decreased dependence on the amino-terminal domain [8]. All the chimeric receptors tested in our present study expressed the extracellular loop 2 from CCR5, but in our hands, the N-terminal of CCR5 was usually not needed for infection. The SIVsm isolates, as well as SIVmac 251 and SIVmac 32H used the FC-1 receptor, with a CXCR4 N-terminal and CCR5 as second extracellular loop. This contrasts the use of chimeric coreceptors by primary HIV-1 R5-isolates that use FC-2 more often than FC-1. In fact FC-1 appeared to be the most restrictively used chimeric receptor by HIV-1 isolates [57,58]. In HIV-1 infection FC-4b using viruses emerge in patients who later switch viral phenotype from R5 to X4. In SIVsm infection, a surprisingly high number of the SIVsm reisolates infected cells expressing FC-4b. However, CXCR4-using SIVsm isolates were not more common among the isolates capable of using FC-4b compared to the total number of isolates. Our results indicate that SIVsm uses CCR5 in a different mode than HIV-1. HIV-1 isolates seem to be more restricted than SIV in the interaction with different chimeric coreceptors. The differences between HIV-1 and SIV are even more pronounced when viruses isolated on mPBMC are tested, since eight out of 11 mPBMC isolates could use all three chimeric coreceptors. It is possible that the SIV envelope is less dependent on conformational changes than the HIV-1 envelope and therefore less dependent on the exact structure of the coreceptor. It is also possible that the ability to use different variants of the CCR5 receptor can predispose the virus for CD4-independent entry. Again, viruses isolated on mPBMC were more CD4-independent than viruses isolated on hPBMC, indicating that human cells may select for CD4-dependence.

Conclusion

Taken together, in this study we could show that CD4-independent infection of CCR5 expressing cells was a common characteristic of primary SIVsm isolates. Different phenotypes were observed among the virus isolates and in many cases CD4-independent infection was at a very low level and was only rescued after cocultivation of infected NP-2/CCR5 cells with hPBMC, defined as CD4-independent-LOW phenotype. Isolates obtained early in infection were often of CD4-independent-HIGH-phenotype. Changes towards a more CD4-dependet phenotype occurred over time; faster in macaques with a more progressive disease than in long-term non-progressors. While these changes were parallel with changes in sensitivity to neutralization (Figure 5) the ability to productively infect monocyte derived macrophages remained at steady high levels.

CD4-independent infections may conceivably have important consequences. First, cell and tissue tropism of SIVsm may broaden and lead to establishment of latently infected cell reservoirs in SIV infections. Second, intracellular maturation may provide the virus with a hideaway from the immune system, including the possibility of direct transfer of viruses between cells. One scenario has been reproduced by our experiments: the low or non-productive infection of CD4 negative cells was amplified by contact with PBMC. Our results also suggest that the SIV envelope is less dependent, than HIV-1, on the exact structure of the coreceptor compared to HIV-1 and that the ability to use different variants of the CCR5 coreceptor may influence CD4-independent entry. This difference may indicate that a higher dependence on CD4 for cell entry as well as a more specific binding to the coreceptor could have evolved in humans infected with HIV-1 and led to the more the pathogenic HIV-1 virus (first suggested by Edinger *et al.* in 1999 [59]).

Methods

Animals and disease progression

Female cynomolgus macaques (*Macaca fascicularis*) of Chinese origin were housed at the Swedish Institute for Infectious Disease Control. Housing and care procedures were in compliance with the general guidelines of the Swedish Animal Welfare Agency and all procedures were approved by the Local Ethical Committee on Animal Experiments. The SIVsm isolate SMM-3 (kindly provided by P. Fultz and H. McClure, Yerkes Regional Primate Research Center, Atlanta, GA, [60]) originated from a naturally infected sooty mangabey (*Cercocebus atys*) was used to infect thirteen macaques intravenously (IV) or intrarectally (IR) with 10 MID_{50} of cell free virus stocks produced in cultures of peripheral blood mononuclear cells (PBMC) from cynomolgus macaques [61]. The macaques were monitored for general clinical status. Blood samples for virus isolation, viral load and CD4+ T-cell count determinations were collected at regular intervals. The animals were kept until development of AIDS, or if asymptomatic, until the end of the study period, when they were euthanized. SIV RNA levels in plasma were measured using a highly sensitive quantitative competitive (QC) RT-PCR assay with a lower detection limit of 100 RNA equivalents/ml plasma, as described in details elsewhere [62]. The animals were monitored for changes in their CD4+ cell counts using two-color flow cytometric analysis as reported previously [63].

Based on the rate of disease progression, CD4 decline, time of death and, whenever available, viral load, the macaques were divided into three groups: progressor (P), slow progressor (SP) and long-term non-progressor (LTNP) [31]. Clinical status of the macaques are supplemented [see Additional file 1]. There was no difference in either the CD4 decline or viral load when comparing macaques inoculated by different routes of infection. As expected, decline of CD4+ T-cells was more pronounced

in the first three months of infection, while decline was less thereafter. For the majority of animals the pattern of viral load was consistent with the observations by Ten Haaft *et al.* in that a threshold plasma virus load which was greater than 10^5 RNA equivalents/ml of plasma 6 to 12 weeks after inoculation could predict a faster disease progression [62]. In most of the P macaques plasma viral load was high initially (>10^6 RNA copies/ml) and stayed high [31]. The P macaques showed the fastest decline in CD4+ T-cell count and all animals developed simian AIDS or AIDS-related symptoms. Mean CD4 decline in the P group (seven animals) was -4.27% CD4+ T-cells/month (range -8.05 to -0.69% CD4+ T-cells/month). All P macaques were euthanized within 27 months post-infection and the mean time to AIDS in this group was 17 months. Virus isolation frequency was high (≥ 83%) with one exception (macaque C39: 58%). Macaques in the slow progressor (SP) group (two animals) did not show disease symptoms during the study period, however, the macaques showed a higher rate of CD4 cell decline and had a higher virus isolation frequency (-1.0% decline of CD4+ T- cells/month and virus isolation frequency ≥ 91%) than the macaques in the LTNP group (four animals, mean CD4+ T- cell decline/month -0.3% cells and mean virus isolation frequency 51%, range 10–72%). None of the four LTNP macaques evaluated in this study showed any signs of disease.

Virus isolates

In the present study two to four isolates from each macaque were used depending on virus isolation frequency and the length of the macaque's survival time [see Additional file 1]. The virus isolates have previously been tested for coreceptor usage and neutralization sensitivity [30,31]. The first isolate was usually obtained as early as two weeks post infection, the second isolate by three or four months, the third isolate was chosen at a time in between the second and the last isolate and the last (fourth) isolate was collected by the time of euthanization. Virus isolation was performed by cocultivation of cynomolgus macaque PBMC (mPBMC) with mPBMC or with human PBMC (hPBMC) stimulated with phytophaemagglutinin (PHA-P) [64]. Due to the restricted availability of blood from macaques, limited numbers of isolates were available from cocultivation with mPBMC, thus the need for us to work with isolates obtained on hPBMC. Reisolates were passaged no more than two times. Cell-free supernatants were screened for the amount of reverse transcriptase (RT) with Cavidi HS-kit Lenti RT (Cavidi Tech, Sweden), and stored frozen at -80°C until used. SIVmac 32H and SIVmac 251 were used as reference virus [14].

Infection of NP-2 and U87.CD4 cell lines

NP-2, a human glioma cell line, was engineered to stably express human CD4 and/or human CCR5, as described elsewhere [13,35,65]. Human glioma U87.CD4 cells, stably expressing CD4 and the chemokine receptors CCR5, or chimeric receptors between CCR5 and CXCR4 were previously described [2,33]. Chimeric receptors were constructed by replacing successively increasing portions of CCR5 with corresponding regions of CXCR4 by a modification of the single-overlap and extension PCR approach. The resulting three chimeric constructs and wild-type receptors were stably expressed in U87.CD4 cells. Parental U87.CD4 cells, engineered to express CD4 but no chemokine receptor, were also included in the experiments.

Cells in 500 μl of medium per well (without selective antibiotics) were seeded into 48-well plates 1 or 2 days prior to infection to obtain a 50%-confluent cell layer by the time of infection. For infection, medium was first removed, after which virus was added to duplicate wells in a volume of 350 μl/well. Cells were infected with virus stocks containing 2.7–3.5 log10 pg RT/well (median 3.2 log10 pg RT/well). After an overnight incubation, cells were washed with 1 ml PBS (0.12 M NaCl, 0.03 M phosphate [pH 7.2]), 1 ml of medium with polybrene (2 μg/ml) was added to each well, and the plates were further incubated. The cultures were kept for 7 days, and inspection for syncytia formation was performed daily. Supernatant culture fluids were collected at day 1 and 7 and production of RT was analyzed with Cavidi HS-kit Lenti RT (Cavidi Tech, Sweden). Supernatants were run undiluted and therefore values above 1000 pg RT/ml were over range. An infection was considered positive when RT activity at day 7 was at least twice the value of day 1, or alternatively positive when reaching above the threshold limit (50 pg RT/ml) when day 1 supernatant was below threshold.

