Effect of Hepatitis C Virus Core Protein on the Molecular Profiling of Human B Lymphocytes

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Hepatitis C virus (HCV) core protein features many intriguing properties and plays a pivotal role in cellular immunity, cell growth, apoptosis, cell transformation, and eventually in tumor development. However, the role of B cells, the primary players in the humoral immune response, during HCV infection is largely unknown. To explore the molecular effects of HCV core on human B cells, we conducted gene expression profiling of serial RNA samples from B cells that were infected with adenovirus harboring full-length HCV core protein and β-galactosidase as a reference using a microarray platform containing 22,149 human oligo probes. The entire experiment was performed in duplicate in B lymphocytes that were isolated from two individual donors and incubated for up to 3 days after infection with adenovirus expressing HCV core protein to identify dynamic gene expression patterns. Differential expression of representative genes was validated by quantitative RT-PCR. We found that HCV core significantly inhibited B-lymphocyte apoptosis. We showed a dramatic downregulation of MHC class II molecules in B cells expressing HCV core, whereas the expression of immunoglobulin genes was not significantly altered. Moreover, genes associated with leukemia and B-lymphoma were consistently upregulated by HCV core. In contrast, downregulation of caspase-1 and caspase-4 was found to be associated with core's ability to prevent B-lymphocyte apoptosis. In summary, we have identified several clusters of genes that are differentially expressed in human B lymphocytes expressing HCV core, suggesting a potential impairment of antigen processing and presentation, which may provide more insights into HCV infection in B lymphocytes.

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INTRODUCTION

Hepatitis C virus (HCV), with 1.8% prevalence of infection in the United States and 170 million worldwide (1), is a major cause of cirrhosis and potentially leads to hepatocellular carcinoma (HCC). The incidence of HCC is increasing in North America, Europe, and Japan, largely because of the high rates of chronic HCV infection (2). HCV replicates in T lymphocytes and suppresses T-cell proliferation and cytokine production (3). For instance, in patients with chronic HCV infection, the frequencies of antiviral CTLs are relatively low (4), and the proliferative response of HCV-specific $CD8^+$ T cells is impaired (5). In addition, the production of Th1-type cytokines (i.e., IL-2 and IFN-γ) is dramatically suppressed in peripheral T cells of chronic HCV patients (6,7). These observations suggest that HCV chronic infection may be the result, at least in part, of an inability to mount effective T-lymphocyte responses, indicating that HCV gene products might be involved in modulating or suppressing host immune responses. In comparison, the evidence of HCV infection in B cells both in vivo and in vitro has been controversial; recent studies have shown that HCV can infect and replicate in B cells from HCV-infected patients (8), which suggests the direct pathological effect of HCV on B cells.

HCV infection is associated with B-cell lymphoproliferative disorders, including mixed cryoglobulinemia, usually a benign condition, and overt B-cell lymphoma (9,10). Epidemiological studies suggest that HCV infection may play a direct role in the genesis of B-cell lymphoproliferative disorders, and clonal B lymphocytes are frequently detected in the blood and liver of patients with chronic HCV infection (11). Furthermore, antiviral treatment for HCV is associated with the regression of B lymphoma

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(12,13). Nevertheless, how HCV induces B-cell lymphoproliferative disorders and whether HCV core plays any role in B-cell immunity is still unclear.

Among the viral proteins encoded by HCV, core protein is the first to be synthesized during the early phases of HCV infection. It can influence cellular immunity, cell growth, apoptosis, cell transformation, and eventually tumor development (hepatocellular carcinoma and possibly B lymphoma) (14). Furthermore, whether HCV core protein plays any role in gene deregulation in B cells and whether HCV core exerts any molecular effects on apoptosis, immunoglobulin production, or antigen-processing need to be addressed.

