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). Intracellularly 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 the 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, pHHJFH1am. 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

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**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>
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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</td>
<td>IgG1</td>
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<td></td>
<td>115–123</td>
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<td></td>
<td>149–157</td>
<td></td>
</tr>
<tr>
<td>2G3</td>
<td>71–77</td>
<td>IgG1</td>
</tr>
</tbody>
</table>

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**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).

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.

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Fig. 2. Effects of intrabodies on the production of HCV. (a) Huh7.5.1 cells were transfected with each intrabody-encoding plasmid along with Pol I-driven HCV genome plasmid pHJFH1am. Infectious titres of HCVcc in the supernatants were determined at 5 days post-infection (p.i.). (b) Infectious titres of HCVcc in the supernatants of cells transfected with each intrabody-encoding plasmid in combination with a Pol I-driven HCV genome plasmid were determined at 6 days post-infection (p.i.). (c) Huh7.5.1 cells were infected with adenoviruses at m.o.i. 10, followed by HCVcc infection 24 h after adenoviral infection. Infectious titres of HCVcc in supernatants were determined at 2 and 4 days after HCV infection. All data are presented as mean ± SD. Statistical differences between controls (vector or mock) and each intrabody were evaluated by Student’s t-test. *P<0.05, **P<0.001 versus control.

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 signalling pathway (Fig. 3b). Thus, the antiviral effects of 2H9-L and AA10-H are most likely due to inhibition of the step(s) for infectious particle assembly.

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 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 et al., 2011; Masaki 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.

Fig. 3. Inhibition of particle assembly by intrabodies. (a) Schematic representation of plasmids used for the generation of HCVtcp. HCV polyproteins derived from JFH-1 are indicated by white boxes. HCV UTRs are indicated by bold lines. Adaptive mutations are indicated as asterisks. CAG, CAG promoter; P, Pol I promoter; T, Pol I terminator. (b) Huh7.5.1 cells infected with the indicated adenoviruses were further infected with HCVtcp. Expression of HCV core, NS5B, actin and intrabodies were detected by immunoblot at 3 days post-HCVtcp infection. (c) HCV core antigen in culture supernatants from Huh7-25 cells transfected with intrabody-encoding plasmid or control vector along with Pol I-driven HCV genome plasmid at 4 days post-transfection. (d) HCV infectivity in culture supernatants and cell lysates from Huh7-25 cells transfected with intrabody-encoding plasmid or control vector along with Pol I-driven HCV genome plasmid at 4 days post-transfection. Data represent mean ± SD. Statistical differences between controls (vector) and each intrabody were evaluated using Student’s t-test. **P < 0.001 versus control.
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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 immunofluorescence.

Preparation of viruses. HCVTCP and HCVcc derived from the JFH-I 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-1α 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 SwaI site of a cosmids vector. The resultant cosmids 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 multiwell 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-N5A 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 NaVO₃, 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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