Expression of GB virus C NS5A protein from genotypes 1, 2, 3 and 5 and a 30 aa NS5A fragment inhibit human immunodeficiency virus type 1 replication in a CD4+ T-lymphocyte cell line

Qing Chang,1 James H. McLinden,1 Jack T. Stapleton,1 M. Aslam Sathar2 and Jinhua Xiang1

1Iowa City VA Medical Center and the University of Iowa, Iowa City, IA 52246, USA
2Nelson R. Mandela School of Medicine, University of Kwazulu-Natal, South Africa

GB virus type C (GBV-C) is a common human flavivirus that has been associated with prolonged survival in HIV-positive individuals in several, though not all, epidemiological studies. There are five distinct GBV-C genotypes that are geographically localized, and it has been speculated that GBV-C genotypic differences may explain variable outcomes observed in different clinical studies. Expression of an 85 aa fragment of the GBV-C NS5A phosphoprotein (genotype 2) in a CD4+ T cell line (Jurkat) resulted in inhibition of HIV replication, mediated in part by decreased surface expression of the HIV coreceptor CXCR4 and upregulation of SDF-1. We expressed the NS5A protein from genotypes 1, 2, 3 and 5 in Jurkat cells, and demonstrated that all genotypes inhibited HIV replication. Further deletion mapping demonstrated that expression of a 30 aa fragment resulted in decreased CXCR4 surface expression, upregulation of SDF-1 and inhibition of HIV replication.

Supporting an interaction between GBV-C and HIV, in vitro co-infection of peripheral blood mononuclear cells or CD4+ -enriched T-cells with GBV-C and multiple HIV-1 clades (A–H and group O) results in inhibition of HIV-1 replication (Jung et al., 2005; Xiang et al., 2001, 2004). GBV-C replication inhibits HIV replication at least in part by downregulating HIV co-receptors CCR5 and CXCR4, and by inducing the relevant ligands for these receptors (the chemokines RANTES, MIP-1α, MIP-1β and SDF-1) (Jung et al., 2005, 2007; Nattermann et al., 2003; Xiang et al., 2001, 2004). Both the GBV-C envelope glycoprotein E2 and the non-structural protein NS5A have been shown to modulate CD4+ T-lymphocyte chemokine receptor expression and chemokine release in vitro, resulting in inhibition of HIV replication (Jung et al., 2005, 2007; Nattermann et al., 2003). Although detailed biochemical studies of GBV-C NS5A are lacking, it is predicted to share features with the HCV NS5A protein (Leary et al., 1996; Xiang et al., 2005a). Like HCV NS5A, GBV-C NS5A exists

GB virus type C (GBV-C, also known as hepatitis G virus) is a positive-sense, single-stranded RNA virus that is the human virus most closely related to hepatitis C virus (HCV). Based on nucleotide and amino acid sequence identity and predicted genome organization, GBV-C has been classified within the family Flaviviridae (Linnen et al., 1996; Simons et al., 1995). GBV-C commonly infects humans, with approximately 1.8 % of healthy blood donors having viraemia detected by RT-PCR at the time of donation (reviewed by Alter, 1997; Stapleton et al., 2004). Although GBV-C was initially thought to cause hepatitis, several large epidemiological studies have found no association between the virus and acute or chronic hepatitis (Alter, 1997). To date, no disease association with GBV-C has been identified.

GBV-C appears to be lymphotropic, and has been shown to replicate in vitro in peripheral blood mononuclear cells, CD4+ and CD8+ T-lymphocytes, and B-lymphocytes (George et al., 2006; Xiang et al., 2000). Blood-borne, sexual and maternal–fetal transmission of GBV-C have been documented, as expected for a lymphotropic virus (reviewed by Polgreen et al., 2003), and, due to shared modes of transmission, 20–40 % of HIV-1-infected individuals are actively co-infected with GBV-C in cross-sectional studies (reviewed by Stapleton, 2003). Several, though not all, published epidemiological studies of HIV-infected cohorts observed an association between GBV-C infection and prolonged survival (reviewed by Stapleton et al., 2004; Zhang et al., 2006). Persistent viraemia appears to be required for the beneficial survival effect (Williams et al., 2004; Van der Bij et al., 2005), and a meta-analysis of several studies of 1294 HIV-infected individuals (Zhang et al. 2006) found a relative risk of mortality among subjects with GBV-C co-infection of 0.41 (95 % confidence intervals 0.23–0.69).

