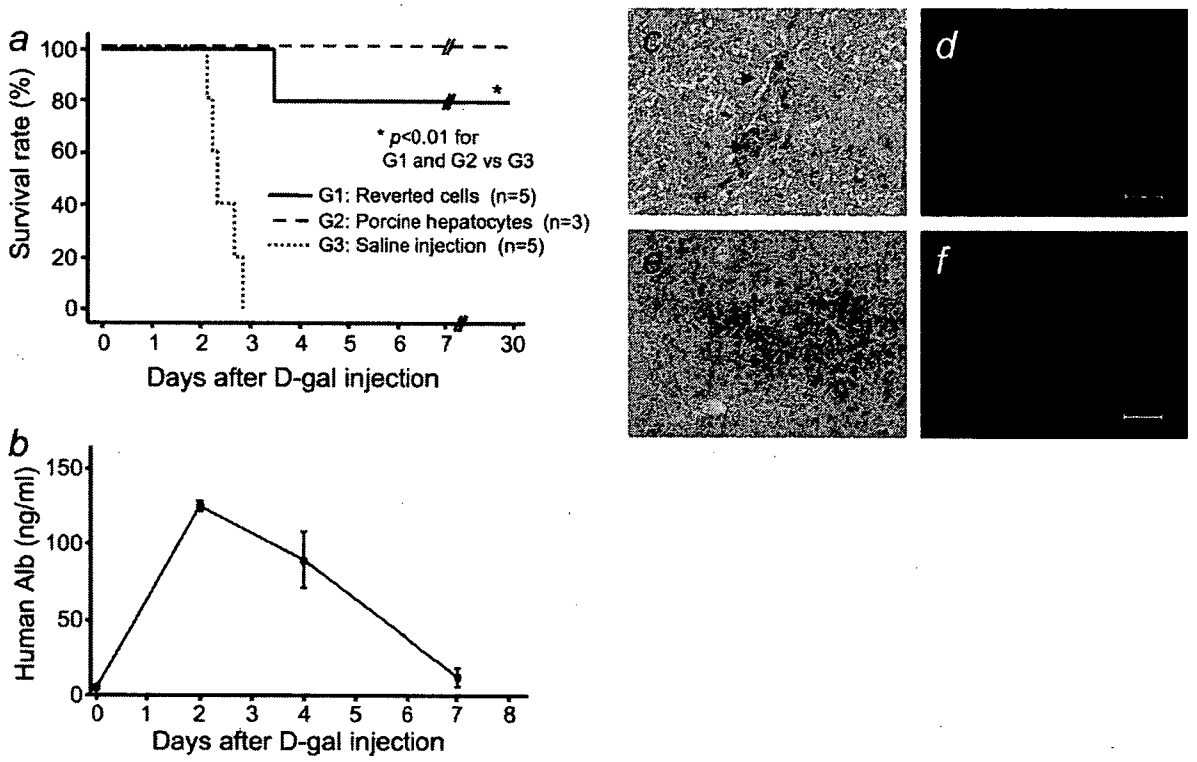

Fig. 5. In vivo 16-T3 cell transplantation experiments in pigs in ALF (a) Comparative survival curves up to 1 month post induction of ALF with D-Gal injection and subsequent intraportal cell transplantation 24 h later. 100% of G1 and 80% of G2 survived long-term, respectively, whereas all control G3 pigs died within approximately 3 days post induction of ALF. (b) The blood level of human specific albumin in the G1 pigs suggested the engraftment of the reverted 16-T3 cells for at least 4 days to support the damaged liver to regenerate. (c) The liver specimen obtained from G1 pigs on day 3 after transplantation revealed clusters of reverted 16-T3 in the portal areas with severe hepatic necrosis induced by D-Gal (magnification × 100). (d) These cells were positive for human specific albumin by immunofluorescence staining with a specific antibody (magnification × 200). (e) In the liver sample taken from G1 pigs 2 weeks after cell transplantation, mild mononuclear infiltrates were observed in the portal areas and no 16-T3 cells or tumor development was identified (magnification × 200) and (f) there were no human specific albumin-positive cells in the samples (magnification × 100).

an siRNA may be effective at rendering the cells resistant to Hepatitis C replication [25]. In the case of liver inborn errors of direct metabolism, autologous hepatocytes could be obtained by limited liver biopsy. Subsequent gene transfer in autologous hepatocytes submitted to the reversible immortalization procedure could allow long-term correction of a genetic disease such as the hemophilias or hypercholesterolemia caused by deficiency in the low density lipoprotein receptor. A potential advantage of this approach as compared to direct in vivo gene therapy is that expression of the therapeutic gene will be restricted to hepatocytes without the risk of triggering a cytotoxic immune response by inadvertant in vivo transduction of antigen presenting cells residing in the liver [26]. In addition, direct gene correction by homologous recombination or gene repair would now be attainable in the immortalized cell population. In clinical situations where the cells are not expected to be rejected by a mismatched host immune system (i.e., autologous and allocompatible cell transplantation), a conditional suicide gene such as Herpes Simplex Virus-thymidine kinase (HSV-TK) could be constitutively expressed in the cells for additional safety [27].

In conclusion, Cre/LoxP-mediated reversible immortalization of human hepatocytes with Tamoxifen-mediated self-recombination made unlimited supplies of reverted cells feasible, allowing a proof-of-principle demonstration of their utility in a pig model of ALF.

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ORIGINAL PAPER

Identification of CTL epitopes in hepatitis C virus by a genome-wide computational scanning and a rational design of peptide vaccine

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Abstract Developing a peptide-based vaccine for the highly variable hepatitis C virus (HCV) remains a challenging task. Variant viruses not only escape antigen presentation but also persist in a patient as quasi-species. Such variants are often antagonistic to the responding T cell repertoire. To overcome these problems, we herein propose a cocktail vaccine consisting of a few epitope peptides, which make it possible to outpace the emergence of variant viruses. To design such a vaccine, we developed a way to identify HLA-A*2402-binding peptides efficiently by means of the computational scanning of the whole genome of the pathogen. Most of the predicted peptides exhibited strong binding to the HLA-A*2402 molecule, while also inducing CD8 T cell responses from the patients' peripheral blood mononuclear cells (PBMCs). Peptide-induced T cells were capable of lysing HCV-expressing HepG2 cells which process antigens endogenously. The amount of HCV core antigen in the patients' livers suggested that the lytic activity of the peptide-induced T cells was clearly in a range suitable for therapeutic use. If T cells were activated under optimal conditions by high density peptides, then they tended to be relatively tolerant of single amino acid variations for cytolysis. Finally, an analysis of the viral population isolated in Japan suggested no obvious changes due to immune evasion in the viral genome even in a host population highly biased toward HLA-A*2402.

Keywords Cytotoxic T lymphocyte · Hepatitis C virus · Peptide · HLA · Vaccine

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Introduction

Hepatitis C virus (HCV) remains a serious threat due to its persistence and the fact that it can also cause liver cirrhosis and cancer. Even the most advanced treatment combining pegylated interferon-α and ribavirin only has a sustained viral clearance rate of just more than 50%, and this falls even further with the HCV genotype 1 and for

patients with other comorbidities (Feld and Hoofnagle 2005; Tsubota et al. 2004). The induction of cytotoxic T lymphocytes (CTLs) specific for HCV-infected cells has been a promising strategy for viral containment. A number of HLA class I-binding peptides have so far been identified (Battergay et al. 1995; Cerny et al. 1995; Kurokohchi et al. 2001; Nakamoto et al. 2003). However, finding HLA-binding peptides and screening them for T cell responses is a laborious, expensive, and time-consuming process. Therefore, HLA-binding has not yet been examined for a number of known T cell epitopes. However, if the affinity is low, even if a high density of exogenously added peptide may stimulate T cells, the infected hepatocytes may not present a sufficient number of peptides to be recognized by such T cells. Therefore, it is important to develop a method to design a peptide-based vaccine more efficiently and effectively.

