Hepatitis C virus (HCV) putative core protein has displayed many intriguing biological properties. Since tumor necrosis factor (TNF) plays an important role in controlling viral infection, in this study the effect of the core protein was investigated on the TNF-α induced apoptosis of human breast carcinoma cells (MCF7). HCV core protein when expressed inhibited TNF-α-induced apoptotic cell death unlike the control MCF7 cells, as determined by cell viability and DNA fragmentation analysis. Additionally, HCV core protein blocked the TNF-induced proteolytic cleavage of the death substrate poly(ADP-ribose) polymerase from its native 116-kDa protein to the characteristic 85-kDa polypeptide. Results from this study suggest that the HCV core protein plays a role in the inhibition of TNF-α-mediated cell death. Thus, the ability of core protein to inhibit the TNF-mediated apoptotic signaling pathway may provide a selective advantage for HCV replication, allowing for evasion of host antiviral defense mechanisms.

Hepatitis C virus (HCV) is an important cause of morbidity and mortality worldwide, causing a spectrum of liver disease ranging from an asymptomatic carrier state to end-stage liver disease. The most important feature of persistent HCV infection is the development of chronic hepatitis in half of the infected individuals and the potential for disease progression to hepatocellular carcinoma (1). Unfortunately, a number of important issues related to HCV-mediated disease progression are unknown at this time. An HCV genome contains a linear, positive-strand RNA molecule of ~9,500 nucleotides encoding a single polyprotein precursor of ~3,000 amino acids (2). The polyprotein is cleaved by both host and viral proteases (3, 4) to generate three putative structural proteins (core, E1, and E2) and at least six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The genomic region encoding the putative core protein is located between amino acids 1–191. HCV core protein may be the fundamental unit for the encapsidation of genomic RNA to facilitate virus morphogenesis. However, in vitro studies suggest that the HCV core protein has many additional biological properties. The core protein transactivates the human c-myc proto-oncogene and unrelated viral promoters and suppresses c-fos, p53, and human immunodeficiency virus type 1 long terminal repeat promoter activities (5–7). HCV core protein transforms primary rat embryo fibroblasts in association with a cooperative oncogene to a tumorigenic phenotype (8), interacts with the lymphotixin-β receptor to possibly modulate immune function (9), and associates with apolipoprotein II for a potential role on lipid metabolism (10). A recent study (11) suggests that missense mutations in the clustering variable region of the hydrophilic domain (residues 39–76) of the core gene may be involved in the pathogenesis of chronic HCV infection during hepatocellular carcinogenesis.

Viral infections may often induce an apoptotic response as a defense mechanism in host cells, and many viruses encode proteins that inhibit this mechanism (12). Alterations in cell survival contribute to the pathogenesis of a number of human diseases including viral oncoeneces (13). Tumor necrosis factor (TNF-α) is a major inflammatory cytokine, secreted primarily by activated macrophages and T lymphocytes, which is thought to limit infections by a variety of microorganisms (14, 15). TNF-α-induced apoptosis requires the activation of one or more of the interleukin-1β converting enzymes (ICEs), which function in the apoptotic response. ICE is a cysteine protease that catalyzes the proteolytic processing of the protein-inflammatory cytokine interleukin-1β from an inactive precursor form to the active mature form. The 116-kDa DNA repair enzyme poly-(ADP-ribose) polymerase (PARP) has been shown to be proteolytically processed to a signature 85-kDa fragment from an aspartate-specific cleavage by an ICE-like protease, which appears to be distinct from ICE. Importantly, PARP cleavage has been associated with a variety of apoptotic responses, including TNF-mediated cell death. In cultured cells infected with different DNA or RNA viruses, TNF may act to inhibit virus replication or induce apoptosis. Some viruses, in turn, have evolved strategies to block the antiviral effects of TNF. Recently, several viral proteins have been reported to interfere with the TNF-mediated signaling pathway leading to apoptosis. For example, CrmA (a cowpox virus protein) or the baculovirus protein p35 directly or indirectly inhibit ICE-like protease and presumably preclude TNF function (16, 17). On the other hand, the adenovirus E3–10.4K/14.5K protein complex inhibits apoptosis by inhibiting TNF-induced translocation of cPLA2 from the cytosol to membrane (18). Equine herpesvirus type 2 E8 protein and molluscum contagiosum virus MC159 protein also block cytokine-mediated apoptosis, probably by interfering with the ability of FLICE/caspase-8 (19, 20).