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in a basal and a hyperphosphorylated form, and the protein inhibits phosphorylation of eIF-2α by inhibiting the function of protein kinase R (PKR; a kinase activated by double-stranded RNA) (Xiang et al., 2005a). Based on comparison with HCV NS5A, it is predicted that GBV-C is anchored in the endoplasmic reticulum (ER) membrane, is a required component of the RNA replication complex and modulates a wide variety of cellular processes including transcription, resistance to apoptosis, and a variety of signal transduction pathways (Macdonald & Harris 2004). Interactions with the ER membrane do not appear to be required for inhibition of HIV, as expression of an 85 aa fragment of NS5A in which the amphipathic anchoring domain was deleted also resulted in potent inhibition of HIV replication (Xiang et al., 2006).

Phylogenetic analysis of different GBV-C isolates from around the world suggests an African origin of GBV-C, with the virus pre-dating human migration from Africa (Smith et al., 1997). At least five GBV-C genotypes have been identified, based on sequence from the 5′-non-translated region of the genome. It has been speculated that biological differences between GBV-C genotypes may result in differences in inhibition of HIV replication, and that this may explain why some studies have not identified an association between GBV-C viraemia and prolonged survival (Schwarze-Zander et al., 2006; Muerhoff et al., 2003). Thus, we investigated the NS5A protein from GBV-C genotype 1, 2, 3 and 5 isolates to determine if there were differences in their ability to inhibit HIV replication.

RNA was extracted from the serum of USA and African subjects, previously shown to be infected with GBV-C genotype 1, 2, 3 or 5 isolates, using the QiAamp Viral RNA mini kit (Qiagen) as described previously (Xiang et al., 2005b). The full-length NS5A sequences were amplified by RT-PCR (nt 6150–7392, 414 aa) using the infectious cDNA sequence (GenBank accession number AF121950) as the reference (Xiang et al., 2000), and these were ligated into the pCR2.1 vector (Invitrogen) as described previously (Xiang et al., 2005a, 2006). No new human samples were utilized in these studies. The NS5A sequences for each isolate were determined (at the University of Iowa DNA Core Facility, using Applied Biosystems automated DNA sequencer 373A), and the nucleotide and deduced amino acid sequences were aligned using the neighbour-joining method (DNAMAN software; Lynnon BioSoft). These full-length NS5A sequences were subcloned into a tetracycline-repressible pTRE2 plasmid (Invitrogen) modified to contain a Kozak sequence followed by an AUG codon in-frame with the NS5A coding region. NS5A sequences were followed by a stop codon, an encephalomyocarditis internal ribosome entry site (EMC IRES) element directing the translation of green fluorescent protein (GFP) followed by a bovine growth hormone polyadenylation site as described previously (Xiang et al., 2006). Stably expressing cell lines were generated by transfecting the modified pTRE2 vector into a CD4+ T-lymphocyte cell line (Jurkat) using the Amaxa Nucleofectin method as described previously (Xiang et al., 2006). Following selection in hygromycin and neomycin (200 μg ml⁻¹), cell lines were cloned at least twice by terminal dilution. Two control cell lines were employed in these studies. One expressed the vector control without NS5A, but containing the AUG, stop codon and EMC IRES directing the translation of GFP (VC-GFP), and the other contained NS5A sequences (nt 6509–6856) containing a +1 frame-shift (FS; sequence numbers from GenBank accession no. AF121950). The FS vector encodes 26 missense amino acids prior to a termination codon (Xiang et al., 2006). Fig. 1(a) illustrates GFP expression detected by flow cytometry and Fig. 1(b) shows NS5A detection by immunoblotting in stably transfected cell lines containing two isolates from genotype 1 (GenBank accession nos EF458005 and EF458006), and a single isolate each for genotypes 2 (DQ177421), 3 (DQ177420) and 5 (EF458004). The immunoreactive proteins were identified using GE3 anti-GBV-C NS5A