HCV is an RNA virus with a high rate of mutation due to the absence of any proof-reading activity in its RNA-dependent RNA polymerase. Viral escape from immune attack by epitope changes has been studied by a number of groups and remains a major concern in vaccine development (Ray et al. 2005; Timm et al. 2004; Weiner et al. 1995). The first type of such mutations leads to poor processing of MHC-binding peptides due to an alteration of the amino acid sequences for proteasome cleavage (Kimura et al. 2005; Seifert et al. 2004). The second type leads to poor binding of epitope peptides to the MHC class I molecules (Chang et al. 1997; Cox et al. 2005; Erickson et al. 2001). The third type induces altered responses known as antagonism or anergy in T cells due to a poor recognition by TCR (Chang et al. 1997; Cox et al. 2005; Erickson et al. 2001; Grakoui et al. 2003; Kaneko et al. 1997). The virus not only changes, but it also exists as a quasi-species, i.e., a mixed population of distinct, but closely related, variants. These variants are often antagonistic to the responding T cell repertoire, and thus, annihilate the T cell responses. The quasi-species of viruses are kept in a dynamic yet lasting balance, and thus, effectively suppress the cytolytic activities of a dominant T cell repertoire (Chang et al. 1997). Therefore, the key to a successful vaccine lies in a design that can eradicate the heterogeneous viral population before escaped mutants become prevalent.

Materials and methods

Cells and antibodies

RzM6 is a HepG2 transfectant with a full genome HCV1b isolate AY045702 (Tsukiyama-Kohara et al. 2004). The expression of HCV can be induced by the tamoxifen-induced expression of the doubly transfected Cre that mediates the removal of an intervening sequence in the 5′ promoter region of the HCV genome. C1R was a gift from Dr. P. Cresswell (Edwards et al. 1982). C1R-A24 is a HLA-A*2402-transfected C1R cell line and was kindly provided by Dr. Takiguchi (Karaki et al. 1993). HepG2 was purchased from ATCC. T2-A24 is a TAP-deficient T2 cell line transfected with HLA-A*2402 and was a gift from Dr. A. Tsuboi (Osaka University).

Peptides

The peptides were manually synthesized using Fmoc chemistry and then were purified by HPLC to a purity of >95% using a C18 Microbondasphere column (Japan Waters, Tokyo). The peptides were examined by mass spectrometry using Voyager DE-RP (Applied Biosystems Japan, Tokyo). The concentrations were determined by a MicroBCA assay using bovine serum albumin (BSA) as the standard (Pierce, Rockford, IL).

Peptide-binding assay

The binding of peptides to the HLA-A*2402 molecule was measured by acid stripping and a reconstitution assay as previously described by Zeh et al. (1994) with minor modifications. Briefly, C1R-A24 cells were exposed to pH 3.3 citrate phosphate buffer and then were reconstituted with graded concentrations of peptide and 0.1 μM human β2-microglobulin (Sigma, M-4890, St. Louis, MS) in DMEM containing 0.25% BSA. An FITC-labeled mAb 17A12 (Tahara et al. 1990) was used to detect the properly folded and peptide-bound HLA-A*2402 molecules. The fluorescence intensity was measured by FACScan (Becton-Dickinson Japan, Tokyo). Both high- and low-binding peptides, HER2-63 TYLPTNASL and Met149 RVWE SATPL, respectively, were always included in the assay, and their binding was used to normalize the variations between experiments. The affinity of a peptide was calculated as previously described (Udaka et al. 2000).

Peptide-specific cell lines

The peripheral blood mononuclear cells (PBMCs) from patients or healthy individuals were stimulated weekly with 1 μM peptide in 10% fetal calf serum (FCS) containing 10 U/ml recombinant human IL-2. After five time stimulations, cells were tested for killing activity. The patients had been diagnosed to be suffering from chronic hepatitis with HCV genotype 1b. All the patients were positive for both the anti-HCV antibodies and viral RNA in the serum by polymerase chain reaction (PCR). Informed consent was obtained from all patients. The study protocol

was approved by the Human Research Committee of Ehime University.

^{51}Cr release assay

A peptide-specific cytolysis assay was conducted against C1R-A24 cells or T2-A24 cells in the presence or absence of 1 μM peptide. An HLA-A*2402-binding peptide, HER2-63 (TYLPTNASL, log Ka; 7.3), was used as a negative control. Target cells were labeled with ^{51}Cr-sodium chromate at either 37 or 26°C, respectively, for 1 h, and then, they were loaded with 1 μM peptide before the addition of effector cells. The *E/T* ratio was 10–20. Percent specific lysis during 3.5 h incubation at 37°C was calculated as (experimental release−spontaneous release)/(total release−spontaneous release)×100. The cytolysis of cells that naturally present HCV peptides was measured using tamoxifen-treated RzM6 cells as a target for 3 h at an *E/T* ratio of 10.

Measurement of HCV core protein

Liver tissue specimens from the patients were taken by a biopsy after informed consent was obtained from all the patients. The cell lines and tissue specimens were lysed in Radioimmunoprecipitation assay (RIPA) buffer (1% sodium dodecyl sulfate (SDS), 1% NP40, 10 mM Tris–HCl, pH 8.0 and 0.14 M NaCl) and the supernatant was subjected to the measurement of core protein using an HCV Ag enzyme-linked immunosorbent assay (ELISA) kit (Ortho-Clinical Diagnostics, Tokyo, Japan). The protein concentrations were determined with a DC protein assay (BIO-RAD, Hercules, CA).

Results

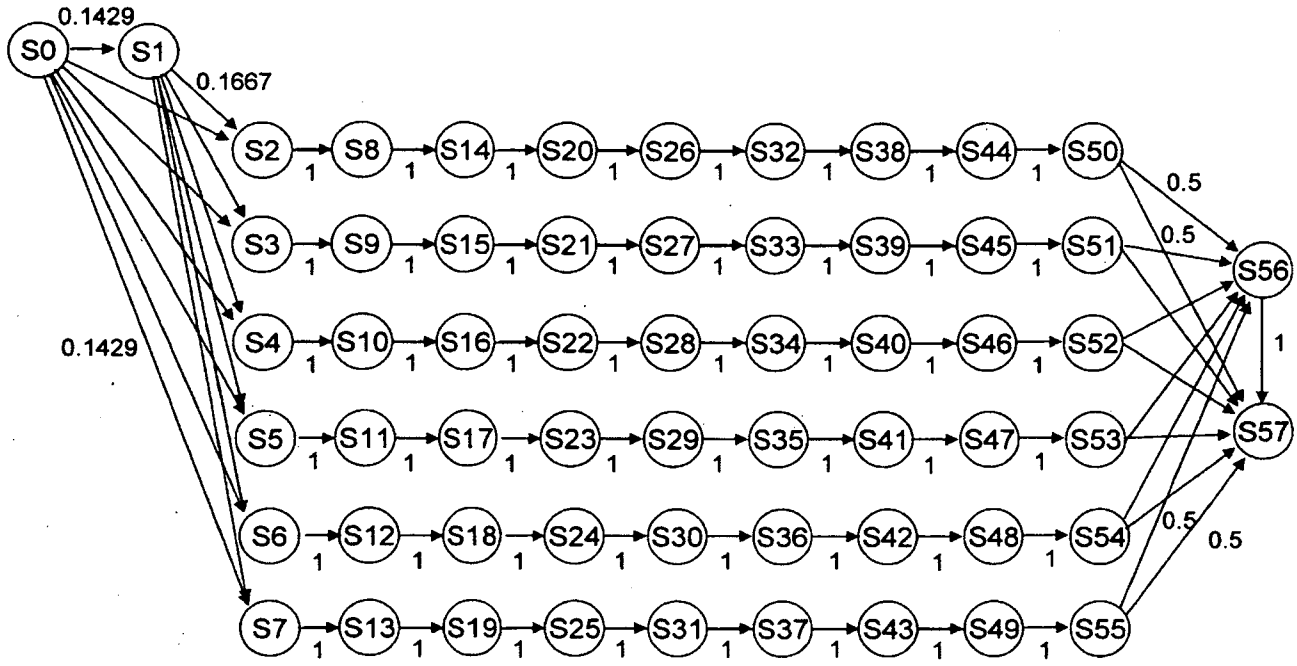
Generation of a program to predict HLA-A*2402-binding peptides

