Hepatitis C virus RNA replication is resistant to tumour necrosis factor-α

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It was demonstrated using self-replicating hepatitis C virus (HCV) RNAs that both types of interferons (IFNs) (in particular IFN-α and IFN-γ) are potent inhibitors of HCV replication in Huh-7 cells. Because IFN-γ and tumour necrosis factor (TNF)-α trigger a partially overlapping set of antiviral defence mechanisms, it is tempting to speculate that TNF-α also inhibits HCV replication. However, this study shows that TNF-α does not affect HCV protein and RNA synthesis, nor does it synergistically enhance the inhibitory effect of IFN-γ. Taken together, these results demonstrate that HCV replication in Huh-7 cells is highly resistant to TNF-α. It is, therefore, unlikely that the increased production of TNF-α, which is seen in many hepatitis C patients, contributes to HCV clearance by inducing antiviral defence mechanisms in infected hepatocytes.

Elevated TNF-α serum levels have been found in patients with hepatitis C (Tilg et al., 1992; Torre et al., 1994). Furthermore, it has been shown that liver infiltrating cytotoxic T lymphocytes and also, to a lesser extent, hepatocytes produce TNF-α during HCV infections (González-Amaro et al., 1994; Koziel et al., 1995; Löhr et al., 1994). To analyse whether TNF-α has a direct antiviral effect on HCV replication, we employed a panel of Huh-7 cell clones containing various subgenomic and genomic HCV replicons (see Fig. 1 for genomic maps of the replicons used in this study). In a first set of experiments, cells containing the bicistronic, subgenomic HCV replicons I377/NS3-3’ or I377/NS2-3’, the monocistronic, subgenomic HCV replicon I389/NS3-3’/Hygubi/5.1 or the bicistronic, full-length HCV genome I350/150-Core/3’/5.1 (Frese et al., 2002; Lohmann et al., 1999, 2001; Pietschmann et al., 2002) were seeded onto glass coverslips and incubated with various doses of up to 10 000 IU recombinant human TNF-α ml⁻¹ (Sigma-Aldrich). After 3 days of treatment, the cells were fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100 and the non-structural protein NS5A was immunostained using the mouse monoclonal antibody...
(mAb) 3924-4940-8858 (Biogenesis) and goat antibodies conjugated to the cyanine dye Cy3 (Dianova). In contrast to previous experiments in which the treatment with IFN-α or IFN-γ inhibited HCV protein synthesis efficiently (Frese et al., 2001, 2002), TNF-α did not affect the expression of NS5A in any of the cell clones tested (Fig. 2A). To substantiate this finding, we treated additional cell clones containing the subgenomic replicon I389/NS3-3’ or the full-length genome I389/Core-3’/5.1 with TNF-α concentrations of up to 100 000 IU ml⁻¹ before analysing the cells for NS5A expression. Again, TNF-α had no effect on the expression of NS5A (data not shown). These results indicate that HCV protein synthesis in Huh-7 cells is not affected by TNF-α.

Next, we analysed whether TNF-α inhibits HCV RNA accumulation. Huh-7 cells containing replicon I377/NS3-3’ or I389/Core-3’/5.1 were seeded at low densities into multiple cell culture dishes. After 3 days of cultivation in the absence of cytokines, the cells were either treated with 5000 IU TNF-α ml⁻¹ for 24 or 48 h or left untreated. Finally, total RNA was prepared and analysed by Northern blotting, as described previously (Frese et al., 2002). We detected similar amounts of HCV RNA in cells treated with TNF-α and in untreated control cells (Fig. 2B, compare lanes 1 and 2 with lanes 13 and 14, respectively), suggesting that TNF-α alone does not affect HCV RNA replication in Huh-7 cells. However, TNF-α has been reported to enhance the antiviral activity of IFN-γ against certain virus infections, even if it has no antiviral activity alone (Feduchi et al., 1989; Lucin et al., 1994; Mayer et al., 1992; Paez et al., 1990; Pasquetto et al., 2002). These findings prompted us to determine whether TNF-α also enhances the inhibitory effect of IFN-γ on HCV replication. Cells containing the replicons I377/NS3-3’ or I389/Core-3’/5.1 were treated with 5, 50 or 500 IU IFN-γ ml⁻¹ (Roche) alone or with a combination of 5000 IU TNF-α ml⁻¹ and 5 or 50 IU IFN-γ ml⁻¹, respectively. Total RNA was prepared and levels of HCV RNA were analysed by Northern blotting. As expected, IFN-γ caused a strong and dose-dependent decrease of