In this study, we examined whether HCV (genotype 1a) core
protein has a role in TNF-a-mediated cell death by utilizing a TNF sensitive MCF7 cell line. Our result suggests that the core protein inhibits the onset of TNF-mediated apoptosis and the associated PARP cleavage.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfections—The TNF-sensitive MCF7 breast carcinoma cell line (16) was kindly provided by Dr. V. M. Dixit (University of Michigan Medical School, Ann Arbor). The plasmid pHBe core, containing the entire HCV-1a core genomic region, has been described previously (21). The MCF7 cell line was transfected with pBabe core plasmid DNA using LipofectAMINE™ (Life Technologies, Inc.), and stable clones were selected using puromycin as described previously (21). The vector DNA transfected MCF7 cell line as a positive control (MCF7-V), a pool of HCV core stable transfectants (Core-pool), and three individual clones (Core-7, Core-10, and Core-12) as the experimental cells were grown for further studies.

RNA Analysis—Total RNA was isolated using an acid-phenol extraction method (22) from the control and experimental cells. Approximately 2 μg of RNA from each cell line was used for a reverse transcription polymerase chain reaction. A reverse transcriptase reaction was performed using random primers and avian myeloblastosis virus reverse transcriptase at 45 °C for 30 min. Subsequently, cDNA was amplified by polymerase chain reaction using synthetic oligonucleotide primers (sense 5′-CGTGAACGGGTGCTGAGCAG-3′ and antisense 5′-GAAGGCGTTCAGAACAGAACAGAA-3′) at 94 °C for denaturing, 60 °C for annealing, and 72 °C for extension for 30 cycles. The amplified products were analyzed by agarose gel electrophoresis and followed by ethidium bromide staining.

Assessment of TNF-cytotoxicity and Apoptosis—To determine the level of TNF-induced toxicity in HCV core-transfected MCF7 cells, various doses of TNF-a were initially used to determine parent MCF7 cell death in a dose-dependent manner. To further examine TNF-associated cell death, approximately 5 × 10⁵ cells were exposed to 15 ng/ml TNF (3.75 × 10⁻⁷ units/mg, Promega) for 18 h in the presence of cytohexamide and incubated an additional 48 h in TNF-free medium. Surviving cells were trypsinized and collected for counting by trypan blue exclusion. The sensitivity of MCF7 cells to TNF in the absence of cytohexamide was also determined by the modified tetrazolium salt (MTT) assay as described earlier (23). Briefly, cells (3 × 10⁵/well) were incubated for the indicated time frame in the presence or absence of different concentrations of TNF in a final volume of 0.2 ml for 72 h. Analysis of cell viability was carried out using the addition of 0.02 ml of various concentrations of TNF in a final volume of 0.2 ml for 72 h.

Detection of PARP Cleavage Following TNF Treatment—The preparation of cell lysates and immunoblotting of TNF-a-treated cells was performed as described earlier (16). Similar amounts of cellular proteins transferred onto nitrocellulose membrane were incubated with a rabbit polyclonal antiserum (1:1000 dilution) to the HCV core protein (kindly provided by Michael M. C. Lai, University of Southern California, Los Angeles) for 2 h at room temperature. An anti-rabbit immunoglobulin coupled with horseradish peroxidase was used as the second antibody for detection of HCV core protein by chemiluminescence (ECL, Amersham). For detection of PARP cleavage following treatment of cells with TNF-a, the anti-PARP monoclonal antibody (kindly provided by S. Chatterjee and N. Berger, Case Western Reserve University, Ohio) was used at a dilution 1:1000. The detection of the second antibody was followed (anti-mouse IgG horseradish peroxidase, Amersham) was used at a dilution of 1:10,000. The peroxidase signal was visualized by chemiluminescence (ECL). The molecular weight of HCV core protein bands in the immunoblot or PARP cleavage products was estimated from the migration of standard protein molecular weight markers (Life Technologies, Inc.).