We have previously described a computational method to analyze the specificity of MHC class I-binding peptides (Udaka et al. 2002). The method utilizes a data mining technique, a query learning algorithm based on hidden Markov models (HMMs). This algorithm finds peptides whose binding properties are hard to predict by using a prototype prediction program established with the existing binding data (supplementary material 2). We synthesized such peptides and measured their binding to MHC molecules. By feeding the newly obtained binding data back into the data pool, the prediction program could thus be improved. This cyclic learning that combines data analysis and peptide-binding experiments is repeated until a satisfactory prediction can be achieved. This time, we examined the HLA-A*2402 molecule, the most frequent allele among Asians, e.g., 33% for the Japanese (Tokunaga et al. 1997) and ~10% in the Western countries (Imanishi et al. 1992). The original HMMs employed a cyclic model (Udaka et al. 2002), but this was switched to a more rational parallel model during the learning (Fig. 1a). After 6 rounds of cyclic learning, examining 400 peptides altogether, i.e., 222 before active learning and 178 newly synthesized during learning, a final prediction program was established. However, further learning was still possible when necessary. The binding data for an additional 105 peptides that had been set aside to monitor the progress of the learning were also added to the data pool to generate the final prediction program. This program predicts the binding affinity by approximating the log K_a values, and thus, gives each peptide a score in real numbers. The performance of the program was assessed by 10-fold cross-validation using the binding data on 505 peptides described above. The coefficient of the correlation between the predicted scores and actual affinity was 0.80 (Fig. 1b). Most of the known T cell epitope peptides have an affinity of 5.5 or higher in the log K_a terms. Among the peptides whose predicted scores were 5.5 or higher, 93% (117/126) actually exhibited a log K_a value of 5.5 or higher. Therefore, the accuracy (sensitivity) of the prediction was 93%. On the other hand, among those peptides whose affinity was 5.5 or higher, 60% (113/187) had scored 5.5 or higher. Therefore, the coverage was 60%. If the threshold had been raised to 6.0, then the accuracy would have been 93% (67/72) and the coverage thus would have fallen to 43% (67/156).

Genome-wide screening of hepatitis C virus for HLA-A*2402-binding peptides

Using the program developed above, we scanned the entire genome of hepatitis C virus (HCV) genotype 1b for HLA-A*2402-binding peptides. We chose a prototype Japanese isolate GenBank D20908 and a subclone of D89815 for the analysis. The latter was chosen due to our initial plan to use cells transduced with that clone as a target. Several amino acids differ between D89815 and its subclone pBRT703′X (Dr. Y. Matsuura, personal communication). The high-scoring peptides were synthesized and subjected to HLA-binding assays. Some peptides (~7% of the peptides synthesized) were hard to synthesize, and thus, were excluded. Several known epitope peptides also scored high, and their binding was examined. The results are shown in Table 1. High-binder peptides (log K_a >5.5) are in bold. The major anchor amino acids identified by Rammensee et al. for HLA-A*2402 are Y/F at P2 and I/L/F at P9 (http://www.syfpeithi.de/). The peptides in italics do not fulfill

a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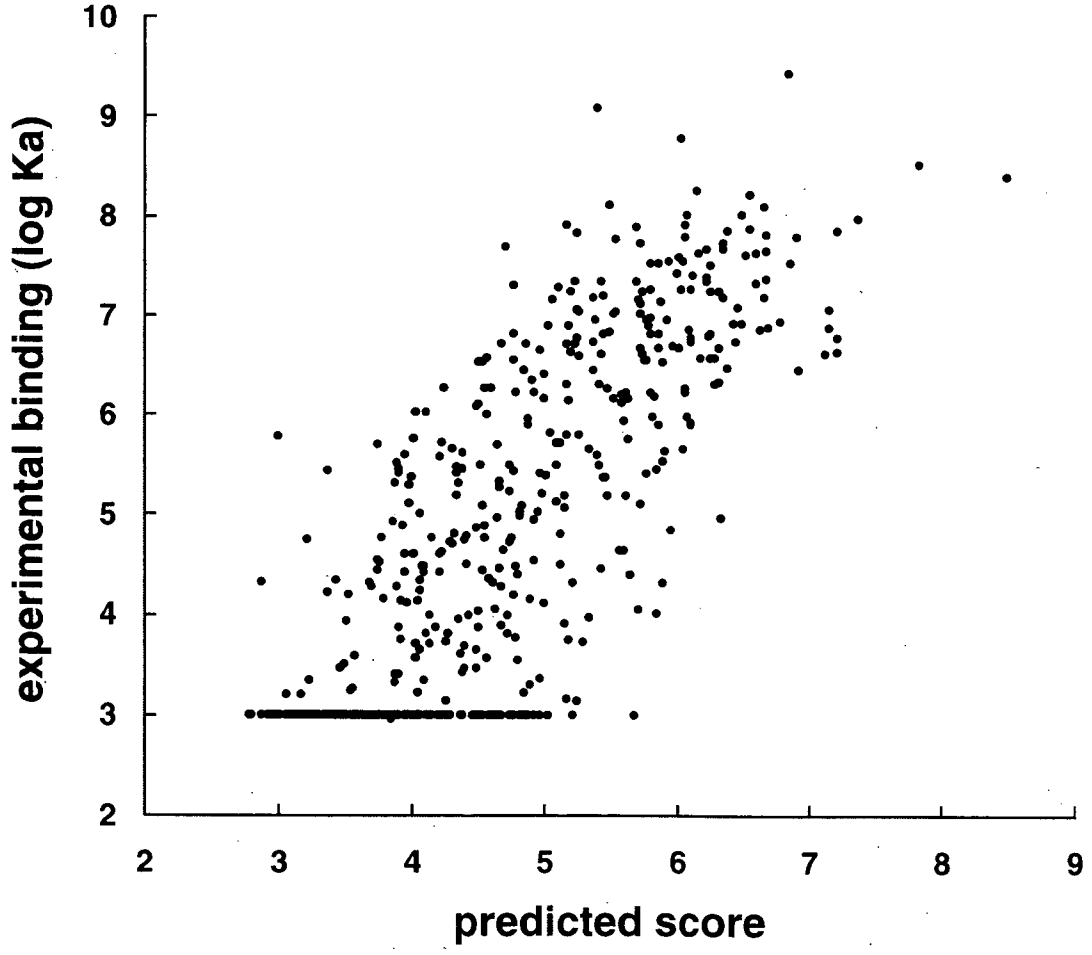
experimental binding (log Ka)
predicted score
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◀ **Fig. 1** The analysis and prediction of HLA-A*2402-binding peptides. **a** A hidden Markov model used for analyzing the specificity of HLA-A*2402-binding peptides. A general description of the model and the data mining algorithm are given in the reference (Udaka et al. 2000) and supplementary material 2. Six independent paths were designed. The first node S0 depicts the initial state and the last node S57 designates the final state. In the states from S1 to S56, the frequency of every amino acid is calculated to give the sum of the probabilities for 20 amino acids to be one in each state. *Arrows* depict transition probabilities. The states S1 and S56 were introduced to accommodate lateral shifts of the binding motif by one amino acid in both directions. Nine states in each row depict amino acid positions in a peptide of nine amino acids starting from the N-terminal amino acid on the left. The initial state can take any path and S1 in the case of an N-terminal shift of the binding motif. The frequency of transition was tentatively given as 0.1429 (=1 / 7) for each *arrow*, but it is replaced by an actual frequency once the model is trained using peptide-binding data. The final state can be reached from any path and from S56. The probability of transition from a row to the final state is tentatively given as 0.5 for each *arrow* in the illustration, but this too is replaced by actual probability. **b** The correlation of the predicted HLA-A*2402-binding scores and experimentally determined binding of peptides. The correlation was examined by a tenfold cross validation using the peptide-binding data obtained during cyclic training of the models

two anchor requirements. The high-binder peptides could also be identified among such peptides (underlined peptides).

