Inhibition of hepatitis C virus core protein expression in immortalized human hepatocytes induces cytochrome c-independent increase in Apaf-1 and caspase-9 activation for cell death

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Abstract

Hepatitis C virus (HCV) core protein has multifunctional activities. We have previously reported that the core protein of HCV immortalizes primary human hepatocytes, which may relate to multistage hepatocarcinogenic events. These immortalized human hepatocytes (IHH) served as a model to study the mechanism of HCV core protein-mediated cell growth regulation. Inhibition of core protein expression in earlier stages after hepatocyte immortalization leads to the induction of apoptosis. Here, we have observed that introduction of antisense core (AS-Core) sequences for inhibition of core protein expression enhanced the expression of E2F1 and p53 levels in early passage IHH. Inhibition of core protein expression also altered the expression level of Bcl-2 family proteins, displaying an increase of the proapoptotic Bax and a decrease in the level of the anti-apoptotic Bcl-xL proteins. These alterations, however, did not result in the release of cytochrome c from the mitochondria. Apaf-1 is frequently deregulated under various pathologic conditions, and examination of AS-Core-expressing apoptotic cells indicated a significant increase in the level of Apaf-1, which coincided with caspase-9 activation. Knockdown of Apaf-1 or the transcriptional regulatory proteins, E2F1 or p53, by small interfering RNA (siRNA) duplexes inhibited the activation of caspase-9 and enhanced cell viability in AS-Core-expressing cells. These findings may contribute to the understanding of the pathophysiology of HCV core protein-mediated hepatocyte growth regulation and disease progression.

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Introduction

HCV is a major public health problem, with more than 170 million people chronically infected worldwide. The most important feature of HCV infection is the development of chronic hepatitis in ~80% of the infected individuals and the potential for disease progression to cirrhosis and hepatocellular carcinoma (Jeffers, 2000; Saito et al., 1990). Studies on HCV are challenging due to the lack of a suitable cell culture system and the absence of a small animal model.

Overproduction and release of core protein have been observed in the blood stream of HCV infected hosts (Maillard et al., 2001). HCV core protein immortalizes primary human hepatocytes, the natural host for virus replication (Ray et al., 2000). These immortalized human hepatocytes (IHHs) exhibit activation of telomerase function and continuous growth in cell culture. Hepatocarcinogenesis involves alterations in the concerted action of proto-oncogenes, growth factors, and tumor suppressor genes. HCV core protein regulates p53, p21, c-myC promoter activities, and cyclin E (Cho et al., 2001; Kwun and Jang, 2003; Ray et al., 1997, 1998; Siavoshian et al., 2004).
Moreover, HCV core protein constitutively activates AP-1, which correlates with the activation of JNK and MAPKK (Marusawa et al., 1999; Shrivastava et al., 1998). Premature senescence involving p53 is activated in response to constitutive MEK/MAPK mitogenic signaling (Lin et al., 1998). We observed a weak level of core protein expression in IHH, which is sufficient for the maintenance of cell growth, and below which early passage immortalized hepatocytes fail to survive (Basu et al., 2002).

A fundamental aspect of p53 is its ability to participate in cell-cycle checkpoint and apoptosis functions that regulate homeostatic tissue renewal. Exogenous p53 expression can suppress the transformed phenotype of many cell types by inducing growth arrest and apoptosis (Soddu and Sacchi, 1998). The modulation of p53 by adenovirus E4 or E6 proteins, HTLV-1 Tax protein, SV40 T antigen, and the E6/E7 gene products of HPV-16 and 18 plays a role in cell growth regulation (Herzig et al., 1999; Nevels et al., 1999a, 1999b; Steegenga et al., 1999; Uittenbogaard et al., 1995). A constant expression of SV40 T-antigen is necessary for the maintenance of immortalized human fibroblasts (Rada et al., 1989), and antisense E7 RNA of HPV-18 causes death of an oral cancer cell line−1483−after a lag period (Steele et al., 1992). Results from our previous studies have suggested that HCV core protein can modulate p53 gene expression, which may contribute to the immortalization of primary human hepatocytes and growth (Basu et al., 2002; Ray et al., 2000).