Fig. 1. Maps of HCV replicons. (A, B) The bicistronic, subgenomic replicons I377/NS3-3’ and I377/NS2-3’ are composed of the HCV 5’ non-translated region (NTR) plus nt 342–377 of the core-encoding region, the neo gene (encoding the neomycin phosphotransferase), the IRES of encephalomyocarditis virus (EMCV IRES), the coding region of the HCV non-structural proteins NS3–NS5B or NS2–NS5B (I377/NS3-3’ or I377/NS2-3’, respectively) and the HCV 3’ NTR. (C) The monocistronic, subgenomic replicon I389/NS3-3’/Hygubi/5.1 has the same NTRs as the bicistronic replicons described above but contains only a single open reading frame consisting of nt 342–389 of the core-encoding region, the hyg gene (encoding the hygromycin phosphotransferase), the ubiquitin-encoding sequence (Ubi) and the HCV non-structural proteins NS3–NS5B. (D) The design of the bicistronic, genomic replicon I389/Core-3’/5.1 is analogous to that of the bicistronic, subgenomic replicons described above. The main difference is that the internal EMCV IRES directs the expression of all HCV proteins. (E, F) The characteristic feature of the bicistronic, subgenomic reporter replicons I389/NS3-3’/LucUbiNeo-ET, Luc-ET and Luc-wt/GND is the luciferase gene of the firefly Photinus vulgaris. Note that only I389/NS3-3’/LucUbiNeo-ET contains a marker for selection (neo) and that the GDD motif of the NS5B polymerase in Luc-wt/GND has been mutated to GND. Arrows point to positions of cell culture-adaptive mutations that are each specified below. Furthermore, note that the cell culture-adaptive mutations in replicon I377/NS3-3’ and I377/NS2-3’ are the result of an evolutionary process that took place during the establishment of cell clones 9-13 and 11-7, respectively. In contrast, the cell culture-adaptive mutations in replicon I389/NS3-3’/Hygubi/5.1, I389/Core-3’/5.1, I389/NS3-3’/LucUbiNeo-ET and Luc-ET were introduced by recombinant DNA techniques to enhance RNA replication.
Fig. 2. Effect of TNF-α on HCV protein and RNA synthesis. (A) Immunofluorescence analysis of NS5A expression in Huh-7 cells harbouring the bicistronic, subgenomic replicon I377/NS3-3′ (cell clone 9-13), the bicistronic, subgenomic replicon I377/NS2-3′ (cell clone 11-7), the monocistronic, subgenomic replicon I389/NS3-3′/Hygubi/5.1 (cell clone 43-1) or the bicistronic, full-length replicon I389/Core-3′/5.1 (cell clone 21-5). Cells were seeded onto glass coverslips, cultured for 16 h and incubated for a further 72 h in the presence or absence of given concentrations of TNF-α (IU ml⁻¹). Subsequently, cells were fixed, permeabilized and immunostained using an NS5A-specific mouse mAb. Bars, 50 μm. (B) Northern blot analysis of HCV RNA accumulation in Huh-7 cells harbouring the subgenomic replicon I377/NS3-3′ (cell clone 9-13) or the genomic replicon I389/Core-3′/5.1 (cell clone 21-5). Cells that had been cultivated for 3 days were incubated for a further 24 or 48 h in the absence (lanes 1 and 2) or presence of given concentrations of TNF-α and/or IFN-γ (lanes 3–14). Total RNA was prepared and samples of 10 μg RNA were analysed using a 32P-labelled negative-sense riboprobe complementary to nt 8376–9440 of the HCV sequence and a 32P-labelled riboprobe specific for β-actin mRNAs. Hybridization signals were quantified by phosphoimaging and HCV-specific signals were corrected for the amounts of total RNA loaded in each lane of the gel using the corresponding actin signals. PSL, arbitrary unit.
replicon RNA levels but TNF-α did not further enhance the inhibitory action of IFN-γ (Fig. 2B).