In this study, we performed a systematic analysis for gene expression profiling in primary human B lymphocytes overexpressing HCV core protein from an adenoviral transfection construct. A total of 22,000 human expression features were analyzed with a modified version of GoMiner, a recently developed quantitative and statistical gene ontology software package with a built-in control on false discovery rate (FDR) (15). This approach identified significant changes in gene expression in several ontology categories, including apoptosis, leukemia/lymphoma, antigen processing, and antigen presenting. These results suggest that HCV core protein may play a role in B-cell escape from apoptosis and thus contribute to the development of B-cell lymphoma, and may impair antigen processing and presentation during HCV infection in B cells.

MATERIALS AND METHODS

Construction of Flag-HCV Core Protein

Because the availability of antibodies is limited, the flag-tagged HCV protein was generated from PCR amplification of the appropriate regions of pCV-J416S, an infectious cDNA clone of HCV genotype 1b, followed by restriction digesting and subcloning into pCMV-TAG. The Flag-tagged construct was inserted into the adenoviral expression vector pZERO-TG (designated as Ade-core). Adenovirus generation and purification were conducted at the Massey Cancer Center Virus Vector Shared Resource, Virginia Commonwealth University (Richmond, VA, USA). Likewise, the β-galactosidase and HCV-NS3 (an HCV nonstructural protein) were generated in adenovirus to be used as control constructs (designated as Ade-β-gal and Ade-NS3, respectively). Viral amplification was conducted by the Gene Therapy Center Virus Vector Core Facility, University of North Carolina (Chapel Hill, NC, USA) as previously described (16).

Human B-Cell Preparation and Viral Infection

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood obtained from normal non-atopic donors (approved and provided by The Department of Transfusion Medicine at NIH) by density-gradient centrifugation on Ficoll Hypaque (Amersham Pharmacia Biotech). Cells were then washed and resuspended in RPMI 1640 containing 10% heat-inactivated fetal calf serum (JRH Biosciences), 2 mM L-glutamine, 50 μg/mL streptomycin, and 100 units/mL penicillin (complete medium). Purified B-cell populations were isolated from PBMCs using a positive magnetic sorting system (Miltenyi Biotec, Auburn, CA, USA) with magnetic beads conjugated to CD19 according to the manufacturer's instructions. The purity of B cells was detected with FACS analysis using anti-CD20 antibody (Miltenyi Biotec). Cell viability assessment of freshly isolated primary B cells from donors was examined by Trypan blue dye exclusion. Twenty million B cells were infected with viral stocks of Ade-core, Ade-NS3, or Ade-β-gal at 50 times multiplicity of infection (MOI). Total RNA was extracted 24, 48, and 72 h after viral infection using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The entire experiment was conducted in duplicate using B lymphocytes derived from two donors.

Apoptotic Cell Death Assay

To quantify apoptosis, cultured B cells were double-stained with Annexin V-FITC conjugate and propidium iodide (PI) using TACS Annexin V Kits from Oncogene (San Diego, CA, USA), according to the step-by-step protocol as provided by the manufacturer, and immediately analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Briefly, 0.5 million B cells were incubated in annexin binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$) and stained with annexin V-FITC for 10 min on ice in the dark. Apoptotic cells were counted on a flow cytometer using a dual filter set for FITC and PI. Early apoptotic cells were positive for annexin V-FITC conjugate but did not stain with PI because their membranes were still intact. Late-stage apoptotic cells or dead cells that had damaged permeable plasma membranes stained concurrently with annexin V-FITC conjugate and PI. For data analysis, FlowJo software (Tree Star) was used.

Western Blot

After RNA isolation, protein was isolated according to the manufacturer's protocol (Invitrogene). Protein concentration was determined by the Bradford Assay according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA). An identical amount of total protein was loaded in each lane of a 16% PAGE gel, followed by transferring and blocking in 5% nonfat dry milk. Blots were probed with anti-actin followed by incubation with horseradish peroxidase–conjugated secondary antibody. Membranes were stripped and reported with anti-HA antibody (Roche, Indianapolis, IN, USA) to detect HCV-core expression. Antibody-antigen complexes were detected by enhanced chemiluminescence according to the manufacturer's protocol (Amersham, Piscataway, NJ, USA).

