Hepatitis C virus core protein expression in human B-cell lines does not significantly modify main proliferative and apoptosis pathways

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Hepatitis C virus (HCV) chronic infection has been associated with many lymphoproliferative disorders. Several studies performed on hepatoma and fibroblast cell lines suggest a role of the HCV core protein in activation of cellular transduction pathways that lead to cell proliferation and inhibition of apoptosis. However, no data are available concerning the effects of HCV core expression on B-lymphocyte proliferation and apoptosis. B-lymphocyte cell lines permanently expressing full-length HCV 1b core sequences isolated from chronically infected patients were established using B-cell lines at different degrees of differentiation. Clones and pools of clones permanently expressing the HCV core were selected and characterized for protein expression by Western blot and FACS. Expression of HCV core proteins did not significantly enhance cell proliferation rates under normal culture conditions or under mitogenic stimulation. Analysis of NF-κB, CRE, TRE and SRE pathways by luciferase reporter genes did not show a significant influence of HCV core expression on these signal transduction cascades in B-lymphocytes. The effects of HCV core on anti-IgM and anti-FAS-induced apoptosis in B-cell lines was also analysed. In this experimental model, HCV core expression did not significantly modify the apoptotic profile of the B-lymphocyte cell lines tested. These data underline a cell type-specific effect of HCV core expression. In fact, it was not possible to show a significant contribution of the HCV core protein in activation of the major B-cell signal transduction pathways involved in the regulation of proliferation and programmed cell death, which is in contrast with the results reported in hepatoma cell lines.

Introduction

Hepatitis C virus (HCV) infection frequently results in chronic hepatitis, cirrhosis and hepatocellular carcinoma. Several studies have suggested an association between chronic HCV infection and lymphoproliferative disorders (LPDs), namely mixed cryoglobulinaemia, non-Hodgkin’s lymphoma and monoclonal gammopathies (Abel et al., 1993; Andreone et al., 1998; Ferri et al., 1994; Zignego & Bréchot, 1999). This epidemiological association has been confirmed in some, but not all, areas worldwide, supporting the role of virus and/or host-related factors (Zignego & Bréchot, 1999).

The molecular mechanisms underlying HCV-related LPDs are poorly understood. A cooperation between sustained stimulation of lymphoid cells and modifications involving antiapoptotic mechanisms has been suggested to play a role in the pathogenesis of these HCV-related diseases (Zignego et al., 2000, 2002). Indeed, recent reports showed a significant association between HCV-related LPDs (mostly mixed cryoglobulinaemia) and t(14;18) translocation in peripheral blood mononuclear cells (Kitay-Cohen et al., 2000; Zignego et al., 2000, 2002; Zuckerman et al., 2001), resulting in the overexpression of bel-2 in translocated B-cells. The observation of a transient disappearance of translocated B-cells in peripheral blood following effective antiviral treatment and reappearance of translocated B-cell clones following virological relapse was consistent with a direct implication of HCV replication (Zignego et al., 2002).

One may also hypothesize that HCV proteins could directly affect lymphoid cell proliferation and apoptosis. In fact, HCV has been shown to infect lymphoid cells, preferen-
tially B-subset and monocyte/macrophage populations (Causin-Schwemling et al., 2001; Laskus et al., 2000; Morsica et al., 1999; Zignego et al., 1992). Furthermore, several studies performed on hepatoma and fibroblast cell lines strongly suggest that the HCV core protein can induce a pleiotropic activation of cellular transduction pathways leading to cell proliferation and/or inhibition of programmed cell death (Chang et al., 1998; Giambartolomei et al., 2001; Hayashi et al., 2000; Ray et al., 1995; Shrivastava et al., 1998; Tsuchihara et al., 1999). Nowadays, however, no data are available concerning the effects of HCV core expression on B-lymphocyte proliferation and apoptosis and its potential contribution to the pathogenesis of HCV-related LPDs; in fact, there have been no reports to date that address the effect of the HCV core on B-lymphatic cell signalling under stable expression conditions.

This study aimed to define whether HCV core protein expression in B-lymphocyte cell lines with different degrees of differentiation could influence cell proliferation and/or interfere with major apoptotic pathways, as described previously in hepatoma or fibroblast cell lines.

Methods

**Cell lines.** Ramos and WIL2-NS B-cell lines were obtained from the ATCC. Burkitt’s lymphoma-derived BL2 cell line was described previously (Cohen et al., 1987). Cell lines were cultured at 37 °C in a 5% CO2 atmosphere in RPMI supplemented with 10% foetal calf serum (FCS) and antibiotics (complete RPMI medium).