Cocultivation of NP-2/CCR5 with PBMC

PBMC from seronegative human blood donors were purified by Ficoll density gradient separation and were activated with 2.5 μg/ml of PHA-P for four days. Seven days after infection the NP-2/CCR5 cell cultures were washed with PBS and treated with EDTA-trypsine (2.5 mM EDTA and 0.125% trypsin) until detached from plastic. Hereafter all NP-2/CCR5 cells, from two parallel wells in the 48-well plates, were transferred to centrifuge tubes and washed once with PBS. The NP-2/CCR5 cells were further transferred to new wells in a 12-well plate and mixed with 6×10^5 PBMC/well from two PBMC donors in 2 ml RPMI medium. Supernatant culture fluids were collected after additional 6 days and production of RT was quantified with Cavidi HS-kit Lenti RT (Cavidi Tech, Sweden).

In an additional experiment NP-2/CCR5 cells were infected with virus isolates in parallel wells as described above. The infected cells from two parallel wells were lysed and the lysates were titrated on hPBMC in order to detect infectious virus in the cytoplasm. For cell-lysis, we adopted a method previously described by Sharova *et al.* where infectious virus was recovered directly from cytoplasmic lysates of macrophages [32]. Briefly, cultures from each well were first washed with 200 μl PBS and added 200 μl trypsin (0.25%) to exclude possible virus attached on the outer cell membrane and to detach the cells from the plastic. Another washing step with PBS was followed after which the cells were incubated with 200 μl 0.001% Triton X-100 in PBS for 5 minutes and put in -80°C for complete freezing. Cells where then thawed and raptured by mechanical force by extensive pipetting and complete lysis was observed by microscope analysis after staining of cells with tryptan blue. Infections with lysed cell material in three five-fold dilution steps were performed on 10^5 PBMC/well in 96-well plates and cells were mixed from four human donors. Infected cultures were incubated over night (37°C in 5% CO_2 atmosphere) in RPMI medium with 10% FBS, antibiotics and 2 μg/ml polybrene at a total volume of 200 μl. Infections with NP-2/CCR5 cell lysates were carried out. Next day, cultures were washed with PBS and further incubated with fresh medium. Supernatant culture fluids were collected at day 1 and 7 and production of RT was analyzed with Cavidi HS-kit Lenti RT (Cavidi Tech, Sweden). The level of detection for RT production was 50 pg/ml.

Isolation and infection of monocyte-derived macrophages (MDM)

MDM were isolated from PBMC from blood donors or cynomolgus macaques according to a previously established protocol [66]. These experiments involved seven different human MDM donors and every isolate was tested on MDM from 2 to 4 blood donors in independent experiments. After Ficoll separation, 15×10^5 cells/cm^2 were seeded in 24- or 48-well plates in RPMI medium supplemented with antibiotics, 20% FBS and 10% human serum (HS), pooled from nine different normal donors. Cells were cultured at 37°C in 5% CO_2 atmosphere. Six days after seeding, non-adherent cells were removed by extensive rinsing with PBS. In addition, cultures were treated with trypsin to further remove non-macrophage cells. Adherent cells were maintained in medium without HS. Cytochemical staining for non-specific esterase (Sigma, Germany) at day 7 showed over 98% positive cells.

After seven days the MDM cultures were infected with the virus isolates. Prior to infection, cells were washed twice with PBS and fresh medium containing 2 μg/ml polybrene was added to the wells. Macrophages were infected with virus stocks containing 3.0–3.1 log10 pg RT/well (in 88% of the cultures). Sequential isolates from each monkey were tested simultaneously. The day after infection, cultures were rinsed twice with PBS and fresh medium was added. In order to compare the kinetics of virus replication in MDM 20 SIVsm virus isolates were tested on MDM from four different human blood donors in a pilot study. Supernatants of infected MDM were analyzed for virus production by testing reverse transcriptase activity (Cavidi HS-kit Lenti RT) at day 1, 3, 7, 11 and 15 after infection. These experiments with peak replication at day 15 or beginning of steady state replication at day 11 after infection, allowed us to choose day 15 for comparison of macrophage tropism of viruses in following experiments (data not shown).

Plaque reduction neutralization assay

The GHOST(3) cell line-based plaque assay is a single cycle infectivity assay for HIV and SIV, where green fluorescent protein (GFP) expression is a hallmark of infection [67]. The assay was performed in 96-well microtiter plates where infected single cells or syncytia appear as distinct green fluorescent plaques and are counted as plaque-forming units (PFU). The serum (H55:16) used in the study was obtained from an infected monkey that remained asymptomatic and had high neutralizing titers towards SIVsm [30]. For the neutralization assay, virus and heat inactivated sera were diluted and mixed in culture medium, to give 20 to 100 PFU/well and 1:20 serum dilution. The virus and serum mixtures were incubated at 37°C for one hour, followed by further dilution in two 5-fold steps, and distributed to triplicate wells. The day after infection the virus-serum mixtures were replaced with fresh medium. The cultures were checked for expression of GFP using fluorescence microscopy three days after infection and virus titers were calculated as PFU/ml: (average number of plaques in triplicate wells × virus dilution)/volume in the well. The neutralizing property of the serum was calculated as percentage plaque reduction of the virus titration by the formula 1-(PFU with serum/PFU without serum) × 100. Based on intra-assay variation, the cut-off for neutralization was set to 30% (≥ 3 × SD), that is, values below 30% are considered as negative for neutralization.

Statistic analysis

McNemar's test was used to analyze if the proportions of CD4-independent phenotypes of paired early and late isolates are the same.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

AL planned the experiments, carried out cell assays and wrote the manuscript. EV was involved in design of the experiments and carried out cell assays. HH participated in the study design, helped in drafting the manuscript. RT was responsible for the animal studies and helped designing the experiments. EMF conceived of the study and participated in its design and coordination and helped to write the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Table over features of the macaques and virus isolates used in this study. This table summarise disease progression of the macaques included in the study as well as the phenotypic characteristics of the virus isolates from the animals.

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Editor-Communicated Paper

Tandem Repeats of Lactoferrin-Derived Anti-Hepatitis C Virus Peptide Enhance Antiviral Activity in Cultured Human Hepatocytes

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Abstract: **Previously, we found that bovine and human lactoferrin (LF) specifically inhibited hepatitis C virus (HCV) infection in cultured non-neoplastic human hepatocyte-derived PH5CH8 cells, and we identified 33 amino acid residues (termed C-s3-33; amino acid 600–632) from human LF that were primarily responsible for the binding activity to the HCV E2 envelope protein and for the inhibiting activity against HCV infection. Since the anti-HCV activity of C-s3-33 was weaker than that of human LF, we speculated that an increase of E2 protein-binding activity might contribute to the enhancement of anti-HCV activity. To test this possibility, we made two repeats [$(C\text{-}s3\text{-}33)_2$] and three repeats [$(C\text{-}s3\text{-}33)_3$] of C-s3-33 and characterized them. Far-Western blot analysis revealed that the E2 protein-binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ became stronger than that of the C-s3-33, and that the binding activity of $(C\text{-}s3\text{-}33)_3$ was stronger than that of $(C\text{-}s3\text{-}33)_2$. Using an HCV infection system in PH5CH8 cells, we demonstrated that the anti-HCV activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ became stronger than that of the C-s3-33. Furthermore, using a recently developed infection system with a VSV pseudotype harboring the green fluorescent protein gene and the native E1 and E2 genes, we demonstrated that the antiviral activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ were stronger than that of C-s3-33. These results suggest that tandem repeats of LF-derived anti-HCV peptide are useful as anti-HCV reagents.**

Key words: **Hepatitis C virus, Lactoferrin, Anti-HCV peptide, E2 protein-binding activity**

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma (28). HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the *Flaviviridae*. The HCV genome encodes a large polyprotein precursor of about 3,000 amino acids (aa), which is cleaved by the host and viral proteases into at least 10 proteins: the core, envelope 1 (E1), E2, p7, and non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (7, 8, 18). These HCV proteins function not only in virus replication but may also affect a variety of cellular functions, including gene expression, signal transduction, and apoptosis (1, 17).

Approximately 170 million people worldwide are infected with HCV (32). The combination of a pegylated interferon with ribavirin is the current standard therapy for chronic hepatitis C and yields a sustained virological response rate of about 55% (6). This means that about 45% of patients with chronic hepatitis C are still threatened by the progress of the disease to cirrhosis and hepatocellular carcinoma.