Responses of the PBMCs from chronic hepatitis patients

Due to the limits of the cell culture, 15 peptides (log K_a >6) were randomly chosen out of the peptides noted in Table 1 and then were tested for the induction of cytotoxic T lymphocytes (CTLs). PBMCs from patients with chronic hepatitis due to HCV 1b and from healthy individuals were stimulated with peptides and tested for cytolysis by ^{51}Cr release assay. A poor cytolytic activity of CD8 T cells despite a robust production of IFN-γ have been observed among both tumor-bearing patients and patients suffering from chronic viral infections (Appay et al. 2000; Huang et al. 2005; Wherry et al. 2003). Therefore, in this study, with the aim of developing a curative vaccine, we used a cytolysis assay. As shown in Fig. 2a and b, most of the peptide-stimulated cell lines exhibited cytotoxicity. Interestingly, there were several peptides to which many patients responded. These peptides can thus be good targets for immunotherapy. Healthy individuals occasionally exhibited some responses but less frequently than the patients (Fig. 2a,b). Most patients exhibited cytolytic activities, often to several peptides. This strongly indicates that in the patients, a HCV-specific T cell repertoire has expanded and the cytolytic activity can therefore be induced if antigenic peptides are provided in a stimulatory environment in vitro.

Peptide-specific cell lines were further examined for restricting HLA molecules and peptide dependency for

Table 1 Prediction of HLA-A*2402-binding peptides in the HCV 1b genome

Protein	Peptide	Sequence	Predicted score	Actual binding normalized log K_a
Core	C36shpB[a]	***LLPRRGPRL***	5.30	**7.71**
	C77shpB	*AQPGYPWPL*	5.98	5.36
	C169shpB	*LPGCSFSIF*	4.98	4.91
E1	C234sh	**NFSRCWVAL**	5.46	**5.92**
	C360shpB	***AYYSMVGNW***	5.73	**6.47**
E2	C616shpB	***WHYPCTVNF***	5.66	**6.39**
	C666shpB	***LLSTTEWQI***	5.35	**8.34**
	C674sh	***ILPCSFTTL***	6.75	**7.66**
	C688pB	***GLIHLHQNI***	6.00	**5.86**
	C764pB	***GILPFFMFF***	6.26	**7.70**
	C789sh	***ALYGVWPLL***	5.80	**6.98**
	C789pB	**AFYGVWPLL**	6.02	**7.69**
NS2	C834sh	**YYKVFLARL**	5.49	**7.12**
	C838shpB	***FLARLIWWL***	5.24	**6.18**
	C876pB	***LMCAVHPEL***	5.52	**6.59**
	C975sh	**VFSDMETKL**	5.54	**7.31**
	C992shpB	*ACGDIISGL*	5.26	3.91
	C1010shpB	***ILLGPADSF***	5.43	**5.50**
NS3	C1031shpB	**AYSQQTRGL**[b]	5.76	**6.54**
	C1291sh	***ITYSTYCKF***	5.27	**6.98**
	C1291pB	***ITYSTYGKF***	5.16	**6.37**
	C1349pB	***ATPPGSVTF***	6.53	**6.51**
NS4B	C1760shpB	***FWAKHMWNF***[c]	5.93	**8.10**
	C1956shpB	***LLKRLHQWI***	5.20	**6.86**
NS5A	C1976pB	***WLRDVWDWI***	5.27	**6.34**
	C1986pB	***TVLADFKTW***	6.25	**6.52**
	C1987pB	***VLADFKTWL***	5.26	**6.63**
	C2132sh	**RYAPVCKPL**	5.96	**6.15**
	C2132pB	**RYAPACKPL**	5.47	**6.76**
	C2139pB	*PLLRDEVTF*	6.39	5.09
	C2173shpB	***SMLTDPSHI***	5.55	**6.94**
	C2251sh	*VILDSFDPI*	6.05	5.32
	C2251pB	*VILDSFEPL*	6.26	5.00
	C2289shpB	*ARPDYNPPL*	5.47	3.93
NS5B	C2422shpB	**SYTWTGALI**	5.51	**7.13**
	C2593shpB	***ALYDVVSTL***	5.99	**6.39**
	C2841shpB	***RMILMTHFF***[c]	6.01	**7.42**
	C2843shpB	***ILMTHFFSI***	5.57	**7.90**
	C2844shpB	***LMTHFFSIL***	5.20	**5.92**
	C2962shpB	*SQLDLSGWF*	5.66	< 3

High-affinity peptides (log K_a>5.5) are shown in bold. The sequences in italics represent the peptides that do not fulfill two major anchor requirements. The high-affinity peptides that would not have been identified by the criteria of two major anchors are underlined.

[a] The number refers to the position of the first amino acid. The suffix sh stands for a sequence from Genbank D20908, and pB, that from the subclone pBRT703′X of D89815. A substantial number of nucleotide mismatches have been identified between pBRT703′X and D89815 (Matsuura, personal communication).

[b] Epitope reported by Kurokohchi et al. (2001)

[c] Epitopes reported by Nakamoto et al. (2003)

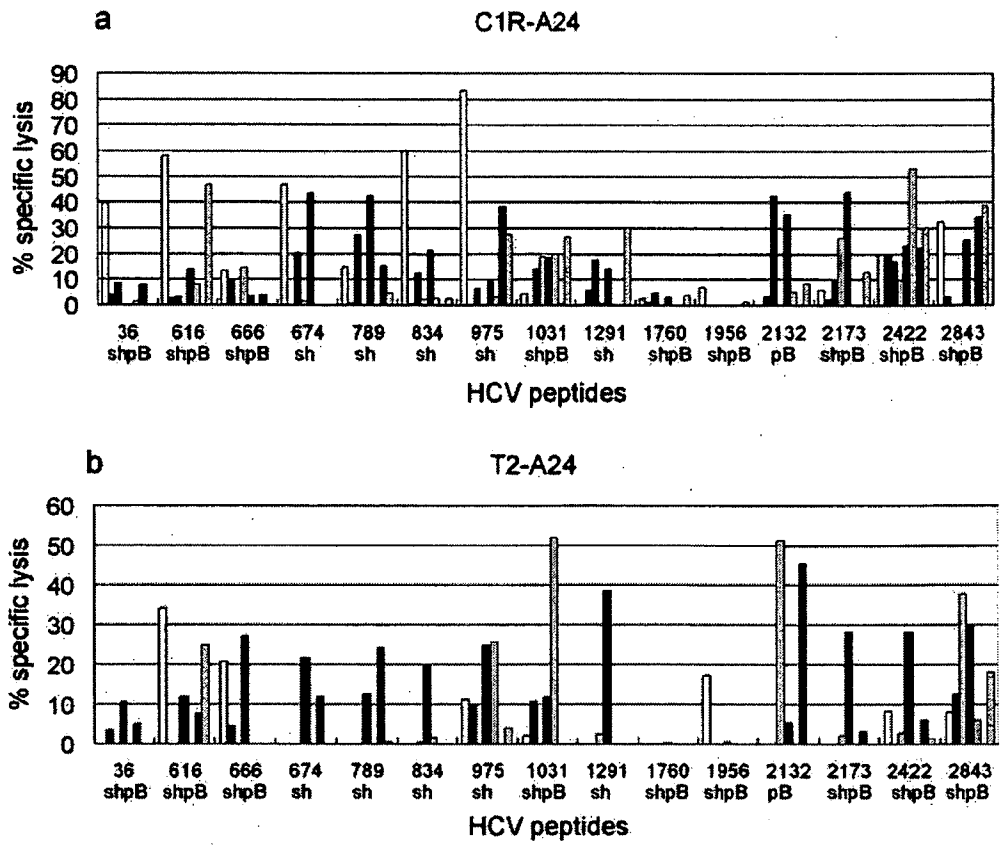

Fig. 2 The cytolytic activities of the peptide-induced cell lines from patients and healthy individuals. Peptide-specific cell lines were established from PBMCs by weekly stimulations with peptides. Each bar demonstrates lytic activity by a cell line from an individual. The assay used allogeneic C1R-A24 in **a** or T2-A24 cells in **b** as a target and was performed in the presence of indicated peptide at a concentration of 1 μM. These target cells exhibited substantial background lysis either without any peptide or with a negative control peptide HER2-63. Therefore, specific lysis is shown as the value from which the background lysis with HER2-63 has been subtracted. Nonoverlapping responders were used for **a** and **b**. The first (*open*) and third (*dark gray*) bars in **a** and the fourth (*closed*) *bar* are cell lines from healthy individuals