Activation of p53 may occur due to an overexpression of E2F1. The p14ARF protein, identified as an E2F target gene, stabilizes p53 and may provide a link between E2F1 and an elevation in p53 levels (Bates et al., 1998). E2F1 is a member of a family of transcription factors and acts to control the G1/S transition in embryotic cells (Harbour and Dean, 2000). In response to mitogenic stimuli, E2F1 is induced in quiescent cells and promotes both cell cycle progression and apoptosis (Nevin, 1998). Apaf-1 is transcriptionally activated by p53 and E2F1 (Moroni et al., 2001), and is a mediator of apoptosis. An increase in Apaf-1 levels has been associated with the direct activation of caspase-9 without mitochondrial damage and leads to the initiation of a caspase cascade (Furukawa et al., 2002).

Immortalization of primary human hepatocytes by HCV core protein may be a result of a regulatory role on cellular gene(s) leading to an alteration in hepatocyte turnover, although the mechanism of core protein-mediated cellular gene regulation is not well understood (Ray and Ray, 2001). HCV core protein has multifunctional activities similar to that seen in a number of other viral proteins with oncogenic potential. The immortalization of a primary human epithelial cell occurs through a multistep process, and many of these steps are not fully understood. Interestingly, inhibition of core protein expression did not alter the growth property of late passage IHH (passage 50) when cells were exhibiting anchorage-independent growth, in contrast to early passage IHH (passage 15). This suggested that HCV core protein is required for the maintenance of this phenotypic change in the early stages after hepatocyte immortalization (Basu et al., 2002; Ray et al., 2000).

In the present study, we have utilized IHH as a model to study the role of HCV core protein-mediated cellular functions, particularly with regard to the mechanism of immortalization. We have investigated the signaling pathway by which IHHs undergo apoptosis upon inhibition of HCV core protein expression. Our results demonstrate a novel mechanism of apoptosis in which hepatocyte death correlates with an increase in Apaf-1. The subsequent activation of caspase-9, leading to the initiation of the intrinsic cell death pathway, occurs in the absence of cytochrome c translocation to the cytosol.

**Results**

**Differentially expressed E2F1 and p53 genes in IHH**

Atlas cDNA expression membrane (Human Oncogene/Tumor Suppressor Array) was used to identify genes induced in response HCV core protein expression in IHH. Total RNAs isolated from HCV core-transfected IHH and from paired donor hepatocytes were used to generate 35P-labeled cDNA probes for the hybridization of Atlas cDNA expression membrane. Modulated genes were selected as described under Materials and methods and a detailed description of the gene expression analyses will be reported separately. Among the genes of interest from our previous studies (Basu et al., 2002; Ray et al., 2000), both E2F1 and p53 were reduced early after immortalization of the primary human hepatocytes by HCV core (passage number15) as compared to primary hepatocytes from matching donors. Each passage number represented cells from ~4 days of culture. Real-time PCR verified an ~35% decrease in E2F1 and ~50% decrease in p53.

p53 is known to play a key role in growth arrest, DNA repair, and apoptosis after cell stress, primarily through its ability to regulate the transcription of downstream cellular target genes. AS-Core gene expression in early passage IHH (passage 15) induces an apoptotic response (Basu et al., 2002). Inhibition of core gene expression led to an ~5-fold increase in protein expression of p53 as compared to vector control in early passage IHH which preceded apoptosis (Fig. 1, panel A). Inhibition of core gene expression also increased E2F1 protein expression by ~2-fold in early passage IHH (Fig. 1, panel B). These results corroborated the data derived from Atlas cDNA expression and real-time PCR, suggesting that the regulation of p53 and E2F1 genes occurred at the transcriptional level.

To examine whether p53 or E2F1 is responsible for AS-Core-mediated apoptosis, we knocked down p53 or E2F1...
expression by a siRNA duplex against specific mRNA and examined their relative protein expression levels, and cell death in response to AS-Core induction. AS-Core cells were incubated in the presence of 50 \( \mu \text{M} \) ZnCl\(_2\) for 5 days, followed by electroporation with the indicated siRNAs. Cells were added on a collagen-coated plate and incubated in medium supplemented with 50 \( \mu \text{M} \) ZnCl\(_2\) until the AS-Core control cells exhibited a significant level (>80%) of death. The introduction of siRNA to p53 (sip53) resulted in \(~20\)-fold decrease in the level of p53 expression in AS-Core-induced cells as compared to AS-Core control cell lysates (Fig. 1, panel A). In a similar manner, siRNA to E2F1 (siE2F1) effectively reduced the level of E2F1 protein in AS-Core-induced cells by 4- to 5-fold (Fig. 1, panel B). Apoptosis was evaluated by cell death ELISA (Fig. 1, panel C), and cell death induced by AS-Core was significantly inhibited with either sip53 or siE2F1 when compared with unrelated siRNA-transfected cells. These results suggest that the regulation of p53 and E2F1 by HCV core protein expression contributes to the suppression of apoptotic response in hepatocytes.

