Short Communication

Interferon resistance of hepatitis C virus replicon-harbouring cells is caused by functional disruption of type I interferon receptors

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Hepatitis C virus (HCV) replicon-harbouring cell lines possessing interferon (IFN)-resistant phenotypes have recently been established. These were divided into two classes: partially IFN resistant and highly IFN resistant. Here, the viral and cellular factors contributing to the IFN resistance of HCV replicon-harbouring cells were evaluated. The results revealed that cellular factors rather than viral factors contributed to a highly IFN-resistant phenotype. The possibility of genetic abnormality of the factors involved in IFN signalling was investigated. As a result, nonsense mutations and deletions in type I IFN receptor genes (IFNAR1 and IFNAR2c) were found in replicon-harbouring cells showing a highly IFN-resistant phenotype, but rarely appeared in cells showing a partially IFN-resistant phenotype. Furthermore, similar genetic alterations were also found in IFN-resistant phenotype, replicon-harbouring cell lines obtained additionally by IFN-β treatment. Moreover, it was shown that ectopic expression of wild-type IFNAR1 in IFN-resistant phenotype, replicon-harbouring cells possessing the IFNAR1 mutant restored type I IFN signalling.

Persistent infection by hepatitis C virus (HCV) is a major cause of chronic hepatitis (Choo *et al.*, 1989; Kuo *et al.*, 1989), which can progress to liver cirrhosis and hepatocellular carcinoma (Saito *et al.*, 1990). Since at least 170 million people are currently infected with HCV worldwide, this infection constitutes a global health problem (Thomas, 2000). HCV is an enveloped RNA virus belonging to the family *Flaviviridae*, the genome of which consists of a positive-stranded RNA encoding an approximately 3000 aa polyprotein precursor (Kato *et al.*, 1990). This precursor protein is processed by the host and viral proteases to generate at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (Kato, 2001).

Since 1998, combined treatment with interferon (IFN)-α and ribavirin has been standard clinical therapy for patients with chronic hepatitis C; however, the effectiveness of IFN is limited to approximately 50 % (Hadziyannis *et al.*, 2004). This clinical result suggests that HCV directly or indirectly attenuates the antiviral actions of IFN (Pawlotsky, 2000).

Although an HCV replicon system carrying autonomously replicating HCV subgenomic RNA containing the NS3–NS5B regions (Lohmann *et al.*, 1999) was considered to be useful in studies on the mechanism(s) of IFN resistance of HCV, all HCV replicons established to date have been highly sensitive to IFN-α, -β and -γ (Frese *et al.*, 2001, 2002; Kato *et al.*, 2003). This seems to contradict the fact that half of the patients with chronic hepatitis C are resistant to current IFN therapy. Therefore, we assumed that prolonged IFN treatment might change HCV replicons from an IFN-sensitive phenotype to an IFN-resistant phenotype.

Based on this assumption, we recently established nine HCV replicon cell lines possessing two IFN-resistant phenotypes: a partially resistant phenotype (αR series: 1αR, 3αR, 4αR, 5αR and αRmix) and a highly resistant phenotype (βR series 1βR, 3βR, 4βR and 5βR) obtained by IFN-α and -β treatment, respectively (Namba *et al.*, 2004). Genetic analysis of these replicons found one common amino acid substitution (Q1737H) in the NS4B region and several additional amino acid substitutions (such as M2174V and T2242N) in the NS5A region of the βR series (Namba *et al.*, 2004). To examine which viral and cellular factors contribute to the IFN resistance of HCV replicons, we evaluated the IFN sensitivity of replicon-harbouring cells (6M.m/6Mc, 1βR.m/6Mc and 4βR.m/6Mc) established by transfection of total RNAs isolated from an IFN-sensitive clone (6M) and from highly IFN-resistant clones (1βR and 4βR) into cured 6Mc cells, from which 50-1 replicons (Kishine *et al.*, 2002) had been eliminated by IFN-γ treatment (500 IU ml^{-1} for

Supplementary material is available in JGV Online.