Oligo microarray

Human operon oligonucleotide chips (v. 2.1, Qiagen Human Array-Ready Oligo Set) were generated by the

National Cancer Institute (Bethesda, MD, USA) microarray facility at the Advanced Technology Center. The oligo array platform contains 22,149 70-mer probes. Detailed hybridization, quality control, data acquisition, and filtering were performed as previously described (17). In a pilot experiment, we had conducted both forward and reverse labeling of Cy3/Cy5 and Cy5/Cy3 dye as a standard procedure. Scatterplot analysis of the ratio of gene expression in forward and reverse labeling of control samples was shown to be highly correlated and ensured dye labeling specificity. For each experiment, fluorescent probes were therefore prepared by an indirect labeling approach of a reference RNA from B cells infected with Ade-β-gal (Cy5), and B cells infected with Ade-core were labeled with Cy3. All microarray data analyses were performed in duplicate in B cells from two donors.

TaqMan Analyses

Total RNA prepared from B cells was used to perform microarray analyses and to monitor cellular RNA by quantitative reverse transcription PCR (qRT-PCR). PCR primers and probes (Assays on Demand) were purchased (Applied Biosystems, Foster City, CA, USA), and qRT-PCR reactions were performed according to the manufacturer's instructions. For each time point of gene detection, an average of four replicate reactions was calculated. Human 18S RNA labeled with VIC reporter dye was used as an endogenous control for normalization in validation assays. Reactions were performed with the ABI PRISM 7700 Sequence Detector System (Applied Biosystems).

Analysis and Statistics

The percentage of apoptotic B cells after Ade-core, Ade-NS3, and Ade-β-gal infection was compared and analyzed with Pearson χ^2 test. For microarray quantification, data processing and cluster analyses were conducted as described (http://nciarray.nci.nih.gov). A class comparison based on univariate *F* tests at a significance level of $P < 0.005$ was used to find genes differentially expressed among various time points. Significant genes were included in the GoMiner analysis, developed by Zeeberg et al. (15), which was performed using a modified program with a built-in control of false discovery rate at $P < 0.05$.

RESULTS

Generation and Expression of Adenovirus Harboring HCV Core Protein and β-Galactosidase

Because human B cells have very low proliferation capacity, adenoviral vectors harboring HCV core protein, HCV NS3, and β-galactosidase were generated to ensure high efficiency of transgene expression in primary B cells. To optimize infection conditions, B cells were infected with Ade-core, Ade-NS3, and Ade-β-gal at MOIs ranging from 5 to 100 for 24 h. An MOI of 50 resulted in efficient expression of HCV core encoded genes as determined by Western blot at 48 h as shown in Figure 1, and the same viral dose was used in further experiments for up to 72 h. The entire experiment was conducted in duplicate in B cells isolated from two normal donors. B-cell purity reached more than 90% as detected by FACS analysis using anti-CD20 antibody (data not shown).

Decreased Apoptotic Cells in HCV Core–Infected B Cells

During 3 days of viral infection with Ade-core, Ade-NS3, and Ade-β-gal in primary B cells, increased apoptotic cells were observed in a time-dependent manner as seen in Figure 2. The percentage of apoptotic cells, especially of the control cells carrying Ade-β-gal, began to rise from 27.9% on day 1 to 44.6% on day 2 and 58.6% on day 3. Likewise, the increase of apoptotic cells harboring HCV-NS3 was similar to the control cells, with up to 64.9% of apoptosis on day 3. In comparison, the cell death of those cells carrying Ade-core was only 27% on day 2 and 35.1% on day 3. The difference of cell death between B cells infected with Ade-β-gal or Ade-NS3 and those with Ade-core became statistically significant ($P < 0.01$ and $P < 0.001$) on days 2 and 3, respectively.