p53 induces the expression of Bax, a proapoptotic member of the Bcl-2 family of proteins (Miyashita et al., 1994; Selvakumaran et al., 1994), while also acting to down-regulate the anti-apoptotic Bcl-2 protein (Haldar et al., 1994). RNase protection assay for certain members of the Bcl-2 protein family indicated an \(~3\)-fold increase in the expression of Bax mRNA level following AS-Core induction, while the levels of the anti-apoptotic Bcl-2 and Bcl-xL mRNAs remained relatively unchanged (Fig. 2, panel A). We further investigated the potential for HCV core protein to modulate members of the Bcl-2 family (Bcl-2, Bcl-xL, Bax) at the protein level by Western blot analysis. Protein loaded in each lane was normalized with the level of cellular actin. Our results suggested an \(~4\)-fold increase in the level of Bax expression after AS-Core gene induction as compared to vector control cells. In contrast, the level of Bcl-xL protein under these conditions was

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**Fig. 1.** Correlation of IHH apoptosis with p53 and E2F1. ZnCl\(_2\)-induced AS-Core or empty vector control cells were electroporated with 100 nM sip53 or siE2F1 (Santa Cruz). Cells were subjected to Western blot analysis for the detection of p53 (panel A) and E2F1 (panel B) levels. siRNA-transfected hepatocytes were also analyzed for apoptotic cell death by ELISA (Roche) following induction with ZnCl\(_2\) (panel C).

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**Fig. 2.** Inhibition of core protein expression in IHH modulates Bax and Bcl-xL expression levels. (Panel A) Analysis of Bcl-2 family mRNA expression level in response to antisense core induction by RNase protection assay. The mRNA expression levels of Bcl-xL, Bax, Bcl-2, and L32 (internal control) were determined in IHH-expressing antisense core gene sequences (AS-Core) and in empty vector control cells. (Panel B) Effect of inhibition of core gene expression on Bcl-xL, Bax, and Bcl-2 proteins in AS-Core or empty vector-introduced IHH. Cell lysates were subjected to Western blot analysis using specific antibodies. The level of cellular actin was used as an internal control for comparison. Molecular weights of the identified protein bands were validated from the positions of prestained molecular weight markers (Bio-Rad).
found to decrease by a similar margin, while Bcl-2 levels remained unchanged (Fig. 2, panel B). The difference between the RNA and protein levels of Bcl-xL could be due to a post-transcriptional alteration, resulting in increased stability for Bcl-xL. These results indicated that the expression of HCV core protein in immortalized hepatocytes might act to suppress an apoptotic response via a modulation of the Bcl-2 family of proteins. In this case, limiting the transcription level of proapoptotic Bax, while encouraging a cellular environment that post-transcriptionally enhances Bcl-xL protein levels.

Apoptosis of immortalized hepatocytes is independent of cytochrome c pathway and relies on the inhibition of core protein expression

The p53 apoptotic target genes can be divided into two groups, the first group encodes proteins that act through receptor-mediated signaling, while those of the second group regulate apoptotic effector proteins and include members of the Bcl-2 family of proteins (Vermeulen et al., 2003). Bcl-2 may act to directly promote cell survival by interfering with the activation of the intrinsic apoptotic pathway (Yang et al., 1997). The positive and negative regulation of an apoptotic response is often determined by the relative ratios and dimerization status of Bcl-2 and Bax (Cory and Adams, 2002). Homodimers of Bcl-2 block apoptosis, whereas Bax homodimers elicit the opposite effect, and Bcl-2–Bax heterodimers are inactive. In a similar fashion, the Bcl-xL protein is able to interact with the pro-apoptotic Bax and Bak proteins, with the potential to affect cell viability. As p53 activates the transcription of Bax, it alters the balance of Bcl-2 and Bax, leading to the potential release of cytochrome c from the mitochondria, activating a caspase-9-associated caspase cascade. Bcl-xL may also further hinder the apoptosis, as well as an apoptotic response from AS-Core gene expression. Prior to the onset of apoptosis, there was only a slight indication of Apaf-1 from AS-Core gene expression. Prior to the onset of apoptosis, there was only a slight indication of Apaf-1 expression was inhibited by a specific siRNA duplex, and the results are presented as % apoptosis. Cells treated with siApaf-1 exhibited a suppression of Apaf-1 expression to a level similar to that found in the vector control