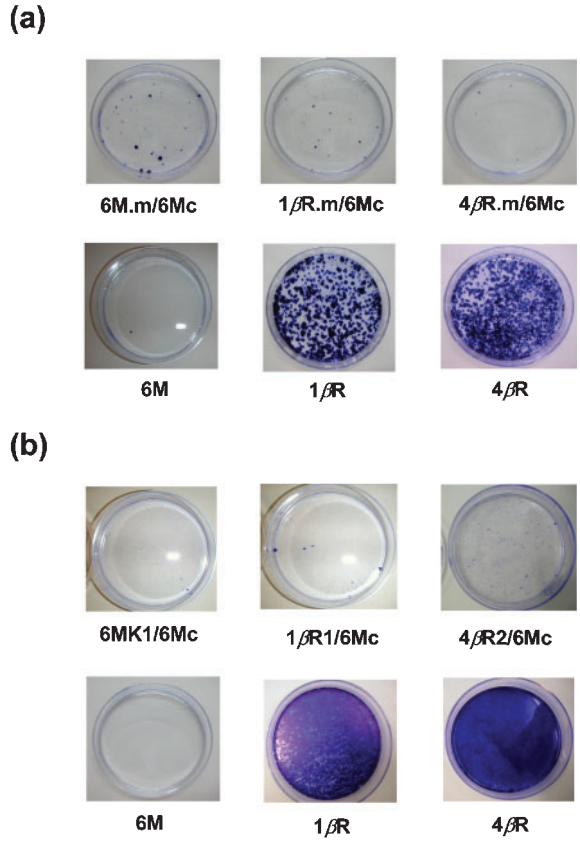

Fig. 1. IFN sensitivities of various HCV replicon-harbouring cells. (a) 6M.m/6Mc, 1βR.m/6Mc and 4βR.m/6Mc cells obtained as G418-resistant mixed colonies were treated with IFN-α (400 IU ml^{-1}) for 3 weeks in the presence of G418 (300 μg ml^{-1}). 6M, 1βR and 4βR cells were also used for control experiments. G418-resistant colonies were stained with Coomassie brilliant blue as described previously (Naganuma *et al.*, 2004). (b) IFN sensitivities of 6MK1/6Mc, 1βR1/6Mc and 4βR2/6Mc cells were examined as described in (a) except that they were treated with IFN-β (200 IU ml^{-1}).

3 weeks). Although many colonies were found to have survived in 1βR and 4βR cells after IFN-α treatment, only a few colonies survived in 1βR.m/6Mc and 4βR.m/6Mc cells or in 6M and 6M.m/6Mc cells (Fig. 1a). Similar results were obtained when these replicon-harbouring cells were treated with IFN-β (400 IU ml^{-1}) (data not shown), although two colonies (named 6βR and 7βR, described below) derived from 6M.m/6Mc and 4βR.m/6Mc cells, respectively, proliferated as highly IFN-resistant clones.

We further examined the IFN sensitivity of replicon-harbouring cells (6MK1/6Mc, 1βR1/6Mc and 4βR2/6Mc) established by the transfection of *in vitro*-synthesized replicon RNAs (6MK1, 1βR1 and 4βR2 obtained from 6M, 1βR and 4βR cells, respectively) (Namba *et al.*, 2004; Kato *et al.*, 2005) into 6Mc cells. The results revealed that few or no colonies survived in 1βR1/6Mc and 4βR2/6Mc cells, as in 6M and 6MK1/6Mc cells, whereas many colonies survived in 1βR and 4βR cells (Fig. 1b). These results suggested that cellular factors rather than viral factors contributed to the highly IFN-resistant phenotype of HCV replicon-harbouring cells. However, the present results obtained under a high concentration of IFN do not necessarily rule out a possible role for HCV mutations in conferring low degrees of IFN resistance, because effects of HCV mutations on IFN activity are presumably weaker than those of the cellular factors.