Although the entry mechanism of HCV remains unclear, to date, several candidates for HCV receptors

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Abbreviations: aa, amino acids; DMEM, Dulbecco's modified Eagle's medium; E2, envelope 2; GFP, green fluorescent protein; HCV, hepatitis C virus; LF, lactoferrin; MBP, maltose-binding protein; NS2, non-structural 2; TF, transferrin; VSV, vesicular stomatitis virus.

have been reported: CD81, the scavenger receptor class B type I, the mannose-binding lectins DC-SIGN and L-SIGN, low-density lipoprotein receptors, etc. (4). Most of them have been identified as interacting materials with a soluble and truncated form of the HCV E2 protein, because of the lack of efficient HCV proliferation in cell cultures, although several culture systems using PCR for detection of HCV infection have been reported (20). However, a major advance in investigating HCV entry has been achieved by the development of pseudotype viruses bearing HCV E1 and E2 proteins assembled onto retrovirus particles (2, 9) or vesicular stomatitis virus (VSV) particles (3, 23, 30). Extensive characterization of the pseudotype viruses has shown that these mimic the early steps of the HCV life cycle. This system has allowed the study of the role of candidate receptors in the early steps of HCV infection (4).

We previously found that bovine and human lactoferrins (LFs) specifically prevented HCV infection in cultured human non-neoplastic hepatocyte PH5CH8 cells using the PCR method for detection of HCV infection (10, 12). Regarding these findings, some clinical studies have demonstrated that monotherapy with bovine LF improves the serum HCV RNA and/or alanine aminotransferase levels in patients with chronic hepatitis C (15, 16, 27, 31).

LF is an 80-kDa mammalian iron-binding glycoprotein and consists of two homologous globular lobes (an N-lobe and a C-lobe), each with a single iron (Fe^{3+}) binding site. It is structurally related to the plasma iron-transport protein transferrin (TF). LF's biological roles include activities in the host defense mechanism as well as in iron metabolism (21, 22). Unlike TF, LF is a primary defense protein against microbial infection. LF possesses strong bacteriostatic and bactericidal activities against pathogenic bacteria, as well as inhibitory activity against pathogenic viruses (5, 21, 22, 33).

LF's preventive mechanism against HCV infection has been thought to be the direct interaction between LF and HCV; indeed, by Far-Western blot analysis using thioredoxin-fused LF fragments expressed in *Escherichia coli* (*E. coli*) and the soluble E2 protein expressed in Chinese hamster ovary cells, we demonstrated that the 93 carboxyl aa of LF (human, bovine, and horse), termed C-s3, specifically bound to the E2 protein (25). On the other hand, Yi et al. (34) independently reported that the E1 and E2 proteins could bind to human and bovine LFs, although the binding region of LF was not identified. Furthermore, we identified the 33 aa of human LF (termed C-s3-33; aa 600–632), which was primarily responsible for the E2 protein-binding activity, and demonstrated that maltose-binding protein (MBP)-fused C-s3-33 prevented HCV infection in PH5CH8 hepatocyte cells (25). However, the E2 protein-binding activity and the anti-HCV activity of C-s3-33 were obviously weaker than those of human LF. Therefore, we presumed that the increase of the E2 protein-binding activity would lead to the enhancement of anti-HCV activity.

To evaluate this idea, we made tandem repeats of C-s3-33, and compared their E2 protein-binding activities and anti-HCV activities with those of the C-s3-33. Here, we report our findings that the anti-HCV activity of the tandem repeats were stronger than that of the monomer when accompanied by the enhancement of the E2 protein-binding activity, by analyses using not only the HCV infection system but also the infection system of a VSV pseudotype bearing the native forms of HCV E1 and E2 proteins.

Materials and Methods

Cell cultures. Simian virus 40 large T antigen-immortalized non-neoplastic human PH5CH8 hepatocytes were maintained as described previously (11, 24). Human hepatoblastoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

Construction of expression plasmids for E. coli. The pMAL-c2X (hLF600–632) (25) expression plasmid for the MBP-fused C-s3-33 LF fragment, was used as a template for the PCR using a primer set of hLFB6 5'-TGATAGGATCCGTGGTGTCTCGGATGATAAGG-3' containing the *Bam*HI recognition site (underlined) (25) and 632R6A 5'-ATCCATCCGAGACACCACAAACTTGTCCGGGCAGTCAGATCC-3' containing an extra 18 nts (underlined) encoding the amino-terminal 6 aa of the C-s3-33 LF fragment. After PCR (20 cycles) using KOD-plus DNA polymerase (Toyobo, Osaka, Japan), the amplified PCR product was used as a template for a second PCR using the primer set of hLFB6 and 632R 5'-TAATAAAGCTTTTAAAACTTGTCCGGGCAGTCAGATCC-3' containing the *Hin*dIII recognition site (underlined) (25). After PCR (35 cycles) using KOD-plus DNA polymerase, amplified PCR products (approximately 200 bp for the two-repeat form and approximately 300 bp for the three-repeat form) were subcloned into the *Bam*HI and *Hin*dIII sites of pMAL-c2X, and were used as expression plasmids for the production of the MBP-fused $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$.

To prepare an expression plasmid for the production of the MBP-fused C-s3-33-relevant fragment (aa 587–619) of human TF, pCXbsr/huTF (29) encoding full-length human TF was used as a template for the PCR using a primer set of hTF587F 5'-TGATAG-

GATCCGTGGTCACACGG-3' containing the *Bam*HI recognition site (underlined) and hTF619R 5'-TAATAAAGCTTTTAAAAGTTGCCCG-3' containing the *Hin*dIII recognition site (underlined). After PCR (35 cycles) using KOD-plus DNA polymerase, the amplified PCR product was subcloned into the *Bam*HI and *Hin*dIII sites of pMAL-c2X, and was used as the expression plasmid.

Expression and purification of the MBP-fused protein. Expression and purification of the MBP-fused LF fragment [C-s3-33, $(C\text{-}s3\text{-}33)_2$, or $(C\text{-}s3\text{-}33)_3$] or the MBP-fused C-s3-33-relevant fragment of human TF were carried out as described previously (25). Briefly, the expression plasmid for MBP-fused protein was transformed into the *E. coli* strain JM109. The transformants were cultured at 37 C for several hours, and the harvested cells were sonicated. After removal of insoluble cellular debris by centrifugation, the supernatant obtained as the soluble fraction was applied onto an amylose resin affinity column (New England Biolabs) to obtain the MBP-fused protein. The purity of the obtained MBP-fused protein was evaluated to be more than 95% by electrophoresis on 10% SDS-PAGE gels. The concentration of the purified MBP-fused protein was determined by using Coomassie protein assay reagent (Pierce). The MBP2 (43 kDa) produced from the pMAL-c2X with a stop codon inserted into the *Xmn*I site was used as a control protein.

Far-Western blot analysis. Far-Western blot analysis was carried out as described previously (25). Briefly, 0.5 μg of human LF, MBP2, and MBP-fused LF fragments were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking with N-buffer (25), a binding reaction was carried out using the secreted form of the E2 protein (E2-681) consisting of aa 384–681 expressed in Chinese hamster ovary cells as a probe (14), and then rat monoclonal antibody, MO-12 (13), against E2 protein was used for the detection of E2 protein-bound MBP-fused LF fragments.

Assay for anti-HCV activity of MBP-fused protein. An assay for anti-HCV activity of the MBP-fused LF fragment was carried out by the method described previously (25). Briefly, 2 μl (2×10^4 HCV) of the HCV-positive serum HCV-O (previously described as 1B-2 (19)) (genotype 1b) and the MBP-fused LF fragment (final concentration, 0.5, 1.0, and 2.0 mg/ml) were pre-incubated for 60 min at 4 C and then inoculated onto to the PH5CH8 cells (1.5×10^4 cells were cultured for 2 days before viral inoculation on a 96-well plate). After incubation of the cells for 90 min at 37 C, the cells were washed three times with PBS and further cultured for 1 day at 32 C. Cellular RNA (0.5 μg) prepared by ISOGEN extraction kit (Nippon Gene Co., Toyama, Japan) was used for the quantitative analysis of HCV RNA using LightCycler PCR as described previously (26). As the positive and negative controls for anti-HCV activity, human LF and MBP2, respectively, were used.

Assay for anti-VSV pseudotype activity of MBP-fused protein. For this assay, the VSV pseudotype VSVΔG*(HCV), bearing the native forms of HCV E1 and E2 proteins from the O strain (19), was used. VSVΔG*(HCV) was prepared by introducing the native form of E1 and E2 proteins into recombinant VSV, VSVΔG*, which harbors the green fluorescent protein (GFP) gene instead of the VSV G envelope protein gene (30). An assay for the anti-VSV pseudotype activity of the MBP-fused LF fragment was carried out by a method described previously (30). Briefly, VSVΔG*(HCV) (Approximately 100 IU/assay) was pre-incubated with the MBP-fused LF fragment (final concentration, 0.1–1.0 mg/ml) at 37 C for 60 min and inoculated onto PH5CH8 or HepG2 cells (1.5×10^4 cells were cultured for 2 days before viral inoculation on a 96-well plate). After incubation of the cells for 90 min at 37 C, the cells were washed with DMEM three times and incubated with fresh culture medium. VSVΔG*G was used as a control in this assay. After 24 hr of incubation, each infectious titer was determined by counting the number of GFP-expressing cells under a fluorescence microscope. As the positive and negative controls for the assay, human LF and MBP2 were used, respectively. Human TF and an MBP-fused C-s3-33-relevant fragment of human TF were also used for the assay.