Apaf-1 expression level is regulated by AS-Core induction

We examined the status of Apaf-1, a central protein in the apoptosis, which has been observed to regulate apoptosis in the absence of cytochrome c release. Cell lysates were examined prior to and after the onset of apoptosis derived from AS-Core gene expression. Prior to the onset of apoptosis, there was only a slight indication of Apaf-1 protein expression, and induction of vector control transfected cells exhibited no increase in the level of Apaf-1. However, we observed an ~3-fold increase in Apaf-1 levels in AS-Core gene-expressing cells that were undergoing apoptosis, as well as an ~10-fold increase in the appearance of an ~84-kDa band associated with a post-apoptotic proteolytic cleavage of Apaf-1 (Fig. 4).

Although inhibition of HCV core protein expression clearly leads to the upregulation of Apaf-1, it is still unclear whether this induction is responsible for the initiation of apoptosis. To address this question, Apaf-1 expression was inhibited by a specific siRNA duplex, and its effect on cell death in response to AS-Core gene expression was examined. Cell death was evaluated by ELISA against uninduced AS-Core cells as a background and the results are presented as % apoptosis. Cells treated with siApaf-1 exhibited a suppression of Apaf-1 expression to a level similar to that found in the vector control cell line (Fig. 5, panel A), and ~2.5-fold lower apoptosis than AS-Core-induced cells (Fig. 5, panel B). A significant decrease in observed cell death, coinciding with a reduction in this protein, suggests that Apaf-1 induction
is a critical step for AS-Core-mediated apoptosis. The role of p53 or E2F1 was examined to further explore the regulation of Apaf-1 in AS-Core-induced cells. The introduction of sip53 into AS-Core cells led to a 2.8-fold reduction in the level of Apaf-1 expression in ZnCl2-induced AS-Core cells, while the induction of siE2F1 sequences led to a 4.4-fold reduction in the level of Apaf-1 (Fig. 5, panel C). These results further suggested that Apaf-1, p53, and E2F1 are associated with apoptosis in IHH.

Antisense core gene expression activates procaspase-9 preceding activation of caspase-7 in immortalized hepatocytes

We determined the activation level of initiator procaspase-9, an upstream activator of procaspase-3 and-7, in control and AS-Core-expressing IHH lysates by Western blot analysis. Upon activation, the proenzyme is cleaved to the active polypeptides of caspase-9 (~35 kDa and ~10
kDa). We used an antibody in this analysis that recognizes both the procaspase-9 and cleaved 35-kDa form. IHH induced to express AS-Core sequences exhibited a significantly increased level of cleaved 35-kDa polypeptide of caspase-9 as compared to vector control (Fig. 6). The presence of sip53 or siE2F1, and siApaf-1, inhibited the activation of procaspase-9. The blot was reprobed with an antibody to actin as a housekeeping protein for comparison. Results from this experiment suggested that apoptosis derived from the induction of AS-Core gene expression is associated with the activation of caspase-9.

We examined whether activation of the downstream effector molecule caspase-3 or -7 occurs in the caspase cascade. Lysates from control and experimental hepatocytes expressing AS-Core gene were prepared after 8 days of ZnCl₂ induction. Lysates were subjected to Western blot analysis using a rabbit polyclonal antibody to caspase-3, and activation of caspase-3 in response to AS-Core induction was not observed. Caspase-7 is expressed in liver tissues and consists of a zymogen form of ~34–38 kDa, and is subject to activation by caspase-9 (Deveraux et al., 1998). Analysis of caspase-7 revealed a significant reduction of the zymogen form of this executioner phase caspase associated with AS-Core-derived apoptosis (Fig. 6, panel B). Results from this set of experiments suggested that AS-Core gene expression promotes caspase-9 activation, which precedes activation of caspase-7 as an effector mechanism for apoptosis of the immortalized hepatocytes.