recognition. The responding cell lines were predominantly $CD8^+$ T cells as is shown in Fig. 3a. When tested against C1R or C1R-A24 target cells, several cell lines exhibited a stronger cytolytic activity against C1R-A24 than C1R in the presence of peptides (Fig. 3b). It was not possible to perform this assay for all the cell lines shown in Fig. 2a,b due to the limited number of cells. For a few cell lines, the cytolysis of C1R was too high for one to see any peptide dependency. This was most likely due to some alloreactivity or NK-like activity because we used a low level of rIL2 during the cell culture.

Cytolytic activity of the peptide induced cell lines against cells naturally presenting the antigen

Although the peptide-induced T cell lines were highly cytolytic against tumor targets heavily loaded with exogenous peptides, it is not clear whether these effecter cells are also capable of killing target cells that present as endogenous antigens through natural antigen processing. To examine this, we tested a HepG2 transfectant RzM6 (Tsukiyama-Kohara et al. 2004) as a target. RzM6 carries a full-length HCV 1b genome (GenBank AY045702). HepG2 naturally expresses HLA-A*2402. The expression of the HCV gene from the CAG promoter can be conditionally induced by the Cre-mediated removal of the floxed intervening sequence that has been inserted in the 5′ terminal region. The RzM6 cells had been doubly transfected with the *Cre* expression construct whose expression could be induced by tamoxifen. The precise 5′ and 3′ trimming at the ribozyme sequences eventually produces a full-length HCV. As is shown in Table 2, the HLA-A*2402-binding peptides expressed in RzM6 differ by several amino acids from the HCV isolates shown in Table 1. Tamoxifen-treated RzM6 cells were lysed by most of the peptide-specific cell lines established as shown in Fig. 4. This indicated that most of the peptides identified as HLA-A*2402-binders from genomic sequences were actually processed endogenously and then presented on the cell surface. Interestingly, most of the peptide-induced CTL lines could lyse RzM6 cells whose epitope sequences carry amino acid substitution(s) compared with the peptides used for stimulation. Although such variant peptides could be antagonistic to part of the CTL repertoire (Kaneko et al. 1997) once the T cells were optimally activated by high-density peptides, then the responding T cells were also found to be cytolytic for such variant peptides. This is encouraging to those developing a peptide vaccine against highly variable pathogens like HCV. Interestingly, the peptide C616shpB is located in a sequence context which is not ideal for proteasomal cleavage (Nielsen et al. 2005; Nussbaum et al. 2001). However, this peptide also seems to

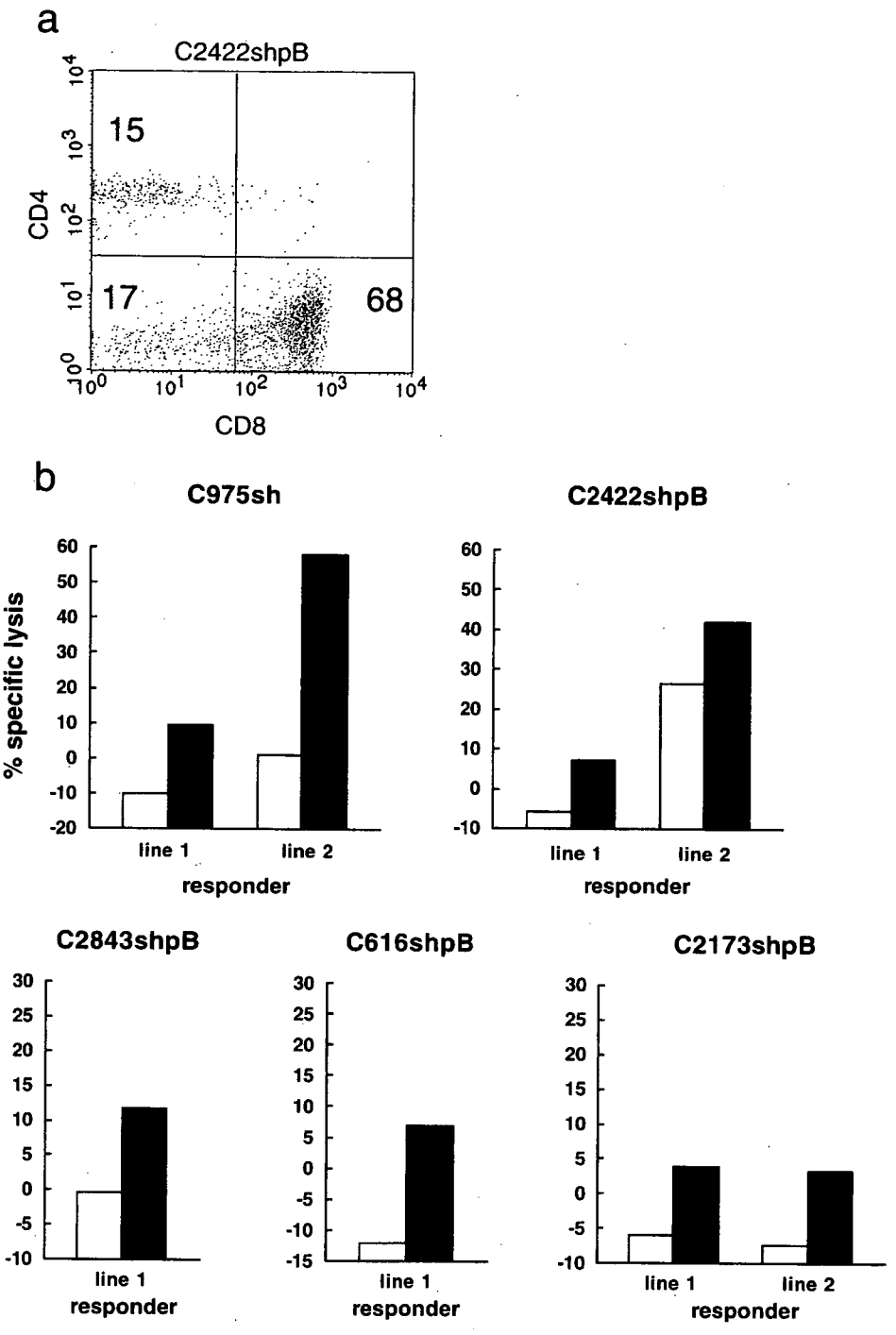

Fig. 3 Peptide-specific cell lines recognize peptides in the context of HLA-A*2402. **a** Cell surface profile of a cell line specific for C2422shpB shown as an example. The enrichment of CD8 cells was obvious for cell lines that exhibited peptide-dependent lysis after 3 to 5 weekly stimulations with HLA-A*2402-binding peptides. **b** Specific lysis of C1R (*open bars*) or C1R-A*2402 (*closed bars*) target cells in the presence of peptides used for the stimulation of the corresponding cell lines. Lysis without peptide has been subtracted

be presented on RzM6. Although inefficient proteasomal cleavage has been reported to be one of the mechanisms for viral escape (Seifert et al. 2004), not all the peptides that are predicted to demonstrate poor cleavage seem to be spared from antigen presentation. Under normal conditions, peptides are in relatively short supply in comparison to the newly synthesized MHC class I molecules in the endoplasmic reticulum (ER) (Lie et al. 1990). Slowly cleaved peptides may still have a chance to bind to empty MHC class I molecules.