![Fig. 1. Characterization of GBV-C NS5A expression in Jurkat cells. The NS5A genes from two GBV-C genotype (GT) 1 isolates (1-1 and 1-2), and from single isolates from GT 2, 3 and 5 were ligated into an expression plasmid containing GFP as described in Methods. Clonal Jurkat cell lines that stably expressed GFP were selected (a; red shaded area, background cellular fluorescence for Jurkat cells without GFP; dark blue and purple, GT 1; green, GT 2; black, GT 3; light blue, GT 5). Cell lysates from the GFP-positive clones were analysed for NS5A expression by immunoblotting (b). FS, ‘frame-shift’ control cells.](image-url)
rabbit serum (kindly provided by Dr Jungsuh Kim, Genelabs Technologies, Redwood City, CA) as described previously (Xiang et al., 2005a). Differences in relative mobility of the immunoreactive proteins have been observed previously with GBV-C NS5A (Xiang et al., 2005a), and this is probably related to differences in phosphorylation and/or degradation.

Deletion mapping of the GBV-C NS5A protein previously demonstrated that 85 aa (NS5A 152–236) were sufficient to inhibit HIV replication (Xiang et al., 2006). This region was examined in further detail by inserting sequences encoding a 30 aa region (residues 152–181) into the pTRE2 vector using synthetic oligonucleotides. This plasmid was transfected into Jurkat cells and a cell line was selected that stably expressed GFP. The anti-NS5A antibody did not recognize this 30 aa fragment (data not shown). To verify that the sequence was present, cellular DNA from the cell line was extracted and the region containing 230 nt upstream of GFP (including the 30-mer coding region) was sequenced, confirming the presence of an open reading frame containing these 30 aa.

HIV-1 isolates (X4) representing clade B (X4 tropic, NIH AIDS Research and Reference Reagent Program, catalogue number 1073) were applied to Jurkat cells (200 pg HIV p24 antigen per 10⁶ cells) containing NS5A sequences or to control cells as described previously (Xiang et al., 2006). Cells were washed extensively 3 h after attachment, and maintained in fresh growth medium (RPMI 1640 containing 10% fetal bovine serum, 100 U penicillin ml⁻¹, 100 μg streptomycin sulfate ml⁻¹ and 2 mM L-glutamine). Cell culture supernatants were collected and HIV replication (p24 antigen in culture supernatants) was monitored as described previously (Xiang et al., 2006). All infections were performed in triplicate, and repeated a minimum of three times with similar results. Fig. 2(a) shows that expression of the full-length NS5A constructs from the genotype 1, 2, 3 and 5 isolates and the 30 aa fragment corresponding to NS5A amino acids 152–181 in Jurkat cells resulted in significant inhibition of HIV replication compared with cells expressing either a vector control (expressing GFP) or a control plasmid that contained NS5A coding sequences altered to have an FS mutation (P<0.01 for all NS5A proteins compared with either control isolate on days 4–7).

Alignment of the predicted NS5A amino acid sequences representing the 30 aa expressed in the deletion mutant from each of the isolates, based on translation of the DNA sequence, found no sequence variation in the genotype 1, 2 and 3 sequences compared to the prototype infectious clone sequence (Fig. 2b; GenBank accession no. AF121950). Genotype 5 had two conservative and one non-conservative amino acid polymorphisms (Fig. 2b). Comparing this 30 aa region with 42 GBV-C full-length sequences published in GenBank found that the valine (position 166) and the histidine (position 173) polymorphisms have not been reported previously; however, the glutamic acid at position 160 is present in 25 of 42 isolates (data not shown). No other GBV-C genotype 5 NS5A sequences have been published to indicate whether these polymorphisms are common to this genotype. Although there is no perfect sequence conservation across genotypes (Fig. 2b), all five NS5A proteins tested representing four separate genotypes inhibited HIV replication. In addition, both the genotype 2 and 3 NS5A isolates inhibited three additional HIV clades (B, C and D; X4 tropic, NIH AIDS Research and Reference Reagent Program, catalogue nos 2521, 317 and 2696; data not shown).