To obtain evidence in favour of the idea that alterations in cellular factor(s) are involved in the emergence of an IFN-resistant phenotype, we attempted to prepare cured cells from the replicon-harbouring cells possessing a highly IFN-resistant phenotype. Since phosphorylation of signal transducer and activator of transcription 1 (STAT1) occurred in the 1βR and 4βR cells treated with IFN-γ (Fig. 2a), these replicon-harbouring cells were treated with IFN-γ (500 IU ml^{-1}) for 3 weeks, and cured 1βRc and 4βRc cells were obtained. Western blot (Fig. 2b) and RT-PCR (data not shown) analyses showed that no replicons were detected in either type of cured cells. Analysis of a luciferase reporter assay indicated that the complete defect of the IFN-α/β signalling was not restored in the cured 1βRc and 4βRc cells (Fig. 2c).

To clarify whether or not the signalling defect in these replicon-harbouring cells was restricted to type I IFN, we examined the phosphorylation status of STAT3 in 6M, 1βR and 4βR cells treated with interleukin-6 (IL6). Since it has been reported that STAT3 is also activated by IFN-α treatment (Pfeffer *et al.*, 1997), the phosphorylation status of STAT3 in these replicon-harbouring cells after IFN-α treatment was also examined. Our results revealed that STAT3 was not phosphorylated in 1βR and 4βR cells treated with IFN-α, while phosphorylation of STAT3 was observed in 6M, 1βR and 4βR cells treated with IL6 and in 6M cells treated with IFN-α (Fig. 2d), indicating that only type I IFN signalling was defective in 1βR and 4βR cells. These results suggested that the initial reaction following the addition of IFN-α/β was defective in replicon-harbouring cells possessing a highly IFN-resistant phenotype.

Following up this suggestion, we examined the genetic status of tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1). Sequence analysis of TYK2 and JAK1 cDNAs obtained from 1βR and 4βR cells was performed after cloning into the pCXbsr vector (Akagi *et al.*, 2000), as described previously (Nozaki *et al.*, 2003). However, the results showed no mutations in these cDNAs (data not shown). We next focused on type I IFN receptors (IFNAR1 and IFNAR2c). Our results showed that the mRNA levels of the two receptors were almost equal among all examined replicon-harbouring cells including 6Mc cells (see Supplementary Fig. S1a, available in JGV Online). More than three independent clones of each cDNA (1708 bp for IFNAR1 and 1582 bp for IFNAR2c) were sequenced as described above. Table 1 shows a summary of sequence analysis of IFNAR1