Next we wanted to find out whether TNF-α inhibits HCV replication in a manner so subtle that it is not readily detectable by Northern blot analysis. A very sensitive and precise quantification of HCV replication can be achieved with replicons encoding the firefly luciferase as a reporter gene (Krieger et al., 2001). Here, we used cells of the Huh-7 cell clone 9B that contain the replicon I_{389}/NS3-3′/LucUbiNeo-ET. The cells were seeded into multiple cell culture dishes and treated for 48 h with TNF-α or IFN-γ or a combination of both cytokines. Finally, cells were lysed and luciferase activities were determined. We observed that TNF-α inhibited slightly the reporter gene activity and enhanced the antiviral activity of IFN-γ (Fig. 3A). However, it should be emphasized that the inhibitory effect of TNF-α was very weak compared to that of IFN-γ. For example, treatment with 5000 IU TNF-α ml⁻¹ caused a 2-fold decrease in luciferase activity, whereas as little as 10 IU IFN-γ ml⁻¹ reduced reporter activity by more than 875-fold. A more detailed mathematical analysis of the data revealed no statistically significant evidence that TNF-α and IFN-γ inhibited luciferase activity in a cooperative manner (data not shown). Taken together, these results corroborate the finding that TNF-α has no or almost no effect on HCV RNA replication in Huh-7 cells.

All experiments described so far were performed using individual Huh-7 cell clones that were selected after the electroporation of HCV replicons and which have been cultivated subsequently for several months or even years. It is, therefore, conceivable that the observed resistance of HCV replication towards TNF-α is a consequence of the selection of particular host cells. To test this hypothesis, we analysed the antiviral effect of TNF-α shortly after the transfection of HCV RNAs into parental Huh-7 cells. To that end, we took advantage of the reporter replicon Luc-ET constructed recently. Naive Huh-7 cells were electroporated with in vitro-transcribed Luc-ET RNAs, as described previously (Lohmann et al., 2001), seeded into multiple cell culture dishes and cultivated for 24 h in the absence of cytokines. The cells were then treated for 48 h with different concentrations of IFN-γ and/or TNF-α. Finally, cells were lysed and luciferase activities were measured, as described previously (Krieger et al., 2001). Similar to the experiments with I_{389}/NS3-3′/LucUbiNeo-ET, we found that the

Fig. 3. Effect of TNF-α on HCV-driven luciferase reporter gene expression. (A) Cells of clone 9B, which contained the replicon I_{389}/NS3-3′/LucUbiNeo-ET, were seeded into multiple cell culture dishes, cultured for 24 h in the absence of cytokines and incubated for a further 48 h in the presence of given concentrations of TNF-α and/or IFN-γ. Finally, cells were lysed and luciferase activity was determined. (B, C) Naive Huh-7 cells were transfected with replicon Luc-ET or Luc-wt/GND, respectively. About 24 h after transfection, cells were seeded into multiple cell culture dishes and cultivated for 24 h in the absence of cytokines. The cells were then treated for 48 h with different concentrations of IFN-γ and/or TNF-α. Finally, cells were lysed and luciferase activities were measured, as described previously (Krieger et al., 2001). Similar to the experiments with I_{389}/NS3-3′/LucUbiNeo-ET, we found that the

- [H]-thymidine incorporation
- CPM (10⁴)
- TNF-α (10² IU/ml)
replication of Luc-ET is almost resistant to TNF-\(\alpha\) but highly sensitive to IFN-\(\gamma\) (Fig. 3B). To exclude that the majority of the transfected RNAs is still present 72 h after electroporation, we analysed luciferase activity in cells after the transfection of Luc-wt/GND, a replicon that lacks cell culture-adaptive mutations and more importantly, carries an inactivating mutation in the active site of NS5B. As expected, we detected high levels of luciferase activity shortly after electroporation but only baseline levels thereafter (Fig. 3C). This indicates that most of the incoming HCV RNAs were degraded within 72 h and in case of Luc-ET, have been replaced by newly synthesized replicons. Taken together, these results demonstrate that the resistance of HCV replication towards TNF-\(\alpha\) is not a consequence of host cell selection.