Discussion

Diverse functional activities have been reported for the HCV core protein (Choi et al., 2001; Lai and Ware, 2000; McLauchlan, 2000; Ray and Ray, 2001), which include encapsidation of viral RNA, a regulatory role on cellular and unrelated viral promoters, interactions with a number of cellular proteins, a modulatory role in programmed cell death under certain conditions, involvement in cell growth promotion and immortalization, induction of hepatocellular carcinoma (HCC) in transgenic mice, and a possible immunoregulatory role. Hepatocellular adenoma or carcinoma has also been noted in transgenic animals encoding a complete HCV protein sequence (Lerat et al., 2002). These intriguing properties suggest that the core protein, in concert with cellular factors, may contribute to the pathogenesis of a chronic HCV infection. In general, development of pathological consequences in the host would vary depending upon the nature of disease, the infecting virus, and the incubation time. Multiple genetic factors, including host innate and acquired immune responses, exert distinct characteristics imposed upon the invading organism. HCV...
core protein immortalizes primary human hepatocytes, the natural host for virus tropism and replication (Ray et al., 2000). In infected liver, HCV core protein may stimulate cells to escape from replicative senescence, allowing for the rise of selective clonal proliferation. Here, we have shown that the inhibition of HCV core protein expression in these immortalized human hepatocytes results in an increase in p53 and E2F1 expression levels preceding the onset of apoptosis. HCV core protein also appeared to modulate anti-apoptotic Bcl-2-related proteins for the maintenance of hepatocarcinoma by antagonizing proapoptotic molecules. Apoptosis derived from the inhibition of HCV core protein expression by antisense sequences correlated with an upregulation of Apaf-1 and the activation of a caspase-9-related cascade in the absence of cytosolic accumulation of cytochrome c.

Apaf-1 constitutes the central element in the intrinsic pathway, which may be triggered by the release of cytochrome c from the mitochondrion (Liu et al., 1996). This translocation of cytochrome c follows a change in mitochondrial membrane potential likely to be associated with the activity of proapoptotic members of the Bcl-2 family. The lack of cytochrome c release in AS-Core-induced IHH is in contrast to the modulation of Bcl-xL and Bax proteins apparent in cells that undergo an apoptotic response related to this induction. Initiation of caspase-9-associated cell death by inhibition of HCV core protein expression appears to be the key mechanism of IHH cell death. The modulation of the intrinsic pathway by this means may be critical for the maintenance of the immortalized phenotype of HCV core-transfected human hepatocytes.

Apaf-1 forms a central element in the mitochondrial death machinery and is responsible for the recruitment and activation of initiator caspase-9 by enforcing a locally high concentration of caspase-9 (Lauber et al., 2001). The close proximity of the caspase precursors then allows the immature proteases to self-activate due to their low intrinsic enzymatic activity (Salvesen and Dixit, 1999). Caspase-9 in turn activates executioner caspase-3 or caspase-7, thus perpetuating the caspase cascade which results in cell death (Slee et al., 1999). Recent studies demonstrated that Apaf-1 might act as an allosteric activator for caspase-9 (Rodriguez and Lazebnik, 1999; Stennicke et al., 1999). Further, caspase-9 remains associated with Apaf-1 in a high molecular weight complex of ~700 kDa, termed as apoptosome, that might also contain executioner caspase-3 and -7 (Cain et al., 1999; Hu et al., 1999). After activation of the downstream caspase pathway, Apaf-1 itself is processed into a proteolytic 84-kDa fragment as a target of caspase proteolytic activity, forming an inactive multimer, which is unable to further bind the caspase recruitment domain (CARD) (Lauber et al., 2001).

E2F-1 enhances the expression of Apaf-1 leading to cell death without the cytosolic accumulation of cytochrome c. Resistance of Apaf-1-deficient melanoma cell lines to the deregulated expression of E2F-1 suggests that Apaf-1 is an essential element of E2F-1-mediated apoptosis (Furukawa et al., 2002). E2F-1 activates caspase-9 by directly inducing the autoactivation of caspase-9 via a localized increase in intracellular Apaf-1 levels. In this manner, the cell may initiate a caspase cascade in the absence of mitochondrial damage. In our study, we observed a significant increase of Apaf-1 in cells undergoing apoptosis associated with AS-Core gene expression. Fortin et al. (2001) reported that p53 up-regulates Apaf-1 as a direct transcriptional target during neuronal cell death. E2F-1 may also transactivate the Apaf-1 gene in the absence of p53 (Furukawa et al., 2002). This may have clinical importance, as the loss of normal p53 function is a common characteristic of a number of cancer cell types. The E2F-1/Apaf-1 pathway (growth-associated intrinsic route) works in parallel with the p53/Bax/cytochrome c pathway (DNA damage-triggered extrinsic route) in promoting apoptotic cell death. The loss of p53 pathway function and deregulated E2F activity infers the possibility that E2F1 may induce apoptosis directly or independently of p53 induction. Apaf-1 and procaspase-9 have a role in