Results

Two and Three Repeats of the Human LF Fragment (C-s3-33) Strengthened the E2 Protein-Binding Activity

Previously we found that bovine and human LFs prevented HCV infection in PH5CH8 cells via direct interaction between LF and HCV (10, 12), and we further identified 33 aa residues (C-s3-33; aa 600–632 of human LF) as an essential and minimum domain possessing binding activity for the HCV E2 protein (secreted form consisting of aa 384–681) and inhibiting activity against HCV infection (25). This result suggested that the E2 protein-binding activity contributes to the anti-HCV activity. However, the E2 protein-binding activity of C-s3-33 was somewhat weaker than that of human LF (25), and the anti-HCV activity of C-s3-33 (IC_{50}=20 μM) in the infection system using PH5CH8 cells was also weaker than that of human LF (IC_{50}=5 μM) (25). To improve these points, we first tried to

enhance the E2 protein-binding activity of C-s3-33 by the multiplication of C-s3-33. Initially, we made pMAL-c2X-based expression vectors encoding two, three, and four repeats of C-s3-33 as MBP-fused proteins, and then expressed them in *E. coli*. We successfully purified two repeats $(C\text{-}s3\text{-}33)_2$ and three repeats $(C\text{-}s3\text{-}33)_3$ of C-s3-33 as soluble forms of the MBP-fused protein; the purification of the four repeats of C-s3-33 failed due to problems with solubility. Using the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$, we performed Far-Western blot analysis to compare their E2 protein-binding activities. The result revealed that the E2 protein-binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ became stronger than that of the C-s3-33, and the binding activity of $(C\text{-}s3\text{-}33)_3$ was stronger than that of $(C\text{-}s3\text{-}33)_2$ (Fig. 1). Although the E2 protein-binding activity of C-s3-33 was weaker than that of human LF, the binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$

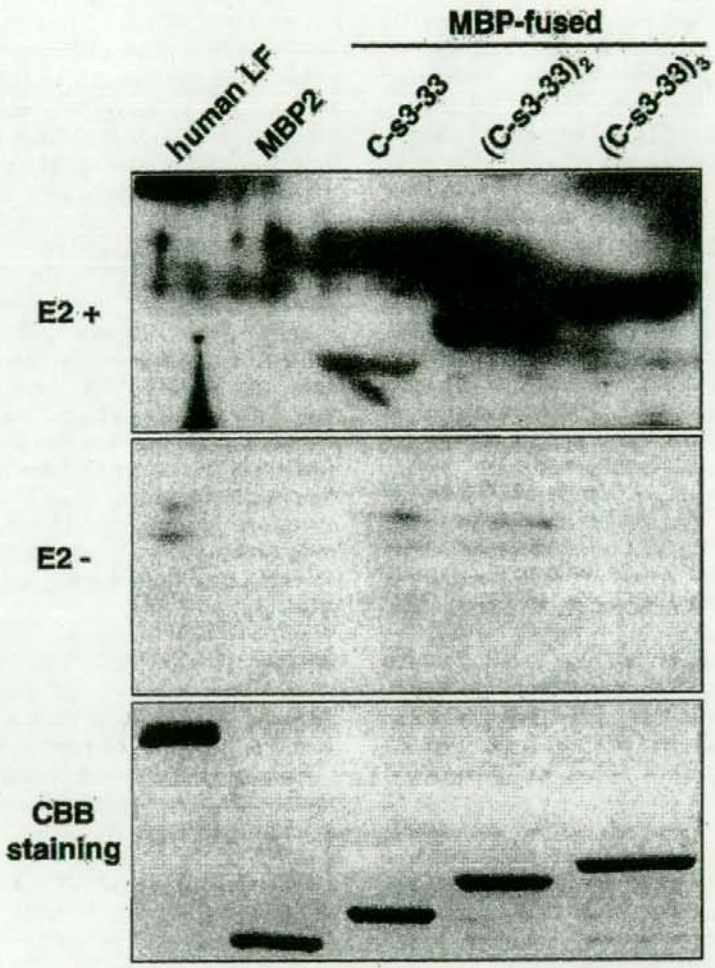

Fig. 1. Comparison of the E2 protein-binding activities of MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$. MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ (0.5 μg each) were resolved by 10% SDS-PAGE. Human LF and MBP2 (0.5 μg each) were also used for the assay as control materials. Far-Western blot analysis using the E2 protein expressed in Chinese hamster ovary cells (14) as a probe was performed as described under "Materials and Methods." Rat monoclonal antibody MO-12 (13) against the E2 protein was used for the detection of the E2 protein bound to MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ as well as human LF. Far-Western blot analysis in the absence of the E2 protein was also performed. The bottom panel shows the results for human LF, MBP2, and MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ detected by staining with Coomassie Brilliant Blue.

became comparable with that of human LF (Fig. 1). To exclude the possibility of cross-reactions between C-s3-33 and the anti-E2 antibody, we performed a Far-Western blot analysis in the absence of the E2 protein. No significant bands were obtained in this control experiment (Fig. 1). The Far-Western blot analysis using normal rat serum instead of anti-E2 antibody also detected no significant bands (data not shown). These results suggest that the specific E2 protein-binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ increase with the degree of multiplication of C-s3-33.

$(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ Efficiently Prevented HCV Infection in PH5CH8 Cells

Since we obtained the expected results that the E2-binding activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ were stronger than that of C-s3-33, we next compared their anti-HCV activities in our HCV infection system using PH5CH8 cells (10, 25). The obtained result (Fig. 2) revealed that the anti-HCV activities of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ (IC_{50}=10 μM in both) became stronger than that of the C-s3-33 (IC_{50}=23 μM), although their activities were somewhat weaker than that of human LF (IC_{50}=5 μM). These results support the previous suggestion that the E2 protein-binding activity of C-s3-33 contributes to the inhibition of HCV infection (inoculum HCV-O) in human hepatocyte cells (25). However, in our HCV infection system, we failed to clearly show a difference in inhibiting activities between $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$, because each standard deviation became somewhat large value due to the low level of cell culture-based HCV infection (20, 25, 31). In order to improve this point, we developed an infection system with VSVΔG*(HCV), a VSV pseudotype bearing the native E1 and E2 proteins derived from HCV-O (30), and this VSV pseudotype was used for further analysis as described below.

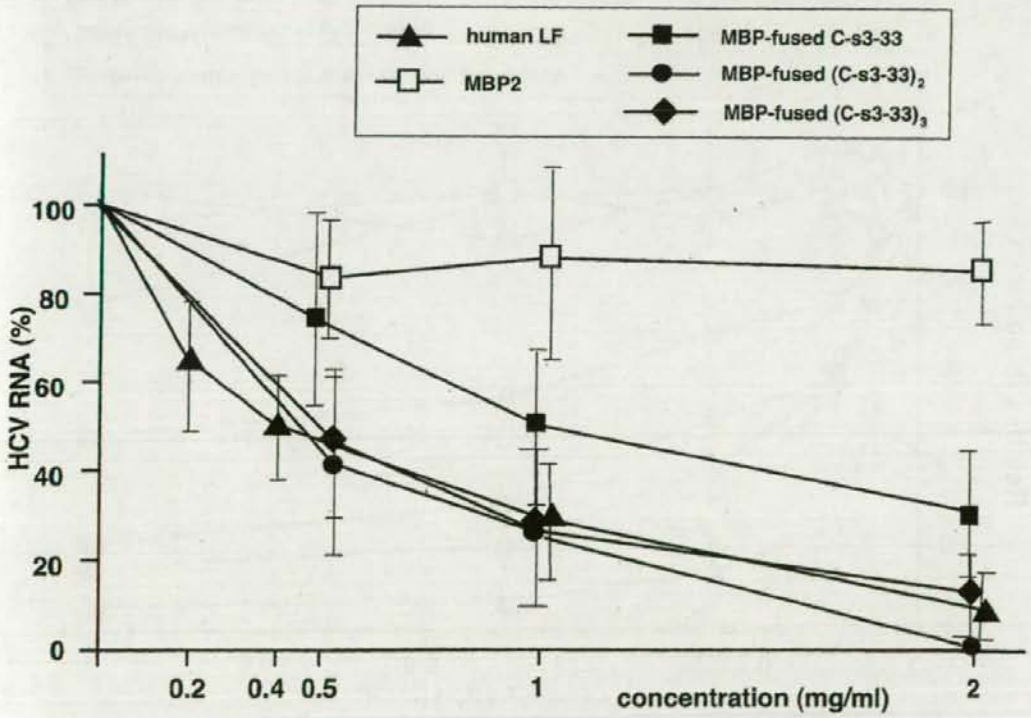

Fig. 2. Anti-HCV activities of MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ in an HCV infection system using PH5CH8 cells. PH5CH8 cells and the inoculum HCV-O were used for the HCV-inhibiting assay, as described under "Materials and Methods." The number in the ordinate axis indicates the percent of HCV RNA determined by real-time LightCycler PCR (26). Approximately 2,000 copies of HCV RNA per μg of cellular RNA were reproducibly obtained using this HCV infection system (10, 26). In addition to the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$, human LF and MBP2 were also used for the assay as control materials. The data are means ± SD of triplicates from three independent experiments.