One might argue that the Huh-7 cells used in our experiments have an impaired TNF-\(\alpha\) signalling. To exclude this hypothesis, we measured the activation of NF-\(\kappa\)B, a key event in the cellular response to TNF-\(\alpha\) (Baeuerle & Baltimore, 1996). Naïve Huh-7 cells and those with the replicons I377/NS3-3' or I389/Core-3'/5.1 were seeded into multiple cell culture dishes, cultivated overnight and co-transfected with p(II-6-kB)3-50hu.IL6p-luc+ (kindly provided by G. Haegeman, University of Ghent, Belgium) and pRL-SV40 (Promega) using OptiMEM (Life Technologies) and the FuGENE transfection reagent (Roche). Note that p(II-6-kB)3-50hu.IL6p-luc+ contains the firefly luciferase gene under the control of three NF-\(\kappa\)B response elements preceding a 50 bp TATA box containing the IL-6 promoter fragment (Plaisance et al., 1997), whereas pRL-SV40 encodes the Renilla luciferase gene under the control of the SV40 early enhancer/promoter region. After part of the transfected cells had been treated with 5000 IU TNF-\(\alpha\) ml\(^{-1}\), the cells were lysed and the luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega). The firefly luciferase activities were then corrected for transfection efficiency using the corresponding Renilla luciferase readings and the effect of TNF-\(\alpha\) on NF-\(\kappa\)B activation was calculated. We observed that treatment with TNF-\(\alpha\) led to an approximately 10-fold increase in reporter gene expression, irrespective of whether or not the cells contained HCV replicons, demonstrating that our Huh-7 cells did indeed respond to TNF-\(\alpha\) (data not shown).

We suspected that non-specific cytotoxic effects of TNF-\(\alpha\) might account for the observed inhibition of HCV replication after the treatment with high doses of that cytokine, although TNF-\(\alpha\) alone usually does not induce apoptosis (Bradham et al., 1998). Thus, we decided to quantify the cytotoxicity of TNF-\(\alpha\). Naïve Huh-7 cells were seeded into 96-well plates and treated for 24 or 48 h with different concentrations of TNF-\(\alpha\) (ranging from 5 to 25 000 IU ml\(^{-1}\)). The metabolic activity of the cells was then measured using either a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 (Roche) or, in a more sensitive way, the incorporation of [6-\(\text{H}\)]thymidine (Amersham). Whereas the WST-1 assay did not reveal any cytotoxicity of TNF-\(\alpha\) (data not shown), we did observe a weak decrease in thymidine incorporation with increasing cytokine concentrations. For example, a 48 h treatment with 5000 IU TNF-\(\alpha\) ml\(^{-1}\) reduced thymidine incorporation to 69% of the levels found in untreated control cells (Fig. 3D). These results demonstrate that TNF-\(\alpha\) does not induce massive cell death in Huh-7 cells but still has a subtle inhibitory effect on the rate of cell proliferation. Thus, we think that non-specific cytotoxic effects of TNF-\(\alpha\) at least contribute to the observed inhibition of the HCV-driven reporter gene activity.

It would be interesting to know whether HCV replication in general is resistant to TNF-\(\alpha\) or whether the resistance is host cell-dependent. At the moment, however, this question cannot be answered because Huh-7 cells are the only ones known to support high level HCV replication in cell culture. Nevertheless, there is accumulating evidence that TNF-\(\alpha\) does not inhibit HCV replication in vivo. For example, TNF-\(\alpha\) alleles with a G\(\longrightarrow\)A substitution at position -308 within the promoter region are associated with increased gene transcription and expression (Kroeger et al., 1997; Louis et al., 1998; McGuire et al., 1994; Wilson et al., 1997), but the genetic capacity to produce higher levels of TNF-\(\alpha\) seems not to prevent a persistent HCV infection (Höhler et al., 1998; Rosen et al., 2002). On the contrary, enhanced TNF-\(\alpha\) expression might even favour virus persistence. In a preliminary analysis of 243 Irish women accidentally exposed to a HCV-contaminated immunoglobulin preparation in 1977, the frequency of TNF-\(\alpha\) alleles with an A at position -308 was significantly higher in woman who developed subsequent chronic hepatitis C (43.5%) than in those who got infected but cleared the virus spontaneously (26.3%) (McKiernan et al., 1999).

In summary, our results demonstrate that HCV replication in the human hepatoma cell line Huh-7 is highly resistant to TNF-\(\alpha\). This finding suggests that the increased production of TNF-\(\alpha\) seen in many hepatitis C patients does not contribute to HCV clearance by inducing antiviral defence mechanisms in infected hepatocytes.

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