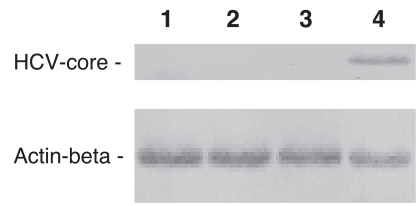

Figure 1. Western blot analysis for HCV core protein expression in human B cells 48 h after introduction with no adenovirus (lane 1) and with adenovirus harboring β-galactosidase (lane 2), HCV NS3 (lane 3), and HCV core (lane 4). Relative amount of total protein of each sample was normalized by β-actin.

Differentially Expressed Genes

Microarray was conducted and analyzed with serial RNA samples from infected B cells isolated from two donors. The B cells were infected with Ade-core and Ade-β-gal over 3 days. As shown in Figure 3, 216 genes were differentially expressed, with a univariate *P* value of < 0.005 and with a greater than 2-fold ratio changes in at least two arrays. Among them, most (90%) genes appeared to be either upregulated (genes shown in red) or downregulated (genes shown in green) on the first 2 days of infection with Ade-core versus Ade-β-gal. These include the cluster of genes related to myeloid/lymphoid leukemia, B lymphoma, transcription regulation, apoptosis, and potential modulators of immune cell activities.

Validation of Microarray Data

To validate the microarray data, six representative genes were chosen to verify the gene expression levels and patterns using qRT-PCR (Figure 4). The quantitations of gene expression were highly reproducible among the duplicate experiments throughout 3 days of viral infection. As shown in Figure 4, when CASP4 was downregulated in

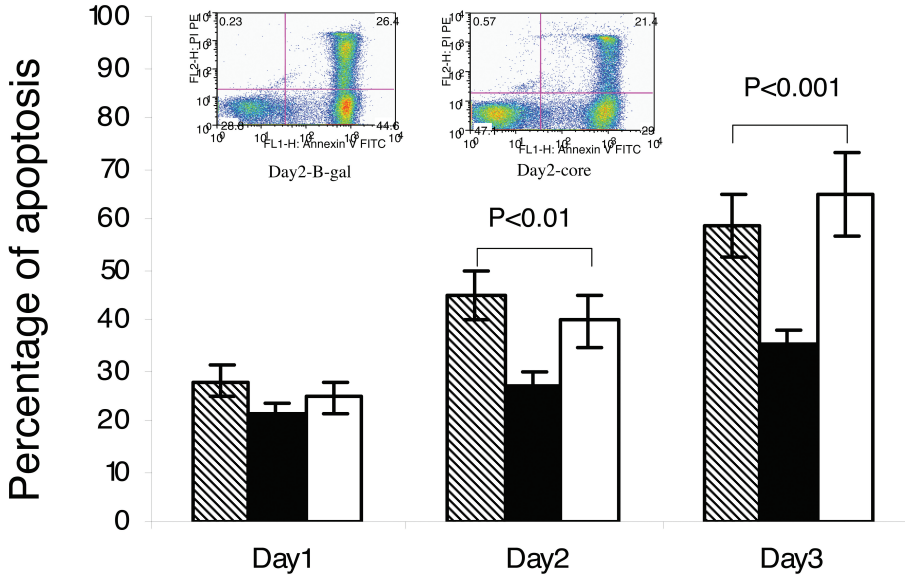

Figure 2. Percentage of apoptotic B cells during 3-day viral infection with adenovirus harboring β-galactosidase (striped bar), HCV core (solid bar), and HCV NS3 (open bar). Data were calculated with average value of duplicate experiments in B cells derived from two donors. Inset represents FACS analysis of annexin V– and IP-stained B cells 2 days after infection of Ade-β-gal (left) and Ade-core (right).