Antiviral Effects of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ against VSVΔG(HCV) Infection in PH5CH8 Cells*

Since PH5CH8 cells showed good susceptibility to our developed VSV pseudotype, VSVΔG*(HCV) (30), we examined the antiviral effects of $(C\text{-}s3\text{-}33)_2$ and $(C\text{-}s3\text{-}33)_3$ against VSVΔG*(HCV) infection in PH5CH8 cells, and compared them with those of the C-s3-33 and human LF. In this experiment, the antiviral effects of human TF and a C-s3-33-relevant fragment of human TF were also examined. The results (Fig. 3) clearly showed that human LF (IC_{50}=0.6 μM) strongly inhibited VSVΔG*(HCV) infection, but that human TF and the C-s3-33-relevant fragment of human TF did not, nor did MBP2, suggesting that inhibition against VSVΔG*(HCV) infection also occurred in an LF-specific manner as observed previously in the HCV infection system (25, 31). These results support previous findings (23, 30) using the VSV pseudotype infection system. Furthermore, we obtained clear results that C-s3-33 showed inhibiting activity against VSVΔG*(HCV) infection, and that its inhibiting activity was increased with multiplication of C-s3-33. The IC_{50} doses of C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ were 17 μM, 5.0 μM, and 3.0 μM, respectively. This result indicates that antiviral activity of C-s3-33 is improved by the duplication and triplication of C-s3-33, although the antiviral activity of $(C\text{-}s3\text{-}33)_3$ is still weaker than that of human LF. We confirmed that these LF fragments did not inhibit VSVΔG*G (control virus without E1 and E2 proteins) infection in PH5CH8 cells (data not shown). In summary, our results suggest that direct interaction of the C-s3-33 fragment with the E2 protein in VSVΔG*(HCV) prevents the virus infection in PH5CH8 cells.

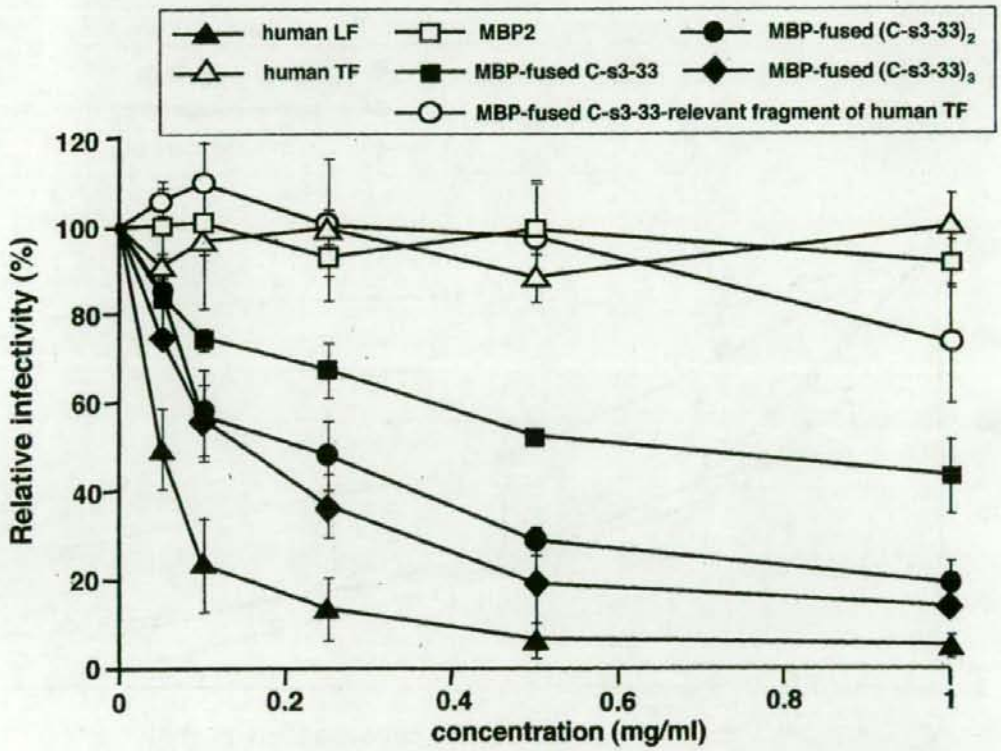

Fig. 3. Antiviral activity of the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$ in the infection system of pseudotype virus using PH5CH8 cells. PH5CH8 cells and the VSV pseudotype, VSVΔG*(HCV), were used for the HCV-inhibiting assay, as described under "Materials and Methods." The number in the ordinate axis indicates the relative infectivity (%) calculated by counting GFP-positive cells. Approximately 100 GFP-positive cells per one assay were reproducibly obtained using this pseudotype infection system (30). In addition to the MBP-fused C-s3-33, $(C\text{-}s3\text{-}33)_2$, and $(C\text{-}s3\text{-}33)_3$, human LF, human TF, MBP2, and an MBP-fused C-s3-33-relevant fragment of human TF were also used for the assay as controls. The data are means ± SD of three independent experiments.

Antiviral Effects of (C-s3-33)$_2$ and (C-s3-33)$_3$ against VSVΔG(HCV) Infection in HepG2 Cells*

We have shown the inhibiting activities of LF fragments against HCV infection or VSV pseudotype infection in PH5CH8 cells; however, it is not clear whether or not the LF fragments used in this study show inhibiting activities against virus infection in cells other than PH5CH8 cells. To clarify this point, HepG2 cells were used for the analysis, because HepG2 cells showed the highest susceptibility to VSVΔG*(HCV) among 25 cell lines examined (30). As a consequence, we obtained similar results (Fig. 4) with those obtained in the infection system using PH5CH8 cells. The IC_{50} doses of C-s3-33, (C-s3-33)$_2$, and (C-s3-33)$_3$ were >12 μM, 7.6 μM, and 3.9 μM, respectively, indicating that, again, the inhibiting activity was increased with multiplication of C-s3-33, although antiviral activity of (C-s3-33)$_3$ was still weaker than that of human LF (IC_{50}=1.2 μM). In conclusion, our results indicated that tandem repeats of C-s3-33 enhanced the inhibiting activity in cell culture-based HCV infection.

Discussion

In our previous (30) and present studies, we showed that pretreatment of VSV pseudotypes with bovine and human LFs reduced the infectivity of VSVΔG*(HCV) and VSVΔG*(E2) bearing only the E2 protein in a dose-dependent manner, whereas pretreatment with TF did not. In contrast, LFs partially inhibited the infectivity of VSVΔG*(E1) bearing only the E1 protein (30). These results suggested that the interaction of LF and the E2 protein is the main contributing factor to the prevention of HCV infection. This idea has been strongly supported by the results obtained in this study. We demonstrated that tandem repeats of C-s3-33, an anti-HCV peptide derived from human LF, enhanced the E2 protein-binding activity and the inhibiting activity