**Plasmids.** Full-length HCV core sequences (573 nt) were obtained from patients chronically infected with HCV genotype 1b (Delhem et al., 2001) and cloned into the EcoRI/XbaI sites of the pcDNA3.1 vector.

**Proliferation assays.** Cells were starved of serum overnight and then plated into 96-well plates at the density of 2 × 10⁴ cells per well. Drugs were added at the following final concentrations: pokeweed mitogen (PWM), 2.5 µg/ml; phorbol myristate acetate (PMA), 1 µM; lipopolysaccharide (LPS), 0.2 µg/ml; Staphylococcus aureus Cowan strain 1 (SAC), 50 µg/ml; and anti-FAS antibodies (FAS), 0.5 µg/ml.

Cells were incubated with conditioned media for 16 h and then 10 µl of Alamar blue solution (Ahmed et al., 1994) was added. The absorbance of each sample was determined at 600 nm at the indicated time-points. Cell count analysis was performed in triplicate for each time-point after staining with Trypan blue and presented results are the average of three independent experiments.

**Western blot analysis.** Western blot analysis was performed as described previously (Simra et al., 1999). Membranes were hybridized with mouse monoclonal anti-HCV core antibodies (clone 215, Biogenesis) at 0.5 µg/ml in PBS with 0.1% Tween 20 and 5% non-fat milk.

**Electroporation and establishment of permanent HCV core-expressing cell lines.** WIL2-NS, Ramos and BL2 cell lines were electroporated in a Bio-Rad cell electroporator in 800 µl serum-free RPMI medium at the concentration of 5 × 10⁶ cells/ml in the presence of a total amount of 20 µg of plasmid DNA. Pulses were performed at 300 V and 900 µF. To establish stable cell lines expressing HCV core proteins, cells were incubated 48 h after electroporation in complete RPMI medium supplemented with 900 µg/ml G418. In transient expression experiments, cells were cultured after electroporation in complete RPMI or conditioned medium for 24 h before analysis.

**Flow cytometry analysis.** All FACS analyses were performed in a Becton Dickinson FACScan flow cytometer. After treatment with anti-human IgM (2 µg/ml for 24 h) or agonist anti-Fas (0.5 µg/ml for 6 h) antibodies, apoptotic cells were detected by FACS analysis of externalized phosphatidylserine (Annexin V-FITC, BD Pharmingen) and confirmed by morphological analysis of nuclei after DNA staining with 2 µg/ml Hoechst 33258 (Sigma). Cell cycle analysis was performed by propidium iodide staining (40 µg/ml) and FACS analysis of DNA content.

BrdU incorporation and FACS analysis were performed as described previously (Dolbeare et al., 1983), with minor modifications. Briefly, after 1 h of incubation with BrdU (20 µM) in culture medium, cells were fixed in 70% ethanol and probed with an FITC-labelled anti-BrdU antibody (BD Pharmingen), according to the manufacturer’s instructions. Samples were then treated with 1 µg/ml RNase A for 30 min, co-stained with propidium iodide (40 µg/ml) and analysed by flow cytometry.

HCV core protein was identified in 4% paraformaldehyde-fixed lymphocytes using anti-HCV core monoclonal antibodies (clone 215, Biogenesis) at 2 µg/ml for 1 h at room temperature, followed by FITC-labelled goat anti-mouse antibody treatment (1 µg/ml).

Results and Discussion

We established B-lymphocyte cell lines permanently expressing the HCV core protein. We used B-cell lines at different degrees of differentiation, namely WIL2-NS, a human, well-differentiated B-lymphoblastoid cell line, Ramos and BL2 (Cohen et al., 1987), two transformed, Burkitt’s lymphoma-derived, Epstein–Barr virus-negative, human cell lines. To express HCV core in these cell lines, we cloned two different HCV 1b core sequences (coreA1 and coreB2) (Delhem et al., 2001) into the mammalian expression vector pcDNA3.1. Isolated clones and pools of clones were characterized for HCV core expression by Western blot (Delhem et al., 2001). Expression levels were constant all along the study in all B-cell lines (Fig. 1a). FACS analysis on pools of clones revealed that the large majority of cells (> 90%) in WIL2-NS and > 60% in BL2 and Ramos core cell lines) scored positive for anti-core staining (Fig. 1b).