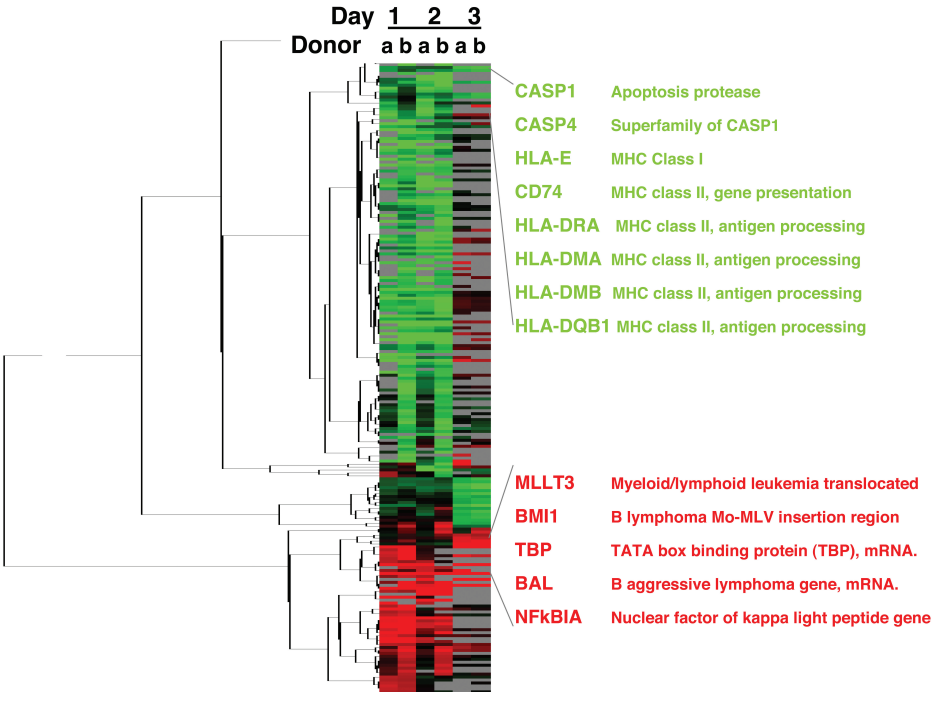

Figure 3. Hierarchical analyses of 216 differentially expressed genes from duplicate experiments in B cells derived from two donors (A and B) as described in Figure 1. The upregulated genes are shown in red and the downregulated ones in green. The representative genes are listed.

microarray analysis from day 1 to day 3 of the experiment, the duplicate qRT-PCR after being standardized with 18S RNA showed the exact same patterns. Likewise, when MLLT3 was upregulated as detected by microarray during viral infection up to day 3, qRT-PCR revealed the identical pattern. The same was true with four other genes tested using qRT-PCR despite some discrepancy in terms of fold of gene expression level compared with microarray analysis (Figure 4). The overall correlation of gene expression for each of the six genes after normalization with 18S RNA between microarray and qRT-PCR was highly significant ($R^2 = 0.86$, $P < 0.01$).

Identification of the Gene Clusters Affected by Core Protein

Genes related to leukemia or lymphoma. Among the most differentially expressed genes, several of them related to myeloid/lymphoid leukemia and B lymphoma, such as MLLT3, BAL, and BMI1, exhibited the greatest enhancement. The microarray analysis showed that MLLT3 expression increased at the first day of experiment and continued to increase up to more than 10 fold. The expression pattern was confirmed by duplicate quantitative RT-PCR. Similarly, BAL, a B-lymphoma related gene, started going up on the second day of infection as detected by both microarray and quantitative RT-PCR.

Immunoglobulin genes. Because HCV is frequently associated with mixed cryoglobulinemia, it was noteworthy to see if any changes in immunoglobulin genes occur during early viral infection with HCV core protein. We examined the entire immunoglobulin gene families, and found that none of them showed any significant changes in transcription levels throughout the 3 days of Ade-core infection in duplicate experiments (data not shown).