RESULTS AND DISCUSSION

Transfection of MCF7 Cells with HCV Core Gene—TNF-sensitive MCF7 cells have been used earlier to study the role of viral proteins in apoptotic cell death (16, 17, 19, 20). We selected the MCF7 cell line as a model in our study due to its sensitivity to TNF-mediated apoptosis. This cell line is amenable to viral proteins for regulation of this important biological process. MCF7 cells stably transfected with HCV core gene under the control of murine leukemia virus long terminal repeat in the pBabe-puro plasmid (21) were analyzed for the level of TNF-a-mediated apoptosis indicated in the cell line. A pool of stable transfectants and three individual colonies were selected following treatment with puromycin and arbitrarily included in this study. mRNA synthesis for the core protein in stable transfectants was characterized by reverse transcription polymerase chain reaction. HCV core gene-transfected pooled cells (Core-pool) and the three clones exhibited core specific mRNA expression, and the results are shown in Fig. 1, panel A. Stable core transfectants of MCF7 cells displayed protein expression when studied by immunoblot analysis, and the results are shown in Fig. 1, panel B. Individual clones exhibited differences in the level of protein expression, and the clone, Core-7, appeared to have the maximum core protein expression among the cell lines included in this study. A study to assess cell viability by the trypan blue exclusion method suggested that HCV core protein expression in MCF7 cells did not have an apparent effect upon the growth rate as compared with the vector-transfected control cells except clone 12. The reason for higher growth of clone 12 is not clear at this time.

HCV Core Protein Inhibits TNF-a-induced Cytotoxicity—Various doses of TNF-a were initially used to determine the dose-dependent response of positive control MCF7-V and core-transfected MCF7 for cell death. At 15 ng/ml TNF-treated cells, 53% cell viability was observed in MCF7-V positive control cells (Fig. 2). Under similar conditions, HCV core transfection inhibits TNF-induced cytotoxicity to a varying degree in selected clones and pooled stable transfectants. The protection provided by HCV core from TNF-a-induced apoptosis correlated with the level of protein expression as determined by immunoblot assay. Thus, the expression of HCV core protein appeared to inhibit TNF-a-induced death in MCF7 cells.

HCV Core Protein Inhibits TNF-a-induced DNA Fragmentation—Endonucleolysis is considered a key biochemical event of apoptosis, resulting in the cleavage of nuclear DNA into oligonucleosome-sized fragments. DNA isolated from control MCF7 cells following treatment with TNF-a (15 ng/ml) for 24 h exhibited a typical oligonuclear fragmentation pattern on agarose gel electrophoresis (Fig. 3), whereas DNA isolated from core-
Results from this study indicated reduced the time of incubation from 24 to 3 h for onset of PARP cleavage, respectively. Use of cyclohexamide in this experiment Core-12, and Core-pooled cells showed a 16, 24, and 27% PARP cleavage within 24 h of TNF-α treatment. However, experimental cell clone (Core-7) are shown in Fig. 4. Densitometric scanning of the autoradiogram suggested an 88% PARP cleavage. 

Transfected MCF7 cell clones treated with TNF-α inhibited DNA fragmentation. Similar inhibition of DNA fragmentation was also observed following TNF-α treatment of core gene-transfected pooled cells. Results from this study indicated that HCV core protein inhibits TNF-α-induced fragmentation of cellular DNA.

**HCV Core Protein Inhibits TNF-α-induced Cleavage of Poly-(ADP-ribose) Polymerase Substrate**—Recent studies have pointed to a role for the family of caspase proteases (ICE/ced-3 proteases) in apoptosis, which act upstream of endonuclease (24). Proteases in apoptosis came to the forefront with studies on the proteolysis of PARP, initially described in cells induced to undergo apoptosis by various chemotherapeutic agents including etoposide (25, 26). This event was later determined to be catalyzed by a protease resembling ICE. The human homolog of this protease has been cloned and is now known (27, 28) as caspase-3 (CPP 32/yama/apopain). To determine whether the core protein has any effect on activation of caspase-3 following TNF-α treatment, PARP cleavage activity was examined. Analysis of the integrity of the death substrate PARP in control MCF7-V cells showed almost complete cleavage of the native 116-kDa PARP to the signature 85-kDa proteolytic fragment within 24 h of TNF-α treatment. However, experimental MCF7 cells exhibited a low level of PARP cleavage. The typical results from control MCF7-V cells and a core-expressing experimental cell clone (Core-7) are shown in Fig. 4. Densitometric scanning of the autoradiogram suggested an 88% PARP cleavage in MCF7-V cells after 24 h of incubation, whereas the Core-7 clone showed only 7% PARP cleavage. The Core-10, Core-12, and Core-pooled cells showed a 16, 24, and 27% PARP cleavage, respectively. Use of cyclohexamide in this experiment reduced the time of incubation from 24 to 3 h for onset of PARP cleavage (data not shown). Results from this study indicated that MCF7 cells stably transfected with HCV core gene inhibit TNF-α-mediated PARP cleavage under our experimental conditions. Inhibition of PARP cleavage has also been shown in cowpox virus CrmA protein and baculovirus p35. However, CrmA is a serpin capable of inhibiting ICE family members. Similar to p35, HCV core protein possesses no homology to any known protease inhibitor.

