Single-domain intrabodies against hepatitis C virus core inhibit viral propagation and core-induced NFκB activation

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Hepatitis C virus (HCV) core plays a key role in viral particle formation and is involved in viral pathogenesis. Here, constructs for single-domain intrabodies consisting of variable regions derived from mouse mAbs against HCV core were established. Expressed single-domain intrabodies were shown to bind to HCV core, and inhibit the growth of cell culture-produced HCV derived from JFH-1 (genotype 2a) and a TH (genotype 1b)/JFH-1 chimera. Adenovirus vectors expressing intrabodies were also capable of reducing HCV propagation. Intrabody expression did not affect viral entry or genome replication of single-round infectious trans-complemented HCV particles. However, intrabody expression reduced intracellular and extracellular infectious titres in CD81-defective Huh7-25 cells transfected with the HCV genome, suggesting that these intrabodies impair HCV assembly. Furthermore, intrabody expression suppressed HCV core-induced NFκB promoter activity. These intrabodies may therefore serve as tools for elucidating the role of core in HCV pathogenesis.

INTRODUCTION

Over 160 million people worldwide are chronically infected with hepatitis C virus (HCV), and are at risk of developing chronic hepatitis, cirrhosis and hepatocellular carcinoma (Lavanchy, 2011). Currently, successful treatments for HCV infection include directly acting antivirals, as well as pegylated-IFN and ribavirin; however, therapeutic agents targeting HCV structural proteins remain unavailable. HCV core protein, whose amino acid sequence is highly conserved amongst different HCV strains, is involved in the formation of the HCV virion and has a number of regulatory functions, including modulation of signalling pathways, immunological responses, cellular and viral gene expression, cell transformation, apoptosis, and lipid metabolism (Lai & Ware, 2000; Ray & Ray, 2001). Intra-cellularly expressed antibody fragments (intrabodies) have been used to inhibit the function of various proteins. Single-chain variable fragments (scFv), composed of the heavy chain and light chain variable regions (VH and VL), have been shown to bind to antigens, thereby regulating the target's function, and have been shown to be applicable to life cycle regulation of several viruses (Corte-Real et al., 2005; Gal-Tanamy et al., 2010; Marasco et al., 1993; Yamamoto et al., 1999). The same binding activity was demonstrated for single variable-domain fragments consisting of either VH or VL alone (Sato et al., 2013; Tanaka et al., 2007). In this study, we constructed several single-domain intrabodies derived from the anti-core mouse mAb. These single-domain intrabodies were shown to bind to HCV core. Amongst these, expression of two
Expression of intrabodies, and interaction between core and each intrabody was assessed (Fig. 1b, c). All but one of the intrabodies were co-immunoprecipitated with FLAG-core; the exception being CA7-L. The levels of precipitated protein differed amongst the different intrabodies, with CA7-H, 2H9-H, 2H9-L, AA10-H, 2G3-H and 2G3-L exhibiting high levels of core binding in cells.

In order to evaluate the effects of intrabodies on HCV production, Huh7.5.1 cells were co-transfected with the intrabody plasmid and an RNA polymerase I (Pol I)-driven HCV genome plasmid, pH/HFH1am. The infectious viral titre of genotype 2a cell culture-produced HCV (HCVcc) was reduced by expression of 2H9-L, AA10-H and 2G3-L (Fig. 2a). Expression of 2H9-L and AA10-H also led to marked reduction in production of genotype 1b HCVcc, whereas expression of 2G3-L yielded moderate inhibition (Fig. 2b). It should be noted that co-transfection with both H and L chain-encoding plasmids (Fig. S2A) or scFv-encoding plasmid (Fig. S2B) showed no greater reduction in HCV production. Therefore, we used 2H9-L and AA10-H intrabodies in subsequent experiments. To improve the efficiency of transduction, adenoviruses expressing 2H9-L or AA10-H were created. Cells infected with the resulting adenoviruses were further infected with HCVcc and infectious titres in the supernatants were determined after 2 or 4 days of culture (Fig. 2c). As expected, HCV production was reduced by expression of 2H9-L and AA10-H, demonstrating the utility of anti-core intrabodies as an antiviral strategy.