Apoptosis genes. To pursue the cause of decreased cell death during Ad-HCV core infection in B cells, we examined the transcriptional levels of all apoptosis-related genes. Among the genes down-

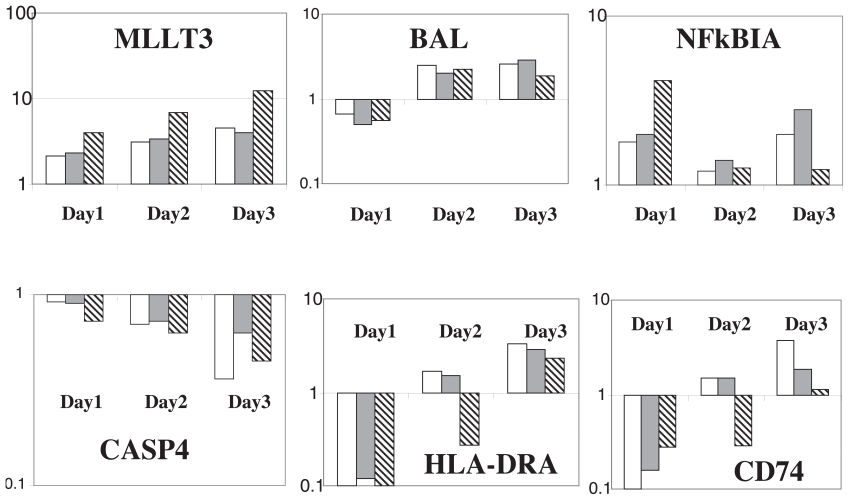

Figure 4. Comparison of fold changes in gene expression of six representative genes between duplicate quantitative RT-PCRs after normalization with 18S RNA (open and gray bars) vs. the mean value from two microarray experiments (hatched bar) during 3-day viral infection. The fold change is expressed on a logarithmic scale. If a gene expression value is greater than 1, the gene is upregulated; if less than 1, the gene is downregulated.

regulated by HCV core protein, CASP4 was shown to be suppressed more than 2 fold as early as the first day, and sustained through day 3. CASP1 showed a gene expression pattern similar to that of CASP4, especially in the first 2 days. In contrast, changes in other apoptosis genes including CASP2, CASP3, CASP7, CASP8, and CASP10, as well as BCL2, BAD, and Granzyme B, were not detected (data not shown).

Transcription regulator genes. Among the early response genes, several transcription elements were identified in the first day of HCV core infection. NF.BIA, a nuclear factor of κ light peptide inhibitor gene, and TBP, a TATA box binding protein, appeared to be upregulated by more than 4 fold (Figure 3), whereas other genes, such as STAT1, were downregulated at the beginning of HCV core infection (Data not shown).

GoMiner Data Analysis

With the advent of GoMiner, a resource for biological interpretation of genomic and proteomic data, we were able to explore and display the major gene family of MHC class I molecules, and especially MHC class II molecules among significant genes detected in microarray. As shown in Figure 5, all MHC class II molecules including HLA-DMA, HLA-DMB, HLA-DQB1, and HLA-DRA, together with CD74, an MHC class II molecule related gene, were found to be dramatically suppressed on the first day of Ade-core infection even though some B cell–specific molecules like CD79B were somewhat upregulated. As two of six representative genes chosen for validation with quantitative RT-PCR, HLA-DRA and CD74 appeared to be as much as 10-fold downregulated in both microarray and qRT-PCR analyses on day 1 (Figure 4).