Our results suggest that the putative core protein of HCV inhibits TNF-α-mediated apoptosis and PARP cleavage in MCF7 cells. TNF is directly cytotoxic to cells infected with both DNA and RNA viruses (29, 30), and overexpression of the cytokine by a recombinant vaccinia virus leads to rapid virus clearance from infected mice (31). The TNF superfamily of cytokines and receptors can activate nuclear factor aB and effect cell growth, differentiation, or death. The mechanism of HCV core protein-mediated inhibition of TNF-α-mediated apoptosis remains to be elucidated. Replication of HCV in its natural host produces HCV polypeptides within the cell. It is possible that other viral proteins may antagonize or sensitize TNF-α-mediated cell death. The significance of inhibition of TNF-α-induced apoptosis by HCV core protein cannot be elucidated at this time due to the lack of a convenient in vitro HCV culture system.

Failure to mount an efficient immune response to HCV antigens, either because of selective defects in the host immune system or because of viral interference with a function(s) of immune cells, could account for the inability of HCV-infected subjects to eradicate viral infection (32). The fact that the majority of patients who acquire HCV develop chronic infection suggests that even though virus-specific cytotoxic T lymphocyte activity is present, it is insufficient to eliminate HCV in most cases. Liver-infiltrating lymphocytes isolated from HCV positive cases exhibited weak cytotoxic T lymphocyte activity against Epstein-Barr virus or concanavalin A-transformed lymphoblast targets infected with vaccinia-HCV recombinant virus (33). Immunization with HCV core plasmid DNA alone failed to induce a strong cytotoxic T lymphocyte response in immunized mice, and independent studies demonstrated <10% lysis in unprimed (spontaneous) and <30% lysis following in vitro stimulation of spleen cells from experimental animals even after multiple immunizations (34–36). Taken together, these results and the physiochemical characteristics of this viral protein, it appears that the HCV core protein may have an immunomodulatory role. In a recent study (37), transgenic mice expressing HCV core protein have been shown to develop progressive hepatic steatosis (fatty change). Apoptosis and an increase in the level of TNF-α have been observed in hepatitis B virus infection, and the majority of the hepatic cell death that occurs during hepatitis B virus infection is mediated by the immune system (38). TNF-α killing depends upon the level of hepatitis B virus expression (39) and may contribute to virus
clearance. On the other hand, a very low number of hepatocytes showing apoptosis in chronic HCV, despite the presence of HCV-specific CTL activity, suggests that HCV may directly or indirectly modulate the apoptotic pathways (40). We have previously demonstrated that HCV core protein suppresses cisplatin-mediated apoptosis in human cervical epithelial cells and apoptosis induced by etoposide overexpression in Chinese hamster ovary cells (21). In a different study (41), HCV core protein from genotype 1b was found to make HepG2 cells sensitized to apoptotic death without up-regulation of surface Fas expression. This was partly mediated through the cysteine protease CPP32 and not through the interleukin 1β-converting enzyme. Recent studies with Ad2E1B-19K suggest that this viral protein may or may not prevent anti-Fas-mediated cytolysis depending on the cellular context (42, 43). E1B-19K is a member of the Bel-2 family, whose members interact to regulate apoptosis (12). Similar to Bel-2, E1B-19K may interact with Bak and Bad to block p53-induced apoptosis (44, 45). A second set of proteins, the ICE-related cysteine proteases, also regulate apoptosis, and Bel-2 can block ICE-like proteases by preventing the release of cytochrome C from the mitochondria (46–48).

The baculovirus p35 protein can directly inhibit some member of the ICE-like protease family to block apoptosis induced by TNF (16). A recent study demonstrated that p35 can also protect against ionizing radiation, suggesting unique signaling pathways for radiation and TNF-induced apoptosis (49). There are numerous distinct signaling cascades that induce programmed cell death, and HCV core protein is likely to target multiple pathways involved in apoptosis, depending on the cellular context and physiologic status of the cells. Investigation of TNF-α-mediated apoptosis in a short term primary hepatocyte culture should increase our understanding of the cell growth regulatory role of HCV core protein in its natural host.

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