In order to address the molecular mechanism(s) of intrabody-mediated inhibition of HCV propagation, we examined the effects of intrabodies on HCV entry and replication using trans-complemented HCV particles (HCVtcp) (Suzuki et al., 2012). HCVtcp were produced from cells harbouring subgenomic replicon RNA by trans-complementation of single-domain intrabodies inhibited the growth of HCV. Furthermore, intrabody expression suppressed HCV core-induced NFκB promoter activity.

**RESULTS AND DISCUSSION**

We constructed 10 single-domain intrabodies derived from five hybridomas, each of which was developed from mice immunized with bacterially expressed core (K. Yasui, Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, personal communication). Each epitope was defined using a panel of overlapping synthesized peptides (Table 1). The isotypes of mAbs were also determined by ELISA using goat antisera against mouse IgG1, IgG2a, IgG2b, IgG3 and IgM (Sigma-Aldrich). cDNA fragments of each variable region were amplified by PCR with appropriate primers and the resulting fragments were cloned into expression plasmids (Fig. 1a). Each variable region possessed the N-terminal leader signal sequence, which enhanced the steady-state levels of intrabodies in the cells, as shown in Fig. S1 (available in the online Supplementary Material).

**Table 1. Summary of mouse hybridomas producing anti-HCV core mAbs used for preparation of intrabodies**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Epitope (aa)</th>
<th>Isotype</th>
</tr>
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<tbody>
<tr>
<td>CA7</td>
<td>28–32</td>
<td>IgG1</td>
</tr>
<tr>
<td>2H9</td>
<td>29–36</td>
<td>IgG1</td>
</tr>
<tr>
<td>DA3</td>
<td>33–37</td>
<td>IgG2a</td>
</tr>
<tr>
<td>AA10</td>
<td>61–75, 115–123, 149–157</td>
<td>IgG1</td>
</tr>
<tr>
<td>2G3</td>
<td>71–77</td>
<td>IgG1</td>
</tr>
</tbody>
</table>

We expressed intrabodies and their interaction with core. We expressed intrabodies and their interaction with core. (a) Schematic representation of intrabody plasmids. CAG, CAG promoter; S, leader signal sequence; Myc, Myc-tag. (b) 293T cells were transfected with intrabody-encoding plasmids, followed by immunoblotting with anti-Myc and anti-actin antibody. (c) 293T cells were co-transfected with a FLAG-tagged core (F-core)-encoding plasmid and Myc-tagged intrabody-encoding plasmid. Cell lysates were immunoprecipitated (IP) with anti-FLAG antibody and examined by immunoblotting using anti-FLAG or anti-Myc antibody.
Infectious titres of HCVcc in supernatants were determined at 5 days post-infection (p.i.). Infectious titres of HCVcc in the supernatants were reduced by expression of 2H9-L or AA10-H (Fig. 3c), suggesting that these intrabodies reduce production of HCV particles. In addition, we observed that both intracellular and extracellular infectious viral titres were reduced by expression of 2H9-L or AA10-H (Fig. 3d). Thus, the antiviral effects of 2H9-L and AA10-H are most likely due to inhibition of the step(s) for infectious particle assembly.

In addition to its function in nucleocapsid formation, HCV core has been shown to affect a number of host cellular pathways, including NFκB signalling. NFκB signalling is involved in anti-apoptotic and pro-inflammatory pathways, as well as in cell proliferation, all of which are processes that can affect HCV-mediated pathogenesis (Mann et al., 2006; Marusawa et al., 1999; Yoshida et al., 2001; You et al., 1999). We therefore assessed the effects of anti-core intrabodies on core-induced NFκB activation. Expression of 2H9-L or AA10-H, but not that of CA7-H, resulted in significant attenuation in the NFκB activity induced by expression of FLAG-core derived from HCV of either genotype 1a, 2a or 3a (Fig. 4). Collectively, these results indicated that anti-core intrabodies not only block HCV production, but also interfere with the modulation of cellular gene expression induced by HCV core.