DISCUSSION

High-throughput genomic studies have been employed to investigate gene profiling associated with hepatitis virus infection, thereby providing more insights into the molecular mechanism of viral infection (16,18). More recently, a number of microarray analyses have been performed in chimpanzees and in patients infected with HCV virus in an attempt to identify specific gene expression profiling and potential markers (10,19). To our present knowledge, however, the role of HCV, and in particular HCV core protein, in regulation of gene expression in human primary B cells has not been studied by microarray analysis. To systematically examine the molecular effects of HCV core protein on regulating host gene expression in primary human B cells, we have performed microarray and GoMiner analyses for gene expression profiling in the human B lymphocytes that were infected with adenovirus harboring HCV core protein in a time-dependent manner using oligo microarray covering nearly 22,000 human oligonucleotides.

Infection of primary human B cells with an adenoviral vector expressing HCV core protein significantly reduced B-cell death in a time-dependent manner compared with control vectors expressing β-gal or HCV-NS3, an essential HCV

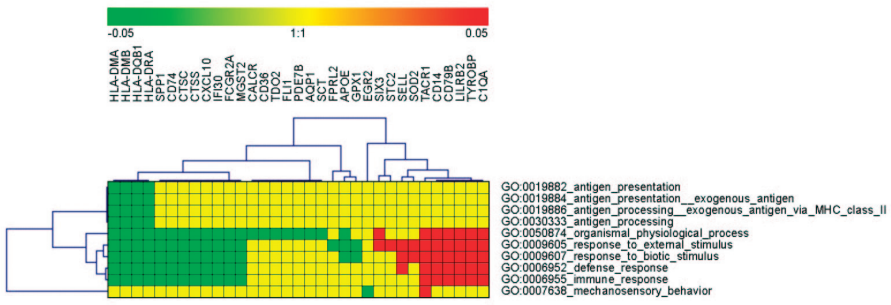

Figure 5. GoMiner analyses of cluster genes associated with antigen processing and presentation 1 day after HCV core infection. The 2-dimensional presentation is visualized for the genes related to their biological process and function. The bar indicates the genes downregulated in green and upregulated in red.

nonstructural helicase protein for viral replication, suggesting that HCV core protein may specifically rescue B lymphocytes from cell death. To identify candidate B-cell genes that might be involved in core-mediated inhibition of apoptosis, we conducted microarray analyses with serial RNA samples from infected B cells isolated from two donors. Under stringent selection criteria, 216 differentially expressed genes were clustered based on significance ($P < 0.005$) and greater than 2-fold ratio changes in at least two arrays. Most genes appeared to be either upregulated or downregulated after 2 days of core expression, indicating that HCV core can modulate cellular gene expression in host B cells. Among the genes downregulated by HCV core protein, CASP4, a member of the CASP1 superfamily, was suppressed more than 2 fold as early as the first day, and this suppression was sustained through day 3 of the experiment. Similarly, CASP1 showed the same pattern as CASP4, especially in the first 2 days. In comparison, changes in other caspase genes including CASP2, CASP3, CASP7, CASP8, and CASP10, as well as BCL2, BAD, and Granzyme B, were not detected. These results suggest that HCV core may rescue B cells from apoptosis possibly through suppression of CASP1 and CASP4. It is plausible that modulation of apoptosis may involve binding of HCV core protein to the intracellular signal transducing portion of death receptors and displacement of signaling molecules. Hence, monitoring caspase activation might provide a reliable tool to estimate the efficacy of HCV therapy, and might open challenging therapeutic strategies in HCV infection (20). Whether these changes in gene expression are due to HCV core's transactivation of cellular promoters, including NF-κB and AP-1, or due to its interaction with c-JNK and MAPK signaling as previously indicated (21), still needs to be fully investigated.

qRT-PCR was used to verify and quantify the microarray data of six representative genes, chosen by their gene expression levels, patterns, and biological functions. Quantification of gene expression levels and patterns were highly reproducible among the duplicate experiments throughout the 3 days of viral infection. The overall correlation of the six genes, standardized with 18S RNA, was highly significant between microarray and qRT-PCR analyses ($R^2 = 0.86$, $P < 0.01$) despite some discrepancy in terms of fold of gene expression level. These results demonstrated that microarray analyses performed in this study using oligo chips made at NCI are valid, although microarray is not adequately quantitative. This observation is also consistent with similar studies from others (22).