HCV peptide presentation in the infected hepatocytes

Importantly, these peptide-specific T cells have, however, not been sufficient to contain viral infections in the patients. The expression of HCV proteins in tamoxifen-treated RzM6 cells (RzM6-Tx) may have been higher than that in the infected hepatocytes of the patients. We, therefore, examined next the expression of core protein as a representative antigen in the patients' livers. As shown in Fig. 5, the expression of core protein of HCV 1b was high

Table 2 Amino acid variations in the epitope sequences of the HVC 1b clones analyzed in this study

Peptides	D20908	pBRT703'X	RzM6
C36	LLPRRGPRL	- - - - - - - - -	- - - - - - - - -
C616	WHYPCTVNF	- - - - - - - - -	- - - - - - A - -
C666	LLSTTEWQI	- - - - - - - - -	- - - - - - - - V
C674	ILPCSFTTL	- - - - - - - - -	V - - - - - - - -
C789	ALYGVWPLL	- F - - - - - - -	- F - - - - - - -
C834	YYKVFLARL	- C - - - - - - -	- - - E - - - - -
C975	VFSDMETKL	- A - - - - V	- - - - - - - - V
C1031	AYSQQTAGL	- - - - - - - - -	- - - - - - - - -
C1291	ITYSTYCKF	- - - - - - G - -	- - - - - - G - -
C1760	FWAKHMWNF	- - - - - - - - -	- - - - - - - - -
C1956	LLKRLHQWI	- - - - - - - - -	- - - - - - H - -
C2132	RYAPVCKPL	- - - - A - - - -	- - - - A - - - -
C2173	SMLTDPSHI	- - - - - - - - -	- - - - - - - - -
C2422	SYTWTGALI	- - - - - - - - -	- - - - - - - - -
C2843	ILMTHFFSI	- - - - - - - - -	- - - - - - - - -

in RzM6-Tx, whereas liver tissues from patients suffering from chronic hepatitis had lower and variable levels of expression. Therefore, the lytic activity of the peptide-specific CTL lines against RzM6-Tx may not necessarily guarantee the lysis of infected hepatocytes. It is not known what proportion of hepatocytes in the patients actually express core antigen. In a report of immunostaining of the liver tissues from chronic hepatitis patients, 1–5% of the hepatocytes express core antigen to a level detectable by specific antibodies (Nouri-Aria et al. 1995). Agnello et al. reported that usually 50% or more, but not all, of the hepatocytes are infected by HCV in chronically infected patients (Agnello et al. 1998). If 50% of the hepatocytes expressed core protein, then the results shown in Fig. 5 would indicate that the expression level of the antigen in individual hepatocytes could be comparable to or within a few fold differences from RzM6-Tx in the patients. If so, there is a good chance that infected hepatocytes are lysed by the peptide-induced CTLs. Therefore, the therapeutic potential of peptide-based vaccine seems to be realistic provided that T cells are activated under optimal conditions with high-density peptides and in an immunostimulatory environment including activated APCs and proper helper activities.

Variability of HCV 1b genomes among Japanese isolates

As HCV is an RNA virus that has a high mutation rate, the sequence variation among HCV isolates is extensive. The

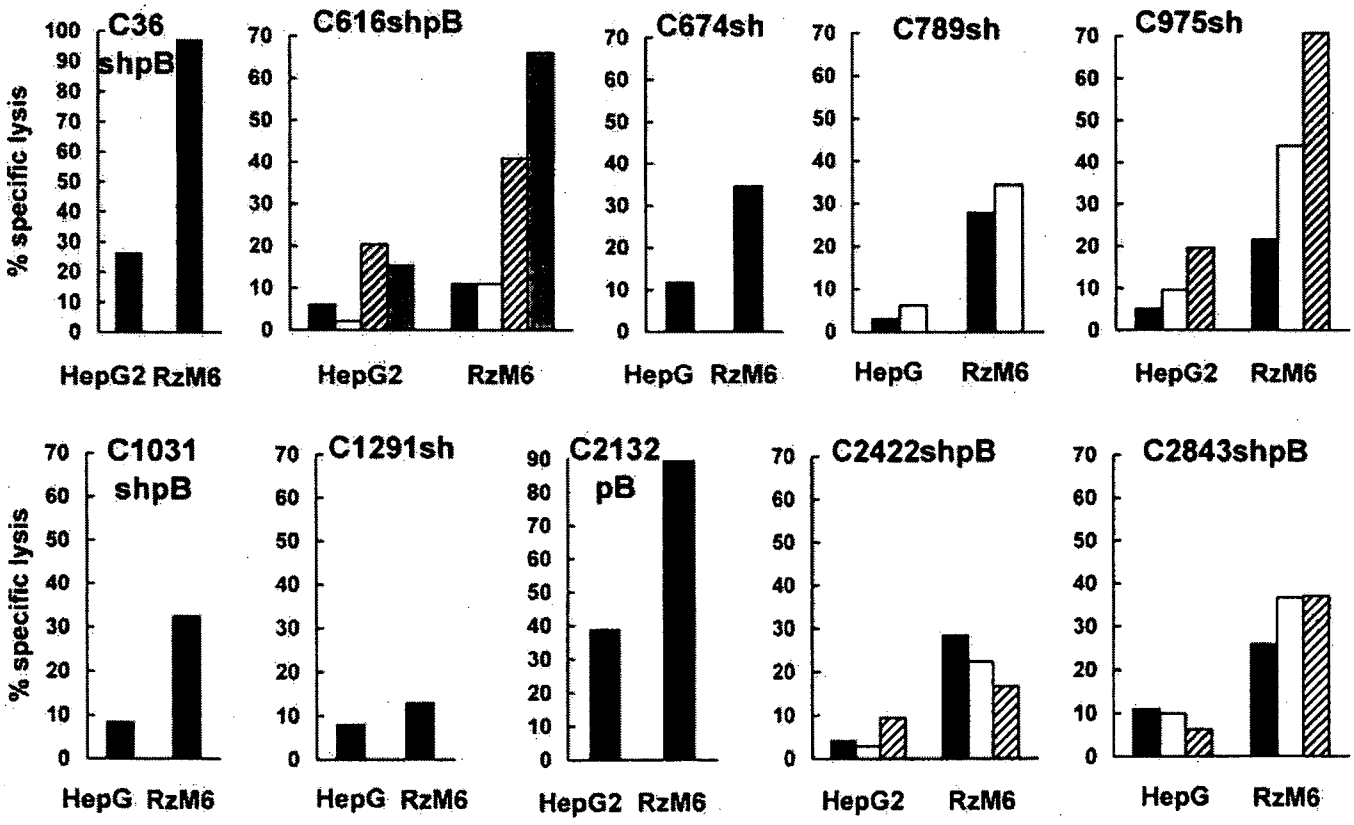

Fig. 4 Lysis of HCV 1b-transfected RzM6 cells by peptide-specific cell lines. The expression of the HCV genome in RzM6 had been induced by tamoxifen-induced Cre expression as described in the Materials and methods section. Peptide-specific cell lines from HCV 1b-infected patients and healthy individuals lysed RzM6 more than the parental HepG2 cells. Individual bars represent the lytic activities of the independent cell lines established from different individuals