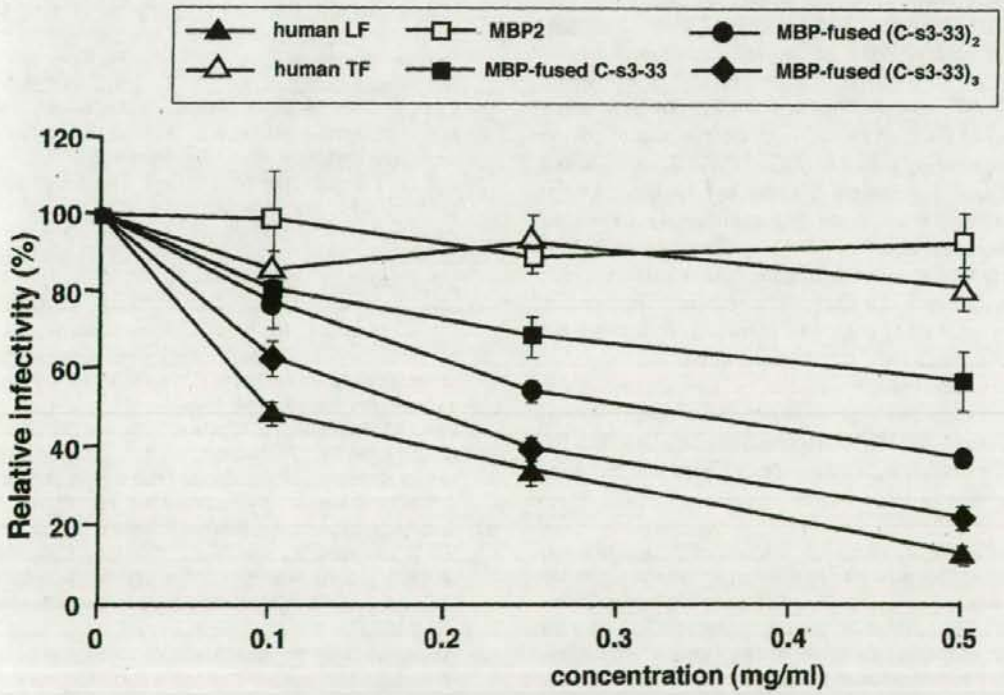

Fig. 4. Antiviral activity of the MBP-fused C-s3-33, (C-s3-33)$_2$, and (C-s3-33)$_3$ in the infection system of the pseudotype virus using HepG2 cells. HepG2 cells and the VSV pseudotype, VSVΔG*(HCV), were used for the HCV-inhibiting assay, as described in "Materials and Methods." The number in the ordinate axis indicates the relative infectivity (%) calculated by counting GFP-positive cells. Approximately 100 GFP-positive cells per one assay were reproducibly obtained using this pseudotype infection system (30). In addition to the MBP-fused C-s3-33, (C-s3-33)$_2$, and (C-s3-33)$_3$, human LF, human TF, and MBP2 were used for the assay as controls. The data are means ± SD of three independent experiments.

against infection by HCV or the VSV pseudotype, VSVΔG*(HCV), in human hepatic cell lines. These results strongly suggest that the direct interaction between C-s3-33 and the E2 protein plays a central role in the inhibition of HCV infection by LF.

Since C-s3-33 or repeated forms of C-s3-33 could prevent HCV and VSVΔG*(HCV) infection, C-s3-33 must bind to a region other than the region (aa 441–500 of E2 protein) required for heteromeric complex formation between E1 and E2 proteins. Our preliminary results suggested that the C-s3-33 bound to aa 411–500 and aa 600–661 of the E2 protein, indicating that the target sites of C-s3-33 may be plural. This result suggests a rather complex interaction between C-s3-33 and the E2 protein. To clarify this point, further comprehensive analysis will be needed.

Although tandem repeats of C-s3-33 enhanced the anti-HCV activity compared with that of the C-s3-33, the fact that their antiviral activities were still several-fold weaker than that of original human LF remains a subject to be resolved. As one approach to increase anti-HCV activity, tandem repeats of C-s3-33-relevant fragment of bovine LF may be useful, because we previously observed that the anti-HCV activity of bovine LF (IC_{50}=1.5 μM) was stronger than that of human LF (IC_{50}=5.0 μM) (26), and that the E2 protein-binding activity of the C-s3 (93 aa)-relevant fragment of bovine LF was stronger than that of C-s3 (25). Since 10 aa out of 33 aa differ between C-s3-33 and its relevant fragment of bovine LF, some aa substitutions between both fragments may help to further increase the anti-HCV activity of LF-derived peptides. Alternatively, some spacer between the C-s3-33 repeats may be needed. Therefore, further trials will be needed to achieve the maximum anti-HCV activity of C-s3-33.

In conclusion, the results of the present study demonstrated that tandem repeats of human LF-derived 33 aa prevented HCV infection more strongly than the 33 aa, and suggest that this repeated form will be useful as a novel anti-HCV reagent.

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Study on the quality control of cell therapy products Determination of *N*-glycolylneuraminic acid incorporated into human cells by nano-flow liquid chromatography/Fourier transformation ion cyclotron mass spectrometry

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Abstract

N-Glycolylneuraminic acid (NeuGc), an acidic nine-carbon sugar, is produced in several animals, such as cattle and mice. Since human cells cannot synthesize NeuGc, it is considered to be immunogenic in humans. Recently, NeuGc contamination was reported in human embryonic stem cells cultured with xenogeneic serum and cells, suggesting that possibly NeuGc may harm the efficacy and safety of cell therapy products. Sialic acids have been determined by derivatization with 1,2-diamino-4,5-methylenedioxybenzene (DMB) followed by liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS); however, the limited availability of cell therapy products requires more sensitive and specific methods for the quality test. Here we studied the use of nano-flow liquid chromatography/Fourier transformation ion cyclotron resonance mass spectrometry (nanoLC/FTMS) and nanoLC/MS/MS for NeuGc-specific determination at a low femtomole level. Using our method, we found NeuGc contamination of the human cell line (HL-60RG cells) cultured with human serum. Our method needs only 2.5×10^3 cells for one injection and would be applicable to the determination of NeuGc in cell therapy products.
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Keywords: *N*-Glycolylneuraminic acid; Nano-flow liquid chromatography; Fourier transformation ion cyclotron mass spectrometry; Cell therapy products

1. Introduction

Sialic acids are a family of acidic nine-carbon sugars found in the non-reducing terminal of *N*-linked and *O*-linked oligosaccharides of glycoproteins and glycolipids [1,2]. There are more than 30 members with different substitutions on the amino group at carbon 5 and on hydroxyl groups at carbons 4, 7, 8 and 9 [2–8]. *N*-Glycolylneuraminic acid (NeuGc), a 5-*N*-glycolylated sialic acid, is produced in several animals, such as cattle, horses, mice and rats [9]. Since human cells cannot synthesize NeuGc due to mutation of the cytidine monophospho (CMP)-*N*-acetylneuraminic acid (NeuAc) hydroxylase gene [10,11], NeuGc is considered to be antigenic and to induce immunoreaction in humans [4,12,13].

Advances in biotechnology and cell culture techniques make it possible to administer human and animal cells directly to patients as cell therapy products. In cell therapy and tissue engineering, human embryonic stem (ES) cells are expected to be useful for the treatment of many diseases. Recently, it was reported that NeuGc is incorporated into ES cells from human and mouse feeder cells and cultivation media containing xenogeneic serum, such as fetal calf serum (FCS) [14,15]. Since NeuGc is a foreign component in humans, it is feared that NeuGc may harm the efficacy and safety of cell therapy products. To

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assess the adverse effects of NeuGc, it is necessary to quantify NeuGc in cell therapy products.

Sialic acids have been determined by labeling with 1,2-diamino-4,5-methylenedioxybenzene (DMB) followed by conventional high-performance liquid chromatography (HPLC) with fluorescent detection [16–20]. The femtomole level of sialic acid can be determined by fluorescent detection [19]. The use of liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) has more advantage in the identification of sialic acid species [18,20–22]. The derivatization of sialic acids with DMB has advantages of good separation of NeuGc from NeuAc in chromatography and enhancement of ionization efficiency in MS. However, more sensitive and specific methods are desired for the quality control of cell therapy products, since in many case only a low number of cell products, approximately 1×10^6 to 1×10^8, should be available for quality tests.

In this study, we studied the use of nano-flow liquid chromatography/Fourier transformation ion cyclotron resonance mass spectrometry (nanoLC/FTMS) and LC/MS/MS to achieve the sensitive and specific determination of NeuGc. The potential of the method for quality testing of cell therapy products was evaluated using substrain of human promyelocytic leukemia HL-60 cells (HL-60RG cells) as model cells. Using this method, we determined NeuGc in membrane fractions from HL-60RG cells cultured with FCS, human serum and serum-free medium.

2. Experimental

2.1. Materials

NeuGc and NeuAc were purchased from Nacalai Tesque (Kyoto, Japan). FCS and normal human serum were purchased from Dainippon Sumitomo Pharma (Osaka, Japan). RPMI1640 medium and ASF104 medium were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Ajinomoto (Tokyo, Japan), respectively.

2.2. Cell culture

Substrain of human promyelocytic leukemia HL-60 cells (HL-60RG cells, JCRB Cellbank, Osaka, Japan) was cultured in RPMI1640 medium supplemented with 10% FCS, 100 unit/ml of penicillin and 100 μg/ml of streptomycin under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. HL-60RG cells were replaced at 2×10^5 cells/100 mm dish in RPMI1640 medium supplemented with 10% FCS or 10% normal human serum, and in serum-free ASF104 medium. The media were replaced four times, and semi-confluent growth cells were harvested.

2.3. Fractionation of the membrane fraction

The cells were washed in phosphate buffer saline (PBS) supplemented with protease inhibitors (protease inhibitor mix solution, Wako, Tokyo, Japan) three times. The washed cells (1×10^6) were suspended in 100 μl of 0.25 M sucrose/10 mM Tris–HCl buffer (pH 7.4) containing protease inhibitors, and sonicated at 4 °C for 30 s, two times (40W, Bioruptor UCW-201, Tosyoudenki, Kanagawa, Japan). After the nuclei were removed by centrifugation at 4 °C, $450 \times g$ for 10 min, the mitochondria and lysosome fractions were removed by re-centrifugation at 4 °C, $20{,}000 \times g$ for 10 min. The membrane fractions were precipitated by ultracentrifugation at 4 °C, $100{,}000 \times g$ for 60 min. The membrane fractions were washed in 100 μl of 150 mM ammonium acetate buffer (pH 7.4) and recovered by re-ultracentrifugation.