To determine the global phenotypic effect of HCV core expression on B-cell proliferation, a cell count of viable cells on neomycin phosphotransferase (neo) clones and permanently core-expressing clones was performed after Trypan blue staining. Fig. 2(a) shows that, in WIL2-NS and Ramos core cell lines, HCV core protein-expressing cells did not show a growth advantage compared to neo controls during 3 days of culture. Similar results were obtained in BL2 core cell lines and in experiments performed under serum-free culture conditions (data not shown).

BrdU incorporation was also carried out to evaluate the cell cycle and replication rate of B-cell core-expressing clones and controls. HCV core protein-expressing cell lines showed a cell cycle profile and replication rate not significantly different from neo clones in all cell lines tested (Fig. 2b and data not shown). To define if the HCV core protein could cooperate with
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Fig. 1. HCV core protein expression in B-lymphocyte cell lines. (a) Western blot analysis of protein extract from WIL2-NS (left), Ramos (middle) and BL2 (right) cells after electroporation with HCV core-expressing plasmid and G418 selection. (b) FACS analysis of WIL2-NS (left), Ramos (middle) and BL2 (right) pools of selected clones expressing HCV coreB2 after immunostaining with anti-HCV core or control antibodies (rabbit anti-mouse immunoglobulins) followed by FITC-conjugated goat anti-mouse treatment. Histograms correspond to anti-core-labelled cells; light grey shadows correspond to control antibody-treated cells.

mitogen stimulation, a viable cell growth assay (Alamar blue cell proliferation assay) (Ahmed et al., 1994) was carried out in core-expressing cells and controls using various lymphocyte mitogens. Expression of the HCV core protein did not significantly enhance cell proliferation rates under normal culture conditions or under mitogen stimulation (Fig. 2c and data not shown).

Igk-luciferase (NF-κB), CRE-luciferase (cyclic-AMP-responsive element), TRE-luciferase (12-O-tetradecanoyl-13-acetate-responsive element) and SRE-luciferase (serum-responsive element) reporter plasmids were used to analyse the effects of the HCV core on main cellular signal transduction pathways in core and neo stable B-cell clones and by transient ectopic expression experiments in BL2, WIL2-NS and Ramos cell lines (Fig. 3 and data not shown). All reporter constructs tested were activated by PMA stimulation (TRE-luciferase, CRE-luciferase and Igk-luciferase) or serum (SRE-luciferase); in contrast, HCV core expression did not significantly modify these signal transduction cascades in B-lymphocytes under either stable or transient expression (Fig. 3 and data not shown).

Since lymphoproliferation may also reflect a reduced clearance of B-cell populations, we analysed the effects of HCV core on anti-IgM and FAS-induced apoptosis in B-cell lines (Cohen, 1991; Hartley et al., 1993; Scaffidi et al., 1999).

Related to a more differentiated phenotype (IgM\textsuperscript{low}IgD\textsuperscript{high}), WIL2-NS-derived cell lines were less sensitive to anti-IgM-induced apoptosis when compared to Ramos and BL2 cell lines (Table 1). In contrast, agonist anti-FAS antibody treatment induced massive apoptosis in the WIL2-NS cell line (Table 2). BL2-derived cell lines showed a strong resistance to anti-FAS-induced apoptosis due to the almost absent expression of FAS molecules on the cell surface, while Ramos-derived cell lines presented an intermediate phenotype (Table 2). Under our experimental conditions, HCV core expression did not significantly modify the apoptotic profile of all B-lymphocyte cell lines tested.

The present study demonstrates that, under our experimental conditions, the HCV core protein does not increase cell proliferation or modify cell cycle or apoptosis profiles when expressed either stably or transiently in B-cell lines at different degrees of differentiation.