Although conventional whole antibodies recognize epitopes via the variable region of heavy and light chain complexes, heavy chain antibodies, which consist of two heavy chains and are observed in the Camelidae family and some cartilaginous fish, possess a single domain as the antigen-binding site (Greenberg et al., 1995; Hamers-Casterman et al., 1993). The variable regions of either heavy or light chain from mAbs have also been shown to bind to antigen (Sato et al., 2013; Tanaka et al., 2007). For therapeutic applications, single-domain antibodies may have advantages over conventional antibodies. The side-effects observed in the use of conventional antibodies are considered to result from target cross-linking due to the bivalent nature of these antibodies or to the presence of the Fc region (Harmsen & De Haard, 2007); such side-effects are...
not expected to occur with single-domain antibodies. In addition, the comparatively low molecular mass of single-domain antibodies may lead to recognition of antigenic sites that are not normally recognized by conventional antibodies (Harmsen & De Haard, 2007). Our findings here show the potential of single-domain intrabodies against HCV core as an antiviral strategy. Technical development to optimize intrabody delivery \textit{in vivo} is a prerequisite for this strategy. Use of recombinant viral vectors such as adeno-associated viruses is a possible future option (Zhang & Rabbitts, 2014) and related studies are currently being planned.

Although 2H9-L and AA10-H intrabodies inhibited infectious viral assembly steps rather than entry or genome replication, as shown in Fig. 3(d), the precise mechanism of action of the single-domain intrabodies that causes decreases in propagation of HCV remains unclear. It is possible that these intrabodies affect processes important for early steps in virion assembly, such as core–RNA binding, dimerization of core, localization of core with lipid droplets or the interaction between core and NS5A (Counihan \textit{et al.}, 2011; Masaki \textit{et al.}, 2008). Furthermore, as anti-core intrabodies interfere with NFκB signalling induced by HCV core, these intrabodies can be applied to dissect the mechanisms underlying the ability of core to regulate cellular gene expression.

Thus, single-domain intrabodies such as 2H9-L and AA10-H are potential inhibitors of HCV production and of signalling pathways activated by the virus. In addition to the possible therapeutic application of intrabodies, structural analyses of intrabody–core interfaces are expected to provide information useful for designing novel structure-based small-molecule inhibitors capable of blocking multiple functions of HCV core.
Intrabodies against HCV core inhibit viral propagation

Fig. 4. Inhibition of core-induced NFκB promoter activity by intrabodies. Huh-7 cells were co-transfected with NFκB-dependent Fluc plasmid, an internal control plasmid pRL-TK or FLAG-core (F-core) plasmid, together with the indicated intrabody-encoding plasmid or with an empty plasmid. Luciferase activities were measured at 2 days post-transfection. Fluc activities were normalized against Rluc activities. Data represent mean ± SD. Statistical differences between controls (vector) and each intrabody were evaluated by Student’s t-test. *P<0.05 versus control.

METHODS

Preparation of hybridomas. Female BALB/c mice were immunized intraperitoneally with purified recombinant HCV core protein expressed in Escherichia coli. Splenic lymphocytes were then fused with P3X63Ag8.653 mouse myeloma cells using 50 % polyethylene glycol. Hybrid cells were screened for antibody production by ELISA. Stable antibody-producing hybrids were cloned and established. All five hybridomas used in this study produced mouse mAbs that reacted to HCV core on immunoblot and immunofluorescence assay (K. Yasui, Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, personal communication).

Epitope mapping. In order to define the epitopes recognized by the mouse mAbs used in this study, a panel of overlapping peptides derived from HCV core was synthesized (Cambridge Research Biochemicals) and antibody reactivity to the peptides was analysed by indirect immunosassay (K. Yasui, Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, personal communication).

Cells. Human embryonic kidney 293T cells and human hepatocellular carcinoma cell lines Huh-7, Huh7.5.1 and Huh7-25 cells were maintained in Dulbecco’s modified Eagle’s medium (Wako Pure Chemical Industries) containing non-essential amino acids, 100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹ and 10 % FBS. Cells were cultured at 37 °C in a 5 % CO₂ incubator.

Plasmids. In order to generate intrabody expression plasmids, total RNA from hybridoma cells was reverse transcribed using a SMART RACE cDNA Amplification kit (Clontech), and cDNAs encoding V₁ or V₂, possessing the N-terminal leader signal sequence and the Myc-tag at the C terminus were amplified by PCR using appropriate primers. The resultant fragments were cloned into pCAGGS. Plasmids for expression of FLAG-tagged core derives from genotype 1a, 2a and 3a were described previously (Saeed et al., 2009). Pol I-driven HCV genome plasmid (genotype 2a) pHHJFH1am was described previously (Suzuki et al., 2012). Transfection with this plasmid yielded an unspliced full-length HCV RNA containing cell culture-adaptive mutations, resulting in efficient RNA replication within the cytoplasm and subsequent production of infectious virions. The cDNA coding core to the first transmembrane region of NS2 (33 aa) in pH1HF1am was replaced with the corresponding sequence of THpa (Suzuki et al., 2012) and was used as the genotype 1b HCV genome plasmid. To examine the effects of HCV core on NFkB promoter activity, NFkB-inducible reporter plasmid (pNF-K-B-Luc; Clontech) containing the firefly luciferase (Fluc) gene driven by the NFkB binding element was used. As a control, a plasmid expressing Renilla luciferase (Rluc) driven by the herpes simplex virus thymidine kinase promoter (pRL-TK; Promega) was used.