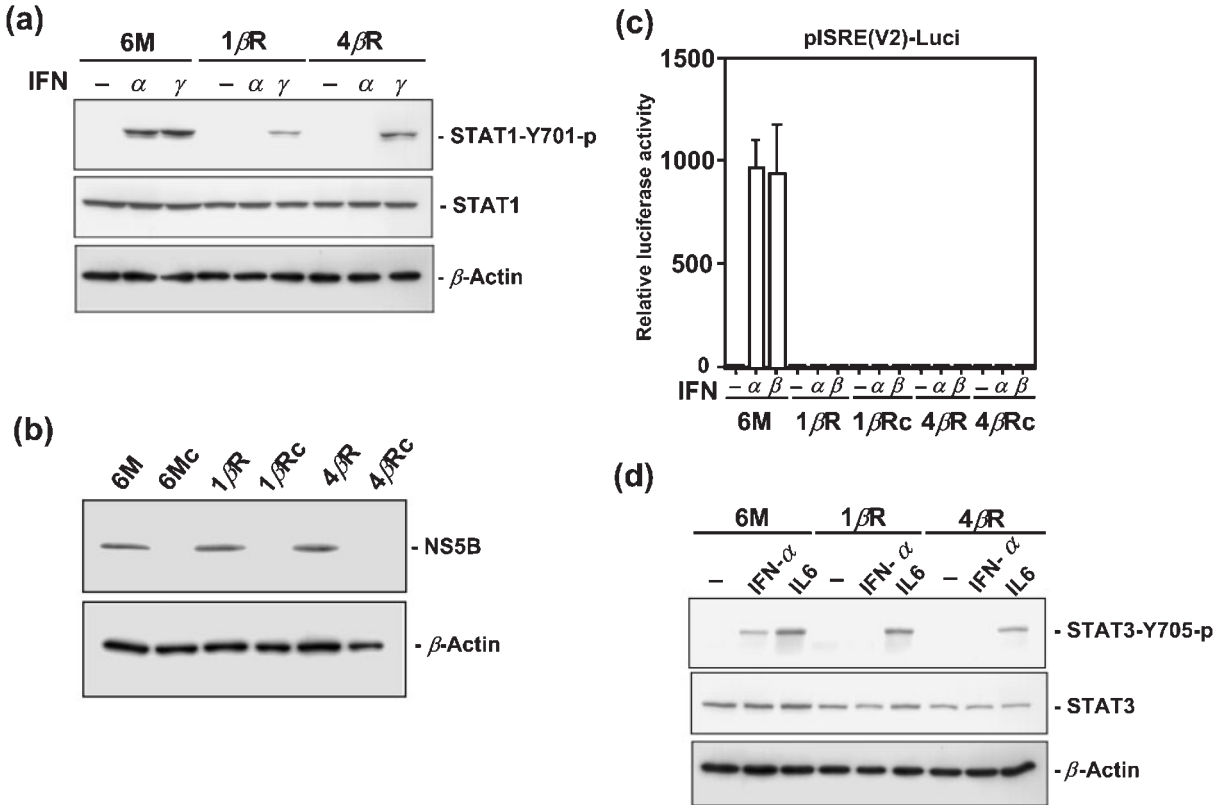

Fig. 2. Cellular factors rather than HCV replicons contribute to defects in type I IFN signalling. (a) Western blot analysis of STAT1 in 6M, 1βR and 4βR cells treated with IFN-α or -γ. The replicon-harbouring cells were treated with or without IFN-α or -γ (500 IU ml^{-1} each) for 30 min, and Western blot analysis for STAT1 and its phosphorylation status (Tyr-701) was then performed as previously described (Hijikata *et al*., 1993). β-Actin was used as a control for the amount of protein loaded per lane. (b) Western blot analysis of NS5B. Anti-NS5B antibody was used for immunoblotting. β-Actin was used as described in (a). (c) Analysis of IFN signal transduction. Dual luciferase assays using pISRE(V2)-Luci (Dansako *et al*., 2003) were performed as previously described (Naganuma *et al*., 2000). Cells were treated with IFN-α or -β (500 IU ml^{-1} each) for 6 h. (d) The defect in the signalling pathway in 1βR and 4βR cells is restricted to type I IFN. Replicon-harbouring cells were left untreated or treated with IFN-α (500 IU ml^{-1}) or IL6 (100 ng ml^{-1}) for 30 min. Western blot analysis for STAT3 and its phosphorylation status (Tyr-705) was performed as described in (a).

and IFNAR2c mRNAs. Surprisingly, we found that nt 475 of IFNAR2c mRNA from 1βR cells had a U substituted for G in 13/13 clones, resulting in a nonsense mutation at codon 159 from glutamic acid (GAG) to the termination codon UAG (see Supplementary Fig. S1b). Furthermore, nt 319 of IFNAR1 mRNA from 4βR cells was also found to have a U substituted for G in 12/12 clones, resulting in a nonsense mutation at codon 107 from glutamic acid (GAA) to the termination codon UAA (see Supplementary Fig. S1c). However, interestingly, IFNAR1 and IFNAR2c mRNAs obtained from 1αR and 4αR cells, which were derived from clone 1 and clone 4 and were counterparts of 1βR and 4βR cells, respectively (see Supplementary Fig. S2, available in JGV Online), did not possess the nonsense mutations found in the mRNAs from 1βR and 4βR cells. In addition, several kinds of deletion and another nonsense mutation (lysine to a termination codon at codon 458) were found in approximately half of IFNAR1 cDNA clones obtained from 3βR and 5βR cells. In contrast to the finding of frequent mutations and deletions in IFNAR mRNAs from the βR series, such genetic abnormalities in IFN receptors were quite rare in the αR series (Table 1).