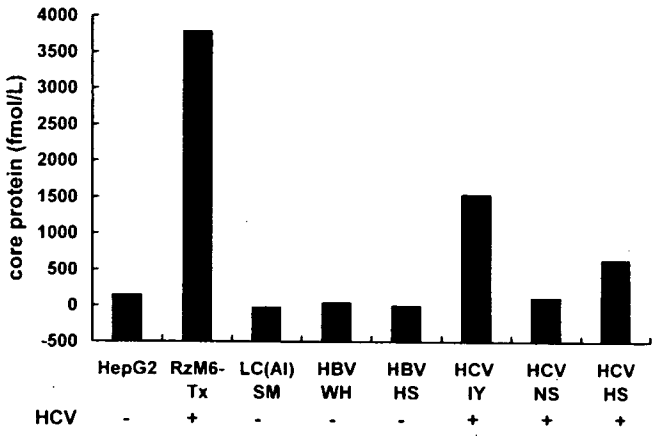

Fig. 5 Core protein expression in a HCV-transfected HepG2 cell line and the liver tissue specimens from HCV-infected patients. The amount of core protein was measured by ELISA in reference to the recombinant core protein. The average value for HCV-negative liver tissues was subtracted as background. The expression of HCV 1b in RzM6 had been induced by tamoxifen (RzM6-Tx). Negative control tissue specimens were from a patient with alcoholic liver cirrhosis; LC (Al) and two HBV-infected patients (WH, HS). Biopsy specimens from three HCV 1b RNA-positive patients (IY, NS, HS) with chronic hepatitis were examined. All samples were measured at a total protein concentration of 0.2 mg/ml

emergence of escape variants during the course of HCV infection has been a subject of serious concern in vaccine development (Chang et al. 1997; Cox et al. 2005; Kaneko et al. 1997; Timm et al. 2004). HLA-A*2402 has a gene frequency of around 30% in the Japanese population (Tanaka et al. 1996; Tokunaga et al. 1997). One-half of all Japanese most likely are at least heterozygous for this allele. Therefore, an HCV virus may thus encounter an HLA-A*2402-bearing host on every other transmissions on average. Such evolutionary pressure over thousands of years may thus have left some footprints on the viral genome. We therefore examined all Japanese isolates of HCV 1b identified to date for whether there is any evidence of amino acid changes in the HLA-A*2402-binding peptides that may have helped the virus escape from immune attack. We analyzed the non-synonymous/synonymous (NS/S) substitution rates (Nei and Gojobori 1986) between HLA-A*2402-binding peptides and the rest of the genome. Hyper-variable regions (HVR) 1 and 2 were analyzed separately because those regions may have been under different evolutionary pressure such as an evasion from antibody responses. The full genome sequence was available for 70 isolates (supplementary material 1).

We first generated an evolutionary tree based on the rapidly changing sequences in HVR 1 and 2 regions (Fig. 6). According to the tree, we calculated and compared the ratio of NS/S substitutions between the HLA-A*2402-binding peptides and then the rest of the genome excluding HVR1 and 2 regions (Table 3). Amino acid substitutions were frequently observed throughout the HCV 1b genome.

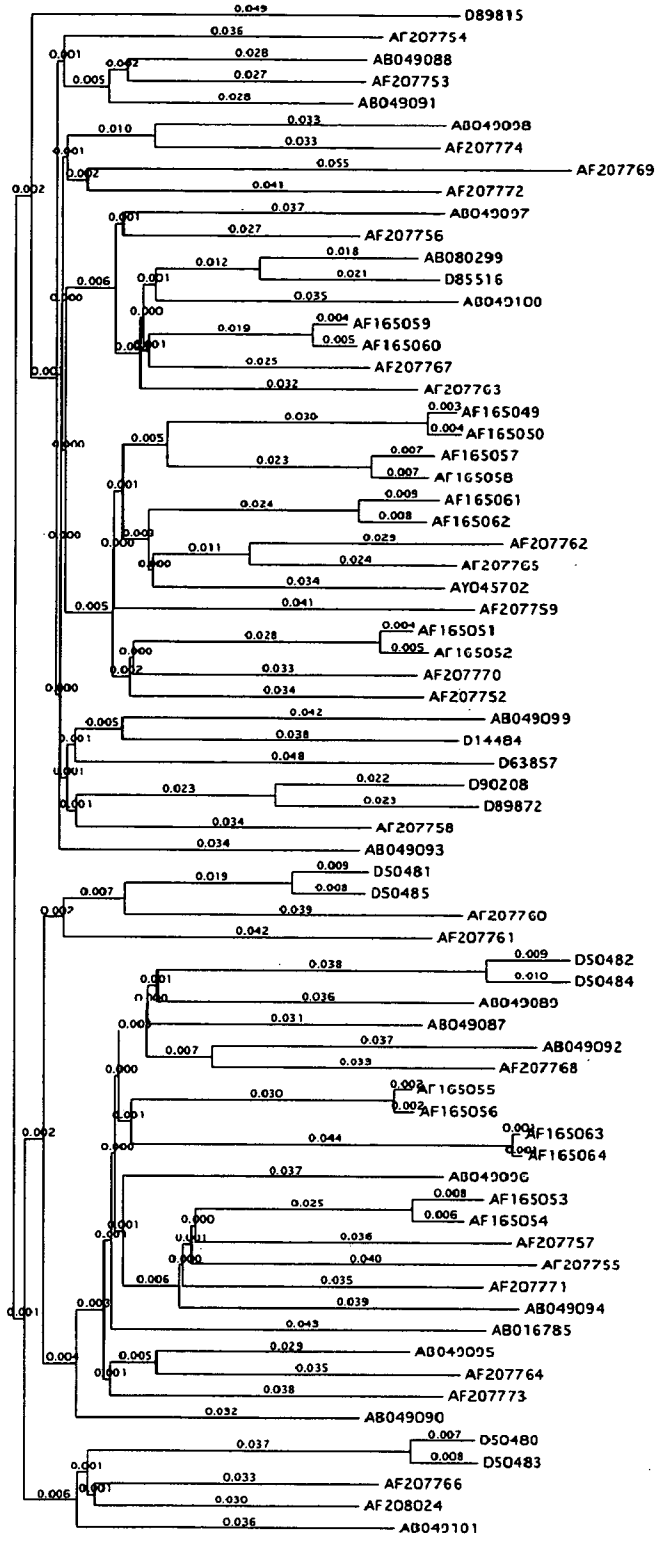

Fig. 6 Phylogenetic tree of the HCV 1b viruses isolated in Japan. An evolutionary relationship among the Japanese isolates of HCV 1b was examined by comparing the sequences in hyper variable regions (HVRs) 1 and 2. The numbers indicate the substitutions per site. The genbank accession numbers of the sequence data are shown

Table 3 Ratio of nonsynonymous versus synonymous substitutions

Region[a]	Nonsynonymous substitutions[b]			Synonymous substitutions[c]			Ratio
	K_N	L_N	d_N	K_S	L_S	d_S	d_N/d_S
Whole	201	6543	0.0307	556	2,418	0.230	0.13
HVR	20.1	58.7	0.342	8.0	22.3	0.359	0.95
ΔHVR	181	6,485	0.0279	548	2,396	0.229	0.12
BS (A24)	12.9	518.4	0.0249	42.2	177.6	0.238	0.11
Non-BS	188	6,025	0.0312	514	2,240	0.229	0.14

[a] *HVR* Hyper Variable Region, *ΔHVR* sequences excluding HVR, *BS(A24)* HLA-A*2402-binding sites, *Non-BS* sequences excluding BS & HVR
[b] K_N, L_N, and d_N stand for the mean number of nonsynonymous substitutions, the mean number of nonsynonymous sites, and the mean number of nonsynonymous substitutions per site, respectively.
[c] K_S, L_S, and d_S stand for the mean number of synonymous substitutions, the mean number of synonymous sites, and the mean number of synonymous substitutions per site, respectively.

HVRs exhibited a marked increase in the NS/S substitution rate, thus, indicating a strong selective pressure for amino acid changes in those regions. In contrast, the NS/S ratio in the HLA-A*2402-binding peptides was not significantly higher than that in other regions of the genome. In several longitudinal studies that followed the changes in individual patients, frequent amino acid substitutions were accumulated in the epitopes for CTLs and antibodies (Cox et al. 2005; Tester et al. 2005; Timm et al. 2004). An elevated NS substitution rate in the HLA class I-binding epitopes (Cox et al. 2005), therefore, suggests that substitutions positively contributed to the survival of the virus. In contrast to longitudinal studies in individual patients, this analysis indicates that such a bias is not obvious in a viral population that has been circulating among a host population which is heavily biased for HLA-A*2402. Ray et al. reported that in the absence of selection, amino acid variation tends to converge toward the consensus sequence, the structure of which is likely better adapted to the function of viral proteins. Such convergence may have rapidly occurred when the virus infected the non-HLA-A*2402-bearing hosts. Judging from the present analysis, it would thus be possible to develop a peptide vaccine for the public where the virus is under continuous selection by one-half of the host population. This is an encouraging result, provided that a strategy is developed to induce immune responses quickly and thoroughly before escape variants emerge.