Preparation of viruses. HCVtcp and HCVcc derived from the JFH-1 isolate (genotype 2a) having adaptive mutations in E2 (N417S), p7 (N765D) and NS2 (Q1012R) were generated as described previously (Suzuki et al., 2012). Recombinant adenoviruses derived from human adenovirus type 5 expressing intrabodies under the EF-1z promoter were generated as described previously (Fukuda et al., 2006). Briefly, intrabody fragments obtained from each expression plasmid by EcoRI digestion and subsequent Klenow treatment were inserted into the SnaI site of a cosmid vector. The resultant cosmid was digested with PacI and transfected into 293 cells to generate recombinant adenoviruses.

Quantification of HCV infectivity. In order to determine the titres of HCVcc, Huh7.5.1 cell monolayers prepared in multwell plates were incubated with dilutions of samples, and then replaced with media containing 10 % FBS and 0.8 % carboxymethyl cellulose. Following incubation for 72 h, monolayers were fixed and immunostained with rabbit polyclonal anti-NS5A antibody, followed by Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen), and stained foci were counted and used to calculate a titre of f.f.u. ml⁻¹.

Immunoblotting. Cells were washed with PBS and incubated with lysis buffer (50 mM Tris/HCl, pH 7.4, 300 mM NaCl, 1 % Triton X-100). Lysates were sonicated for 10 min and added to the same volume of 2 × SDS-PAGE sample buffer. Protein samples were boiled for 10 min, separated by SDS-PAGE and then transferred to PVDF membranes (Merck Millipore). After blocking, membranes were probed with primary antibodies, followed by incubation with peroxidase-conjugated secondary antibody. Antigen–antibody complexes were visualized using an enhanced chemiluminescence detection system (Super Signal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific), in accordance with the manufacturer’s protocols.

Immunoprecipitation. Transfected cells were washed with ice-cold PBS, and suspended in lysis buffer (20 mM Tris/HCl, pH 7.4, containing 135 mM NaCl, 1 % Triton X-100 and 10 % glycerol) supplemented with 50 mM NaF, 5 mM Na 3VO4 and complete protease inhibitor cocktail, EDTA free (Roche). Cell lysates were sonicated for 10 min and then incubated for 30 min at 4 °C, followed by centrifugation at 14 000 g for 10 min. Supernatants were immunoprecipitated with anti-FLAG agarose beads (Sigma-Aldrich). Immunocomplexes were washed four times with lysis buffer. Proteins that bound to the beads were boiled with SDS sample buffer and were then subjected to SDS-PAGE.

Reagents and antibodies. For immunoblotting, anti-β-actin (AC-15; Sigma-Aldrich), anti-FLAG (M2; Sigma-Aldrich) and anti-core (2H9) mouse mAbs (Masaki et al., 2010) were used. Rabbit polyclonal antibodies against Myc were obtained from Santa Cruz Biotechnology. Anti-NS5B antibodies were rabbit polyclonal antibodies against synthetic peptides, as described elsewhere (Suzuki et al., 2012).

DNA transfection. Monolayers of 293T cells were transfected with plasmid DNA using FuGENE 6 transfection reagent (Promega) in accordance with the manufacturer’s instructions. Huh-7,
Hub7.5.1 and Hub7-25 cells were transfected with plasmid DNA using TransIT LT1 transfection reagent (Mirus).

Quantification of HCV core antigen. Concentration of HCV core antigen in culture medium was measured using a Lumipulse Ortho HCV Ag kit (Ortho Clinical Diagnostics).

Luciferase assay. For quantification of NFκB reporter activity, Fluc and Rluc activities of the cells were determined using a dual-luciferase reporter assay system (Promega) in accordance with the manufacturer’s protocols.

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