To evaluate the possibility that genetic mutants might preexist in the cloned replicon-harbouring cells (clones 1, 3, 4 and 5 shown in supplementary Fig. S2) or that genetic mutants had appeared during the IFN-β treatment of the cloned replicon-harbouring cells, we repeated IFN-β treatment of cloned 1, 3, 4 and 5 cells following the method described previously (Namba *et al*., 2004). The result was almost identical to that obtained previously (Namba *et al*., 2004), indicating the good reproducibility of the experiment with IFN-β treatment (data not shown). In the present study, each of the three colonies showing resistance to IFN-β was isolated and proliferated (see Supplementary Fig. S2) and we then performed sequence analysis of IFNAR mRNAs

Table 1. Genetic alterations of type I IFN receptors in HCV replicon-harbouring cell lines possessing IFN-resistant phenotypes

The determined nucleotide sequences were compared with those of 6M and 6Mc cells, which confirmed that the deduced amino acid sequences were identical to the human IFNAR1 (GenBank accession no. NM_000629) and IFNAR2c (GenBank accession no. L41942) sequences. NM, Not mutated.

Cell line	IFNAR1 mRNA			IFNAR2c mRNA		
	Nucleotide change and position	Effect on protein	Mutation frequency*	Nucleotide change and position	Effect on protein	Mutation frequency*
6M	NM	None	0/3	NM	None	0/3
1βR	NM	None	0/3	G to U at nt 475	E to stop at codon 159	13/13
3βR	Deletion of 5 nt at nt 376	Truncated (135 aa)	2/11	NM	None	0/3
	A to U at nt 1372	K to stop at codon 458	3/11			
4βR	G to U at nt 319	E to stop at codon 107	12/12	NM	None	0/3
5βR	Deletion of 176 nt at nt 201	Truncated (78 aa)	4/10	NM	None	0/3
	Deletion of 79 nt at nt 201	Truncated (67 aa)	2/10			
1αR	NM	None	0/3	NM	None	0/3
3αR	NM	None	0/3	NM	None	0/3
4αR	Deletion of 5 nt at nt 376	Truncated (135 aa)	1/3	NM	None	0/3
5αR	Deletion of 79 nt at nt 201	Truncated (67 aa)	1/3	NM	None	0/3
αRmix	NM	None	0/3	NM	None	0/3
6Mc	NM	None	0/3	NM	None	0/3

*Number of mutated or truncated clones/number of examined clones.

as described above. The results revealed that the nonsense mutations or deletions identified at this time (see Supplementary Table S1, available in JGV Online) were quite different from those obtained from the βR series (Table 1). Therefore, it is unlikely that the identified IFNAR mutants pre-existed in cloned 1, 3, 4 and 5 cells when these cells were obtained as colonies surviving IFN-α treatment.

To examine whether or not additional HCV replicon cell lines possessing the IFN-resistant phenotype could be obtained from HCV replicon-harbouring cells other than the parental replicon-harbouring cells used for the isolation of the αR and βR series, 6M.m/6Mc, 1βR.m/6Mc, 4βR.m/6Mc and 50-1 replicon-harbouring cells were treated with IFN-β (see Supplementary Fig. S2). Finally, we obtained four replicon-harbouring cell lines (6βR obtained from the 4βR.m/6Mc cells, 7βR obtained from the 6M.m/6Mc cells, and 8βR and 9βR obtained from the 50-1 cells) showing resistance to IFN-β. These results indicated that HCV replicon-harbouring cells showing the IFN-resistant phenotype were obtained from HCV replicon-harbouring cells established immediately. By sequence analysis of IFNAR1 and IFNAR2c cDNAs as described above, the E107stop nonsense mutation in IFNAR1 cDNA, which was the same mutation found in the 4βR cells, was found again in the 8βR and 9βR cells, while no IFNAR mutations were detected in the 6βR and 7βR cells (see Supplementary Table S1). The observation that IFNAR mutations occurred preferentially after IFN-β treatment is interesting. Since a variety of mutations and deletions in the IFN receptors were obtained from the cloned replicon-harbouring cells surviving after IFN-β treatment, such genetic alterations might occur accidentally in order to impair the antiviral states caused after IFN-β treatment. Thereafter, only replicon-harbouring cells possessing the IFNAR mutants might be able to proliferate in the presence of G418, resulting in the βR series.