Expression of GBV-C NS5A protein from a genotype 2 isolate decreased the surface expression of the HIV...
co-receptor CXCR4, and increased the release of the CXCR4 ligand, SDF-1, compared with control cells (Xiang et al., 2006). To determine if there were differences in the surface expression of CXCR4 on Jurkat cells expressing different GBV-C genotypes or the 30 aa NS5A fragment, CXCR4 surface expression was determined by flow cytometry as described previously (Xiang et al., 2004). All experiments were repeated a minimum of three times. GBV-C NS5A protein expression from all genotypes tested led to a significant reduction in the surface expression of the HIV co-receptor CXCR4 compared with the control cell line containing the NS5A FS mutation (P<0.01 for all NS5A cell lines; Fig. 3a). Jurkat cells expressing the 30 aa fragment (NS5A residues 152–181) also had significantly less surface CXCR4 expression compared with the FS control cell line (P=0.02; Fig. 3a). Although the absolute amount of CXCR4 expression varied slightly between cell lines, this may reflect the differences in the amount of NS5A protein expression demonstrated in Fig. 1(a, b).

Incubation of CD4+ T-cells in medium containing SDF-1 (the natural ligand for CXCR4) results in inhibition of HIV replication in vitro. The mechanism of inhibition relates both to competition between SDF-1 and HIV for binding to the CXCR4 co-receptor, and to inhibition of post-entry DNA transcription (Amella et al., 2005; Cocchi et al., 1995; Copeland, 2005; Lin et al., 2002). Expression of genotype 2 NS5A protein in Jurkat cells has been previously shown to increase the release of SDF-1 into culture supernatants (Xiang et al., 2006). SDF-1 levels were measured in culture supernatants of Jurkat cells expressing the NS5A protein from different GBV-C genotypes and the 30 aa NS5A fragment and compared to vector-control or FS-control cell lines. SDF-1 levels in culture supernatant fluids were determined by ELISA in triplicate (R&D Systems) and repeated at least once. All of the NS5A proteins and the 30 aa fragment induced the release of significant amounts of SDF-1 into culture supernatants (Fig. 3b; P<0.02 for all constructs compared with the vector control 6 and 7 days after HIV infection).

These data demonstrate that the expression of the GBV-C NS5A protein from four different genotypes in Jurkat cells results in the inhibition of HIV replication (Fig. 2a). Thus, genotype-specific amino acid differences in the GBV-C NS5A protein do not appear to explain why some clinical studies have not recorded a beneficial effect. Review of clinical epidemiological studies suggests that confounding variables such as antiretroviral therapy (Birk et al., 2002), clearance of GBV-C viraemia (Bjorkman et al., 2004; Williams et al., 2004; Van der Bij et al., 2005), and the duration of HIV infection at the time at which GBV-C RNA is measured (Zhang et al., 2006) may better explain why GBV-C has not always been associated with better clinical outcomes in HIV-infected people than difference in genotype (Schwarze-Zander et al., 2006; Muerhoff et al., 2003). Furthermore, in the only two longitudinal studies conducted prior to the availability of effective combination antiretroviral therapy, persistent GBV-C viraemia was associated with a statistically significant prolongation of survival (Williams et al., 2004; Van der Bij et al., 2005).

The mechanism by which GBV-C NS5A inhibits HIV replication is mediated, at least in part, by the reduction of HIV co-receptor CXCR4 detected on the surface of CD4+ T cell lines (Fig. 3a) and by the increased production of the chemokine SDF-1 as compared with control cells (Fig. 3b). By further deleting parts of the 85 aa NS5A fragment that has previously been shown to inhibit HIV replication, we found that a highly conserved 30 aa fragment (residues 152–181) is sufficient to inhibit HIV-1 replication (Fig. 2a). This fragment contains four potential phosphorylation sites and is predicted to have a high capacity for binding HIV co-receptor CXCR4, leading to decreased surface expression and increased SDF-1 release. These findings provide new insights into the mechanisms by which GBV-C NS5A protein inhibits HIV replication.
sites (one tyrosine, one threonine and two serine residues) that may be involved in altering cellular factors that contribute to HIV inhibition.

The HIV-inhibitory effect of NS5A expression (and expression of the 30 aa fragment) appears to be mediated by modulation of cellular chemokine and chemokine receptor expression, and there are no data to suggest that NS5A has a direct antiviral effect on HIV replicative enzymes. Consequently, it is tempting to speculate that GBV-C NS5A inhibition of HIV replication should have a lower rate of development of HIV drug resistance compared to current antiretroviral medications. Thus, further characterization of the HIV-inhibitory 30 aa fragment of NS5A appears warranted, and may lead to novel approaches for antiretroviral therapy.

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References


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