Several genes related to myeloid/lymphoid leukemia and B lymphoma were among the most enhanced by expression of core protein. MLLT3 is involved in translocations associated with both acute lymphoblastic and acute myelogenous leukemia (23). BAL (B-aggressive lymphoma) is a novel risk-related gene in diffuse large B-cell lymphomas that enhances cellular migration. A recent study showed that stable BAL-overexpressing B-cell lymphoma transfectants had significantly higher rates of migration than vector-only transfectants, indicating that the risk-related BAL gene promotes malignant B-cell migration (24). Further elucidation of the deregulation of these genes in B cells as an early molecular event following HCV infection, and the potential underlying mechanism, may be important to the development of novel therapeutics for HCV-associated B-cell lymphoma.

HCV is frequently associated with mixed cryoglobulinemia (25,26), raising the possibility that HCV core may influence the regulation of immunoglobulin genes in B cells. Surprisingly, no member of the entire immunoglobulin gene family showed significant transcriptional changes throughout 3 days of Ade-core infection. These results suggest that overproduction of immunoglobulin in HCV-related B-lymphocyte disorders may require other viral HCV proteins that possibly contribute to the regulation in vivo. Alternatively, other cell types, for example, T cells or macrophages, may activate B cells and eventually lead to the overproduction of immunoglobulin in patients. Moreover, most cryoglobulinemia is related to B-lymphocyte proliferation, which we did not observe in this study.

GoMiner is a useful software package that organizes lists of up- and downregulated genes for biological interpretation in the context of the Gene Ontology (15). With the advent of GoMiner, we were able to explore and display the transcriptional expression of the major gene family of MHC class I molecules, and especially MHC class II molecules, from thousands of genes detected in microarray. All MHC class II molecules including HLA-DMA, HLA-DMB, HLA-DQB1, and HLA-DRA, together with CD74, an MHC class II molecule related gene, were suppressed on the first day of Ade-core infection. The data were verified by quantitative RT-PCR, and both HLA-DRA and CD74 appeared to be as much as 10-fold downregulated in both microarray and TaqMan RT-PCR analyses. Our recent FACS analysis indicates that HCV core also downregulates HLA class II protein expression (unpublished data). These results suggest that HCV core inhibits MHC class II molecules, thereby impairing antigen-processing and presentation. Thus, it is plausible that HCV core protein modulation of MHC class II expression and antigen presentation to $CD4^+$ T cells provides HCV with a means of avoiding early immune recognition.

Our novel finding is in agreement with a recent study demonstrating reduced expression of MHC class II molecules in human cytomegalovirus-infected macrophage cultures from 90% of the donors (18). Human cytomegalovirus uses different mechanisms to decrease the expression of MHC class II molecules on infected macrophages either independent of or dependent on viral replication. Moreover, CMV-infected macrophages exhibited a 66% to 90% reduced capacity of stimulating an antigen-specific prolif-

erative $CD4^+$ T-cell response (27). It is generally accepted that genes within the major histocompatibility complex play a central role in the development of the immune response against HCV. MHC class II molecules may be important for viral clearance, because particular alleles are associated with chronic HCV infections in patients, and MHC class II molecules restrict HCV-specific CTL responses (28,29). Furthermore, inhibition of MHC class II molecules, and therefore inhibition of antigen processing and presentation, may account for delayed development of neutralizing antibodies against HCV, as observed both in animal experiments and in HCV-infected patients (30,31). Moreover, MHC class II molecules can influence T-cell function, because HCV core binds directly to gC1qR on $CD4^+$ and $CD8^+$ T cells (32,33). Future studies directed at the mechanism of suppression of MHC class II molecules and regulation of T cell function by HCV core protein may identify new approaches for therapeutic regulation of HCV infection.

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