[Fig. 6. Effect of AS-Core gene expression on downstream effector molecules in IHH. (Panel A) Inhibition of core protein expression in the IHH activates procaspase-9. Cell lysates were prepared after induction of AS-Core gene expression or empty vector control and analyzed for procaspase-9 level by Western blot using a specific antibody. Inhibition of caspase-9 was observed following treatment of cells with sip53, siE2F1, or siApaf-1. (Panel B) Inhibition of core protein expression in the IHH significantly decreases procaspase-9 level. Cell lysates were subjected to Western blot analysis using antibody to procaspase-7. The level of cellular actin was used as an internal control for comparison. Molecular weights of the identified protein bands were validated from the positions of prestained molecular weight markers (Bio-Rad).]
controlling tumor development, and the genes encoding these proteins are strong candidates as tumor suppressors (Moroni et al., 2001). As both E2F1 and p53 transcriptionally activate Apaf-1, these proteins may sensitize cells to apoptosis by a process that would circumnavigate the need for cytochrome c release to initiate the Apaf-1/caspase-9–associated cell death pathway. Further studies will continue to study the underlying biochemical changes apparent in immortalized hepatocytes and how these changes may reflect those apparent in HCV-infected patients.

Materials and methods

Cells and plasmids

Primary human hepatocytes immortalized by transfection with plasmid DNA expressing core protein from HCV genotype 1a under the control of MuLV LTR in the pBabe-puro expression vector were generated as described earlier (Ray et al., 2000). Hepatocytes were seeded on collagen type I-coated plates and maintained at 37 °C in a defined culture medium supplemented with growth factors and antimicrobials (SAGM, Clonetics, CA). The antisense orientation of the core genomic sequence (encoding amino acids 1–191) from the same HCV genotype 1a was cloned under the control of an inducible metallothionein promoter in MTCB6 + vector (kindly provided by Frank J. Rauscher, III, Wistar Institute, Philadelphia).

Transfection and maintenance of hepatocytes

HCV core-mediated immortalized hepatocytes (from passage 15) were transfected with the antisense orientation of the core genomic region (AS-Core) or an empty vector using lipofectamine. Briefly, cells were transfected with an inducible promoter linked with the AS-Core gene or empty vector DNA (mock control). At 48 h post-transfection, cells were treated with G-418 (800 μg/ml) for the selection of stable transfectants. Isolated colonies were pooled to limit the potential for clonal artifacts. For induction of AS-Core gene expression, cells were treated with a predetermined dose of ZnCl2 (50 μM) for 6–8 days. The addition of ZnCl2 displayed no detectable growth inhibition in the absence of AS-Core gene sequences.

Immunofluorescence for cytochrome c

IHHs were treated with ZnCl2 (50 μM) to induce AS-Core gene expression. Upon visual observation of cellular changes associated with apoptosis, cells were treated with mitotracker for 30 min, washed, and fixed with 3.7% formaldehyde in PBS for 30 min. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min. After washing, cells were incubated with a mouse monoclonal antibody to cytochrome c (Promega), followed by staining with an anti-mouse secondary antibody conjugated with Alexa-568 (Molecular Probes). Finally, washed cells were mounted for confocal microscopy (Bio-Rad 1024).

Preparation of cytoplasmic fraction

IHH cells stably transfected with the MTCB6+ vector with or without the AS-Core gene were treated with ZnCl2 for 8 days. Experimental cells were harvested after death, resuspended in ice-cold isotonic buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose, and containing 5 μg/ml aprotinin, 2.5 μg/ml leupeptin, and 3 μg/ml pepstatin as a cocktail of protease inhibitor), and fractionated as described earlier (Bossy-Wetzel et al., 1998). The pellet obtained comprised the mitochondrial membrane fraction, and the supernatant contained the cytosolic fraction. Equal amounts of cytosolic protein extracts and membrane fractions were resolved by SDS–PAGE (15%) and electroblotted onto a nitrocellulose membrane. Cytochrome c was detected by Western blot analysis with a specific antibody to cytochrome c (Santa Cruz), followed by chemiluminescence (Amersham).