2.4. Derivatization of NeuGc and NeuAc with DMB reagent

The membrane fractions were sonicated in 250 μl of H_2O and then incubated with 250 μl of 4 M acetic acid (final concentration, 2 M) at 80 °C for 3 h. The released sialic acids were passed though a solid-phase extraction cartridge (SepPak C-18, Waters, Milford, MA, USA) with 2 ml of H_2O, dried under vacuum, and resolved in 50 μl of H_2O. The solution was incubated with DMB according to the manufacturer's instruction (Takara, Tokyo, Japan), and the reaction mixture was applied on a solid-phase extraction cartridge (Envi-Carb C, Supelco, Bellefonte, PA, USA). After washing the cartridge with 2.5 ml of 5 mM ammonium acetate (pH 9.6) for desalting, the DMB-labeled sialic acids were eluted with 3 ml of 45% acetonitrile/5 mM ammonium acetate (pH 9.6). The collected fraction was freeze dried.

2.5. nanoLC/FTMS

DMB-labeled sialic acids were separated by HPLC using Paradigm MS4 (Michrom BioResource, Auburn, CA, USA) equipped with a reversed-phase C18 column (Magic C18, 50 mm × 0.1 mm, 3 μm, Michrom BioResource, Auburn, CA, USA). Elution was achieved using 0.1% formic acid/2% ace-

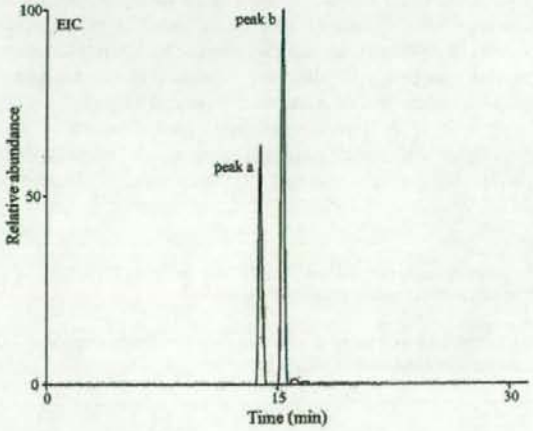

Fig. 1. EIC at *m/z* 426.13–426.17 and *m/z* 442.12–442.16 obtained by SIM (*m/z* 400–450) of DMB-NeuGc and DMB-NeuAc in the positive ion mode.

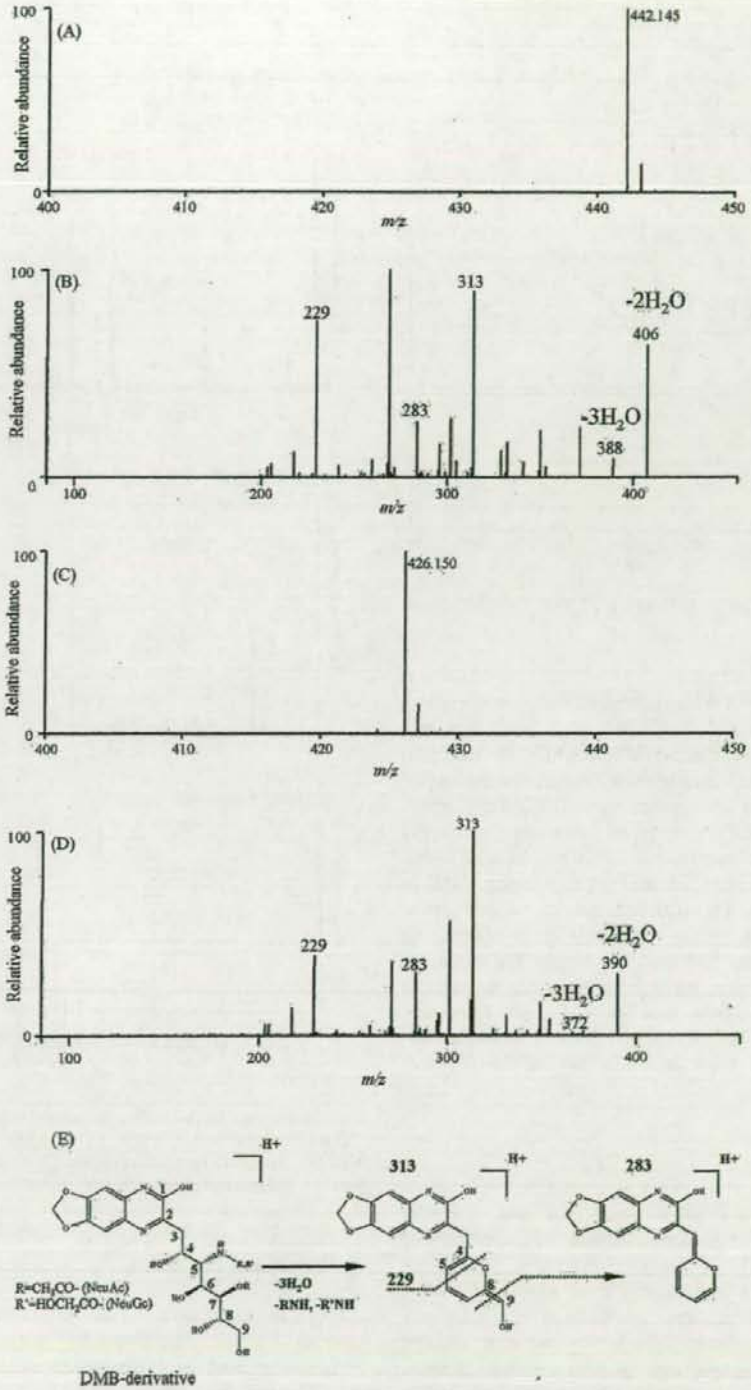

Fig. 2. (A) Typical MS spectrum of peak a. (B) MS/MS spectrum of $[M+H]^+$ (m/z 442.145) acquired from around peak a. (C) Typical MS spectrum of peak b. (D) MS/MS spectrum of $[M+H]^+$ (m/z 426.150) acquired from around peak b. (E) Fragmentation of DMB-NeuGc and DMB-NeuAc.

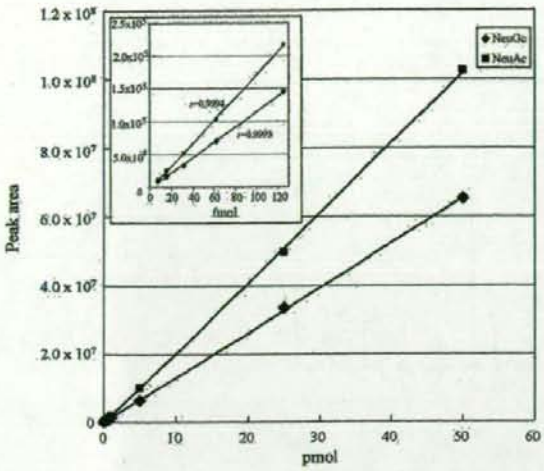

Fig. 3. Calibration curves of DMB-NeuGc ($r = 0.9998$) and DMB-NeuAc ($r = 0.9995$).

tonitrile (pump A) and 0.1% formic acid/80% acetonitrile (pump B) with a linear gradient of 10–90% of B in 30 min at a flow rate of 750 nl/min. On-line MS and MS/MS were performed using an Fourier transformation ion cyclotron resonance (FT)/ion trap (IT) type mass spectrometer (LTQ-FT, ThermoElectron, San Jose, CA, USA) equipped with a nanoelectrospray ion source (AMR, Tokyo, Japan). DMB-NeuAc and DMB-NeuGc were determined by selected ion monitoring (SIM) in the positive ion mode. The analytical conditions were set to 200 °C for capillary temperature, 1800 eV spray voltage, *m/z* 400–450 scan range, and 35% collision energy. The automatic gain control (AGC) value, which is adjusted for the amount of imported ions for FTMS, was set to 5×10^4. Maximum injection times, which are the adjusted times of imported ions, for ITMS and FTMS, were set to 50 and 1250 ms, respectively.

2.6. Method validation

The linearity of the signal intensity peak area of DMB-NeuAc and DMB-NeuGc was assessed by injections of 0.0078–500 pmol DMB derivatives. Correlation coefficients were calibrated using a least-squares linear regression model. The detection limit (DL) and the quantification limit (QL) were calculated using the formulas $DL = 3.3 \times \sigma/slope$ (σ: average of noise on chromatograph) and $QL = 10 \times \sigma/slope$, respectively. Accuracy and precision were determined by measuring three samples, where NeuGc spiked at the concentration of 50 fmol to the membrane fraction of cells cultured in serum-free medium which contains no NeuGc before the derivatization of NeuGc with DMB. Accuracy was calculated by comparison of the mean peak area and the calibration curve. Precision was estimated by relative standard deviation (RSD) from three samples.