Discussion

One of the most troublesome features of HCV for vaccine development is its genetic instability. Patients carry quasi-species of variant viruses which can sometimes be antagonistic to each other for virus-specific T cells. One of the authors and her colleagues have previously shown that the antagonistic inhibition of CTL-mediated cytolysis requires the expression of two closely related peptides on the same antigen-presenting cell that should occur only under limited conditions in a natural infection. More problematically, however, an antagonistic peptide expressed alone on a single antigen-presenting cell can effectively impair antigen-induced proliferation (Kaneko et al. 1997) which, therefore, would normally require a stronger engagement of TCRs than cytolysis (Valitutti et al. 1996). This selectively and effectively limits the expansion of agonist-specific CTLs (Kaneko et al. 1997). When designing a peptide vaccine against a variable target like HCV, it has to be kept in mind that the use of a particular peptide always bears a risk of inducing antagonistic responses in the responding T cell repertoire.

In this study, we observed that the peptide induced CTL lines, if properly stimulated, were cytolytic against the HepG2 transfectant that naturally presents HCV peptides. The transfectant carried amino acid substitutions in some of the epitopes, but it was thereafter effectively killed by CTL lines which were stimulated by wild type peptides. It is not known, however, whether this observation is relevant to natural infections because the transfectant may express more HCV antigens than the liver tissue specimens obtained from chronic hepatitis patients. Under suboptimal conditions, variant peptides may act as antagonists, or they could show a poor presentation in the HLA class I molecules. To overcome these problems, T cells have to be stimulated fully, at least, at the local site of immunization. To provide a stimulatory environment for T cells, the following points should be considered: (1) Epitope peptides should be provided at high density to overcome poor responses of the T cell repertoire that may not have an optimal affinity. An exogenously added synthetic peptide has an advantage over a DNA vaccine in this regard. If a DNA vaccine is used, then, it has to be expressed at a high level and compete for MHC class I presentation with cellular

proteins. For a peptide vaccine, choosing peptides with an optimal affinity for HLA class I molecules is a critical step for achieving a high epitope density. The epitope search program presented herein is thus considered to be a powerful tool to help design peptides. Alternatively, in the future, it may be possible to intentionally avoid a high-affinity dominant epitope against which the T cell repertoire may be anergized. In such a case, it may be better to target subdominant epitopes of intermediate affinity. (2) Antigen-presenting cells at a local site of immunization should be activated fully to induce offensive responses against infected hepatocytes, which may not be an optimal antigen-presenting cell. (3) Helper T cells also need to be activated. In patients chronically exposed to HCV antigens, the immune system may have fallen into a state similar to self-tolerance against HCV (Grakoui et al. 2003; Semmo et al. 2005), or it could simply be exhausted due to the high load of viral replicates circulating in a body, i.e., approximately 10^{10} to 10^{12} newly synthesized virions per day (Grakoui et al. 2003). Helper T cell recruitment and their optimal activation thus remains an important issue to be resolved. Whether or not the helper epitopes need to be of HCV origin or they can be from different antigen sources also remains an important question.

Interestingly, viral changes have been reported to be extensive in the acute phase of an infection, but they appear to subside when the disease enters a chronic phase. In addition, the degree of the immune response against the infected hepatocytes seems to have some correlation with the mutation rate (Chang et al. 1997). In the chronic phase, it takes several months before escaped variants form a visible fraction in patients (Chang et al. 1997; Cox et al. 2005; Erickson et al. 2001; Kaneko et al. 1997). Evolutionary studies on viral changes in HCV also suggest that it takes several months before an amino acid change becomes stabilized in the viral population (Cox et al. 2005; Timm et al. 2004). Considering this time scale, we propose a cocktail vaccine containing a few epitope peptides. One amino acid change in an epitope may occur sooner or later, but the incidence would be much lower for a variant virus which happens to have amino acid changes in two epitopes simultaneously. If three peptides could be used for immunization, then the possibility of a variant virus carrying mutations in all three epitopes of developing would be negligible. If the effector CTLs could be recruited in a timely manner, then a chance to eradicate viruses would thus be obtained. The HLA-A*2402 prediction program is a powerful tool for designing multiple peptide vaccines. A more efficient viral eradication by a broad-ranging T cell repertoire, in contrast to the vulnerability of the T cell repertoire directed against a single major epitope to viral escape, has previously been demonstrated in infected patients (Tester et al. 2005).

In this study, we focused on the development of a HLA class I-binding peptide vaccine. However, such peptides alone would usually not be sufficient to induce CTLs. HCV-specific CD8 T cells can be abundantly found in patients especially at the early stage of an infection. Those CTLs become less cytotoxic along the course of chronic transition. A loss of helper T cell activities has been cited by several groups as a cause for the annihilation of the CTL activities (Day et al. 2003; Grakoui et al. 2003; Semmo et al. 2005; Tester et al. 2005; Thimme et al. 2002). In addition, the suppression of the CTL activities by a population of the $CD4^+CD25^+$ regulatory T cells has also been demonstrated (Boettler et al. 2005; Rushbrook et al. 2005; Sugimoto et al. 2003). A strategy is thus needed to overcome these problems. Helper peptides can be processed by APCs from exogenously added proteins. Therefore, the activation of APCs and helper T cells along with CD8 T cells in a more stimulatory environment than the chronically infected liver is thus considered to be a crucial point for developing a curative vaccine for HCV.

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Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice

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Abstract

Persistent infection with hepatitis C virus (HCV) is a major cause of liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Here we report that inhibition of heat shock protein 90 (Hsp90) is highly effective in suppressing HCV genome replication. In HCV replicon cells, HCV replication was reduced by Hsp90 inhibitors and by knockdown of endogenous Hsp90 expression mediated by small-interfering RNA (siRNA). The suppression of HCV replication by an Hsp90 inhibitor was prevented by transfection with Hsp90 expression vector. We also tested the anti-HCV effect of Hsp90 inhibition in HCV-infected chimeric mice with humanized liver. Combined administration of an Hsp90 inhibitor and polyethylene glycol-conjugated interferon (PEG-IFN) was more effective in reducing HCV genome RNA levels in serum than was PEG-IFN monotherapy. These results suggest that inhibition of Hsp90 could provide a new therapeutic approach to HCV infection.

Keywords: HCV; Hsp90; Replication; Replicon; Chimeric mouse with humanized liver

Infection with hepatitis C virus (HCV), the major causative agent of non-A, non-B hepatitis [1–3], can lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [4]. An estimated 170 million people worldwide are currently infected with HCV [5]. Combination therapy comprising polyethylene glycol-conjugated interferon (PEG-IFN) and ribavirin represents the current standard treatment for chronic HCV infection, although it has demonstrated limited success and causes some serious side effects [6–8]. The development of safer and more effective drugs for the treatment of HCV infection is therefore an urgent necessity.

HCV, a member of the *Flaviviridae* family, has a single-stranded RNA genome of positive polarity. The genome encodes a large precursor polyprotein which is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope1, envelope2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [9,10].

Lohmann et al. [11] have established an HCV replicon system in which HCV subgenomic RNA autonomously replicates in HuH-7, a human hepatoma cell line (HCV replicon cells). This HCV replicon system allows one to investigate HCV genome replication in cell culture.

In this study we performed random screening with natural-product libraries using HCV subgenomic replicon cells and found that inhibitors of heat shock protein 90 (Hsp90)

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