Real-time PCR

Total RNA was treated with DNase I, reverse transcribed using random priming and Superscript II reverse transcriptase following manufacturer’s procedure (Invitrogen). Quantitative PCR was performed by monitoring in real-time the increase in fluorescence of the SYBR Green dye on an ABI PRISM 7000 Sequence Detector System (Applied Biosystems) according to the manufacturer’s protocol. Gene-specific primers (Invitrogen) were designed using the Primer Express software from Applied Biosystems (Table 1). Relative expression levels of the target genes in immortalized hepatocytes were plotted as fold change compared with a matched untransfected primary control; 18 S rRNA was used for normalization. Each real-time quantitative PCR assay was performed twice using triplicate samples.

Gene array

RNA isolation from cells and generation of 32P-labeled cDNA probes were performed as recommended by the

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supplied for gene array (CLONTECH Laboratories, Inc.). In brief, total RNA was isolated from frozen cell pellets and DNase treated. mRNA was isolated from total RNA using oligo(dT)-conjugated magnetic beads. Radiolabeled cDNA probes were generated by reverse transcription of 0.5–1 μg of mRNA (the same amount for stimulated and control in each case) using Moloney murine leukemia virus polymerase in the presence of [γ-32P]dATP. Hybridization of the cDNA probes to Atlas membranes (Human Oncogene/Tumor Suppressor Array) was done overnight at 68 °C in ExpressHyb (CLONTECH) using mRNA isolated from independent experiments on two different filter sets. The results were analyzed using a PhosphorImager (Molecular Dynamics). Differential expression was determined as the percent deviation from control after normalizing all spots according to the average relative intensities of the nine housekeeping genes on stimulated and control membranes. cDNA spots that showed substantial deviation from control membranes were selected. The threshold was set around 60–100% for up-regulation and 40–60% for down-regulation.

Small interfering RNA (siRNA)

The 21-nucleotide siRNA duplexes targeted to p53, E2F1, and Apaf-1 were purchased from Santa Cruz, CA. The relevant siRNAs (100 nM) were introduced into IHH by electroporation (Bio-Rad), and cells were treated with ZnCl2 for induction of AS-Core expression. Cells were collected after induction for subsequent studies.

RNase protection assay

An RNase protection assay was performed using custom made probes (Pharmingen) to investigate the modulation of cellular genes as previously described (Basu et al., 2001). Control human RNA provided with the assay kit was used to ensure the detection of specific bands. Total cellular RNA was isolated from vector-transfected control or AS-Core-transfected cells. Total RNA was hybridized with radiolabeled antisense RNA multiprobes (Bcl-xL, Bax, Bcl2, and L32). The reaction mixture was digested with RNase A and RNase T1. The protected fragments were analyzed by urea-polyacrylamide gel (5%) electrophoresis, followed by autoradiography. The autoradiogram was densitometrically scanned to compare mRNA levels between control and experimental cells after normalizing the values with respect to the housekeeping gene, L32.

Western blot

IHHs expressing the AS-core sequences or vector alone were treated in parallel with ZnCl2 (50 μM) for 8 days until apoptotic induction in the experimental set was apparent. Cells were washed in phosphate-buffered saline and lysed in 0.5% SDS, 125 mM Tris–HCl, pH 6.8, and 10% 2-mercaptoethanol containing 5 μg/ml aprotinin, 2.5 μg/ml leupeptin, and 3 μg/ml pepstatin. Proteins were resolved by SDS–PAGE and electroeluted to a nitrocellulose membrane. The membrane was blocked for 2 h with 5% nonfat dry milk in TBS-tween and incubated with appropriate antibodies. Detection of protein was completed by the addition of an enhanced chemiluminescent substrate, according to the manufacturer’s protocol (Amersham).

Antibodies

Rabbit antibody to Bax, procaspase-9, and procaspase-7, monoclonal antibodies to p53, and Bcl-xL (Santa Cruz, CA), monoclonal antibody to Apaf-1 (R&D System, MN), and monoclonal antibodies to actin, Bcl2, and E2F1 (Oncogene Science, CA) were purchased from the respective manufacturer.

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