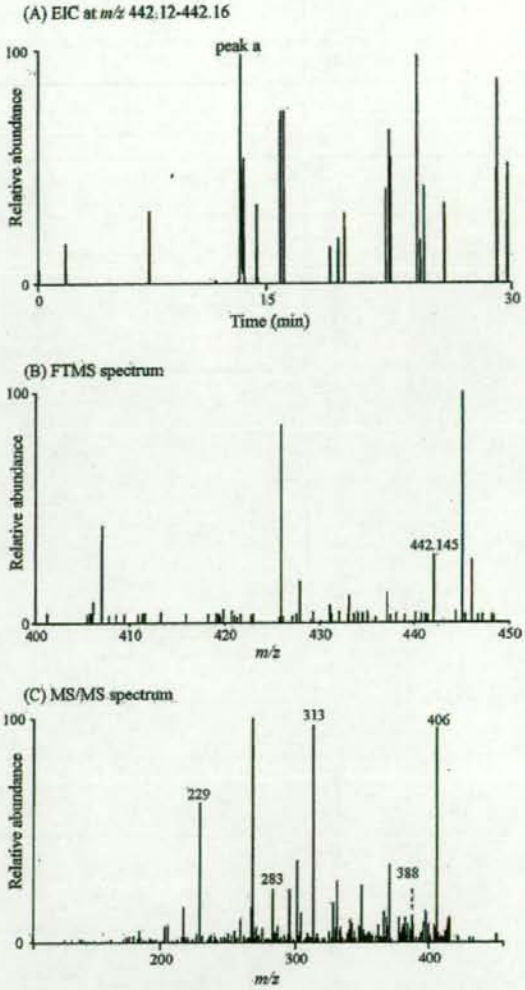

Fig. 4. Detection of DMB-NeuGc in the membrane fractions of HL-60RG cells (2.5×10^3) cultured with 10% FCS. (A) EIC at *m/z* 442.12–442.16 obtained by SIM. (B) Typical MS spectrum of peak a. (C) MS/MS spectrum of $[M+H]^+$ (*m/z* 442.145) acquired from around peak a.

3. Results and discussion

3.1. Analysis of NeuGc and NeuAc by nanoLC/FTMS

It was reported that DMB-NeuGc yielded its dehydrated ion (*m/z* 424) together with molecular ion (*m/z* 442) by MS in the positive ion mode [18,21]. To control the dehydration of molecular ion in the ion trap device, AGC value, which regulates the amount of ions trapped into ion trap device, was set to 5×10^4 (default value, 5×10^5). This value was also useful for the detection of molecular ion of DMB-NeuAc.

Using the AGC value at 5×10^4, SIM (*m/z* 400–450) was carried out in the positive ion mode. When a mix-

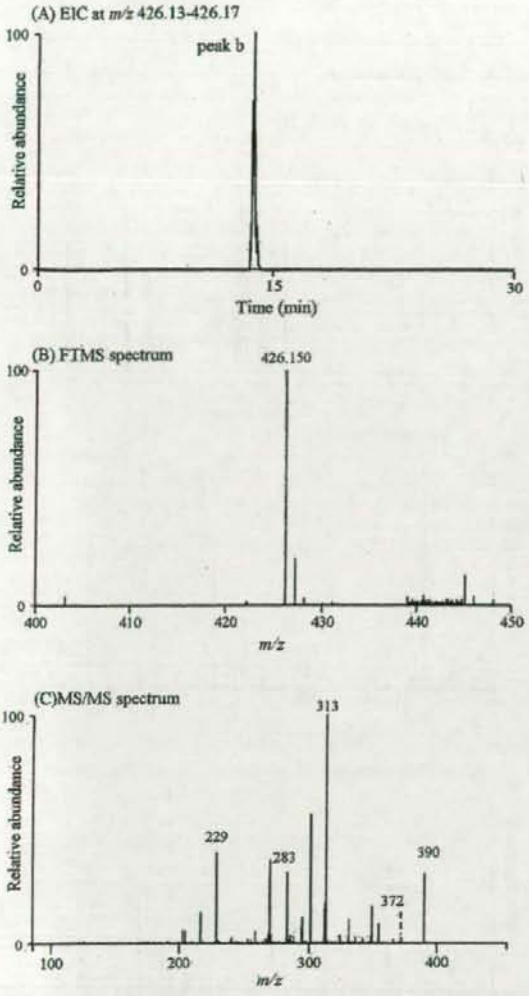

Fig. 5. Detection of DMB-NeuAc in the membrane fractions of HL-60RG cells (2.5×10^3) cultured with 10% FCS. (A) EIC at m/z 426.13–426.17 obtained by SIM. (B) Typical MS spectrum of peak b, (C) MS/MS spectrum of $[M+H]^+$ (m/z 426.150) acquired from around peak b.

ture of DMB-NeuGc and DMB-NeuAc (2 pmol each) was subjected to nanoLC/MS, two peaks appeared at 14 min (peak a) and 15 min (peak b) on the extracted ion chromatogram (EIC) at m/z 426.13–426.17 and m/z 442.12–442.16 (Fig. 1).

As shown in Fig. 2A, the m/z values of molecular ions around 14 min (m/z 442.145) suggest the elution of DMB-NeuGc in peak a. The structure of the DMB derivative at peak a was confirmed by the product ion spectra acquired from $[M+H]^+$ (m/z 442.145) as a precursor ion (Fig. 2B). Product ions missing two and three molecules of H_2O were found at m/z 406 and 388 in MS/MS spectra. Ions losing three H_2O and glycolyl groups (m/z 313), cross-ring fragment ion (m/z 229) and fragment ion

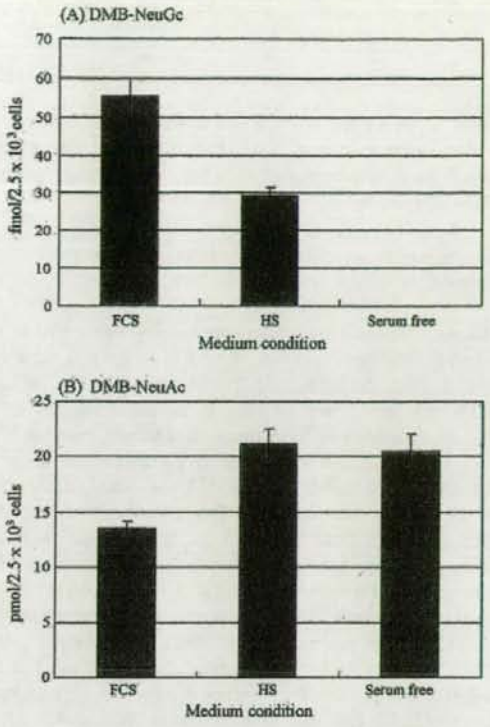

Fig. 6. Levels of (A) NeuGc and (B) NeuAc in the membrane fraction of HL-60RG cells (2.5×10^3) cultured with 10% FCS, 10% human serum (HS) and serum-free medium. Values are the means ± SD ($n = 3$).

yielded by loss of formaldehyde (m/z 283) were also formed by MS/MS (Fig. 2E). The fragment pattern of the MS/MS spectrum from $[M+H]^+$ (m/z 442.145) was consistent with that of DMB-NeuGc in the previous report [21]. Fragments at m/z 406 and 388 are DMB-NeuGc characteristic ions, which could be used for specific determination of DMB-NeuGc. Likewise, peak b was identified as DMB-NeuAc by molecular ions (m/z 426.150) and their product ions (m/z 390, 372, 313, 283 and 229) formed by MS/MS of $[M+H]^+$ (m/z 426.150) as a precursor ion (Fig. 2C and D).

Calibration curves were prepared by the injection of DMB-NeuGc and DMB-NeuAc from 0.0078 to 500 pmol. The linearity of DMB-NeuGc and DMB-NeuAc was confirmed in the range of 0.0078–50 pmol with the regression equations of $Y = 1.31 \times 10^6 X - 9028.5$ ($r = 0.9998$) and $Y = 2.03 \times 10^6 X - 21548.0$ ($r = 0.9995$), respectively (Fig. 3). DL and QL of DMB-NeuGc were 8.6 and 26.3 fmol, and those of DMB-NeuAc were 5.6 and 16.9 fmol, respectively. The use of FT/MS gave an accuracy of 92.4% by eliminating contaminants by using accurate m/z values. The precision of this method for NeuGc was 7.3%. Compared to the former method, in which a micro or semi-micro column and the quadrupole mass spectrometer were used for the detection of picomole levels of DMB derivatives, SIM by using nanoLC/FTMS achieved the specific detection of DMB-derivatized sialic acids at a lower level. The