To clarify whether or not the IFNAR mutations found in the βR series were determinants for the IFN sensitivity of HCV replicons, we prepared 4βR cells (possessing the IFNAR1 mutant) stably expressing wild-type IFNAR1 and examined its IFN sensitivity. Analysis of a luciferase reporter assay (see Supplementary Fig. S3a, available in JGV Online) clearly showed that IFN signalling in 4βR cells was restored by the expression of wild-type IFNAR1 in comparison with those of 4βR cells expressing the IFNAR1 mutant (see Supplementary Fig. S3b). The quantitative RT-PCR analysis of replicon RNA in the cells treated with IFN-β clearly showed that the level of 4βR replicon in cells expressing wild-type IFNAR1 was drastically decreased after IFN-β treatment, as was the level of 6M replicon in cells expressing wild-type IFNAR1 (see Supplementary Fig. S3c). In summary, we demonstrated that the IFNAR mutation found in 4βR cells was a major determinant for a strongly IFN-resistant phenotype of 4βR cells, suggesting that IFNAR mutations, which lead to the impairment of IFN signalling, convert HCV replicon-harbouring cells from an IFN-sensitive phenotype to a highly IFN-resistant phenotype.

IFNAR1 and IFNAR2c belonging to the class II cytokine receptor superfamily are structurally conserved transmembrane receptors located on the cell surface (see Supplementary Fig. S4a, available in JGV Online). However, since both the IFNAR1 E107stop mutant and the IFNAR2c E159stop mutant found in 4βR and 1βR cells, respectively, were N-terminally truncated and probably soluble forms, these truncated proteins may not be functional as IFN receptors or may act as dominant-negative inhibitors, and will lead to the interception of IFN signalling (see Supplementary Fig. S4b). Thus, the cause of the IFN-resistant phenotype of 1βR or 4βR cells appeared to be the functional disruption of IFNAR. The present results suggest that the downstream JAK/STAT pathway is intact, at least in 4βR cells.

Although for the most part we could clarify the mechanism underlying a highly IFN-resistant phenotype of HCV replicon-harbouring cells, at least in the case of 4βR cells, the mechanism underlying a partially IFN-resistant phenotype remains unclear, because IFNAR mutations were rare in the αR series. Since the expression levels of IFNAR, TYK2 and JAK1 were not decreased in the αR series, a functional deficiency of other cellular factor(s) involved in the IFN signalling may contribute to the acquisition of IFN resistance. Alternatively, certain HCV mutation(s) may account for the partially IFN-resistant phenotype of the αR series.

Since Machida *et al.* (2004a, b) recently reported that the frequency of genetic mutation was enhanced by HCV replication in *in vitro*-infected B cells and that the HCV core and NS3 were involved in the induction of a mutator phenotype mediated through the activation of inducible nitric oxide synthase, we cannot exclude the possibility that persistent HCV replication induces some irreversible genetic mutations. To clarify whether or not HCV acts as a mutagen for cellular factors, further study using an HCV RNA replication system (Ikeda *et al.*, 2002, 2005; Naka *et al.*, 2005) will also be necessary.

The HCV replicon-harbouring cells including 1βR and 4βR, in which IFN signalling is impaired, used or obtained in the present study may be useful for future studies, not only of the mechanism(s) underlying the IFN resistance of the replicons but also of the functional characterization of IFN receptors. Furthermore, these replicon cells may also be useful for screening novel anti-HCV reagents that act by mechanisms unrelated to IFN signalling.

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