Previous reports, mostly obtained by transient expression experiments in hepatoma cell lines, depicted the HCV core protein as a pleiotropic activator of many cellular pathways regulating cell growth and apoptosis (Aoki et al., 2000; Chang et al., 1998; Fukuda et al., 2001; Giambartolomei et al., 2001; Hayashi et al., 2000; Ray et al., 1995; Shrivastava et al., 1998; Tsuchihara et al., 1999; You et al., 1999). The HCV core
protein has been also implicated in transformation of primary cells and inhibition of programmed cell death (Jin et al., 2000; Marusawa et al., 1999; Ray et al., 1996a, b). However, published data concerning the biological effects of the HCV core are often contradictory or ambiguous, depending on the experimental conditions and cell type used (Chang et al., 1998; Dumoulin et al., 1999; Honda et al., 2000; Ray et al., 1996a, b; Ruggieri et al., 1997; Zhu et al., 1998). In this report, we used three different B-cell lines, each characterized by a distinct differentiation status. We expressed two different HCV 1b core proteins that previously showed dissimilar biological effects concerning interferon signalling and apoptosis when ectopically expressed in hepatoma cell lines (Delhem et al., 2001). To avoid clonal diversity, we analysed pools of clones with homogeneous and constant levels of HCV core expression; we also checked some isolated clones that did not behave differently from polyclonal populations (data not shown). To test whether expressing higher levels of protein might modify our results, we also transiently expressed HCV core proteins in three B-lymphocyte cell lines and did not observe significant transactivation effects on our reporter constructions (data not shown).

Taken together, our results do not support a direct action of HCV core protein on cell growth and apoptosis pathways in B-lymphocytes. Along this line, a recent study failed to show any immunomodulatory effects induced by HCV core protein expression both in vitro and in vivo (Liu et al., 2002). In addition, other reports provided some evidence supporting an indirect action of HCV in the pathogenesis of LPDs. In fact, a significant association between HCV-related LPDs (mostly mixed cryoglobulinaemia) and t(14;18) chromosomal translocation in peripheral blood B-lymphocytes has been demon-
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Fig. 3. Effect of HCV core expression on major cellular signal transduction pathways in HCV core-expressing B-lymphocyte cell lines. WIL2-NS pools of stable HCV core-expressing clones were co-transfected by electroporation with pCMV-LacZ plasmid and the following reporter plasmids: Igκ-luciferase (a), CRE-luciferase (b), SRE-luciferase (c) and TRE-luciferase (d). Ordinate axes represent relative luciferase units (RLU) normalized with β-galactosidase activity.

Table 1. Percentage of cells undergoing anti-IgM-induced apoptosis in HCV core-expressing cell lines

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<th>10% FCS</th>
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<td></td>
<td>Control (%)</td>
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</table>

Table 1. Percentage of cells undergoing anti-IgM-induced apoptosis in HCV core-expressing cell lines

HCV core-expressing cell lines were incubated in medium containing either low (0.5%) or high (10%) levels of FCS. The percentage of cells undergoing anti-IgM-induced apoptosis was determined in each HCV core-expressing cell line and controls. Control antibodies were rabbit anti-mouse antibodies.

strated (Zignego et al., 2000; Zuckerman et al., 2001). Juxtaposition of the bcl-2 gene to the IgH locus results in the overexpression of bcl-2 in translocated cells. Their prolonged survival could increase the risk of additional genetic modifications (second hit) and development of a malignant phenotype. The observation of a transient disappearance of translocated B-cells in peripheral blood following effective antiviral treatment (Zignego et al., 2002; Zuckerman et al., 2001) and the
reappearance of translocated B-cell clones following virus-induced relapse strongly suggest that these expanded clones need the persistence of circulating HCV particles to be maintained (Zignego et al., 2002). In addition, we recently showed the activation and increase in cell growth in WIL2-NS cells induced by CD81 cross-linking (Mazzocca et al., 2000), suggesting that the interaction of HCV proteins with the putative virus receptor could contribute to the abnormal expansion of B-cell populations frequently reported during chronic HCV infection.

In conclusion, our results underline a cell type-specific effect of HCV core expression. In fact, under our experimental conditions, we were not able to show a significant contribution of HCV core protein in activation of major B-cell signal transduction pathways involved in the regulation of proliferation and programmed cell death, which is in contrast with the results reported in hepatoma and fibroblast cell lines. Thus, although we cannot exclude a direct contribution of HCV proteins when expressed in the context of the whole viral genome and a potentially different effect on primary B-cells, HCV proteins seem more likely to influence B-cell proliferation by an extracellular stimulation rather than a direct activation of cellular signal transduction pathways that regulate cell proliferation and apoptosis.

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### Table 2. Percentage of cells undergoing anti-Fas-induced apoptosis in HCV core-expressing cell lines

HCV core-expressing cell lines were incubated in medium containing either low (0.5%) or high (10%) levels of FCS. The percentage of cells undergoing anti-Fas-induced apoptosis was determined in each HCV core-expressing cell line and controls. Control antibodies were rabbit anti-mouse antibodies.

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### References


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