BAD, a Proapoptotic Member of the BCL2 Family, Is a Potential Therapeutic Target in Hepatocellular Carcinoma

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Abstract

Proteins of the BCL2 family are key regulators of apoptosis. Their expression levels are frequently altered in cancers, enabling tumor cells to survive. To gain insight into the pathogenesis of hepatocellular carcinoma (HCC), we performed a comprehensive survey of the expression of the members of the BCL2 family in samples obtained from surgically resected HCCs. Here, we report the occurrence of a new molecular anomaly, consisting of a strong reduction in the expression of the proapoptotic protein BAD in HCC compared with surrounding nontumoral tissue. We investigate the function of BAD in a panel of HCC cell lines. Using gene overexpression and RNA interference, we show that BAD is involved in the cytoxic effects of sorafenib, a multikinase blocker, which is currently the sole therapeutic drug effective for the treatment of HCC. Finally, we report that ABT-737, a compound that interacts with proteins of the BCL2 family and exhibits a BAD-like reactivity, sensitizes HCC cells toward sorafenib-induced apoptosis. Collectively, our findings indicate that BAD is a key regulator of apoptosis in HCC and an important determinant of HCC cell response to sorafenib. Mol Cancer Res; 8(8): 1116–25. ©2010 AACR.

Introduction

Hepatocellular carcinoma (HCC) is a malignant tumor with a poor prognosis (1). Its progression is marked by the sequential accumulation of genetic/epigenetic abnormalities (2) that give rise to a set of common characteristics, among which is the ability to escape programmed cell death by apoptosis (3). The loss of sensitivity to apoptosis contributes to tumor progression and metastasis, and also determines how cancer cells respond to treatments (3, 4). Therefore, deciphering how apoptosis is regulated in HCC cells is an important goal to understand how these tumors develop and to improve their clinical handling.

Members of the BCL2 protein family function as positive or negative regulators of apoptosis, and they share a number of homology domains (Bcl2-homology domains BH1-BH4; ref. 5). Proapoptotic multidomain members, such as BAX and BAK, promote the release of apoptotic factors from mitochondria into the cytosol. They are regulated through interactions with antiapoptotic members of the BCL2 family, such as BCL2, BCLXL, or MCL1. A proapoptotic subset of BCL2 proteins characterized by a homology restricted to the BH3 domain of BCL2, and thus called the “BH3-only” members of the BCL2 family, plays a sentinel role in apoptosis regulation (5, 6). BH3-only members of the BCL2 family can either directly activate BAX and BAK or sensitize cells to apoptosis by neutralizing the antiapoptotic members of the BCL2 family (5, 6). The balance that results from the interactions between proapoptotic and antiapoptotic members of the BCL2 family determines the sensitivity of the cell to apoptosis (5, 6).

Changes in the expression levels and activation of one or more members of the BCL2 family occur in most types of tumors (7-10). Two antiapoptotic members of the BCL2 family, BCLXL and MCL1, have been shown to be expressed at increased levels in HCC (4, 11). Although the reported changes probably significantly contribute to hepatocarcinogenesis, no study has yet examined the complete BCL2 protein family. It remains unclear to what extent the expression of the other members of the BCL2 family of proteins changes in HCC. In particular, little is known about the variations in the expression of the proapoptotic members of the BH3-only family of proteins, although they have recently emerged as an important barrier against malignant transformation (10). A recent report indicates that BID, a member of the BH3-only subfamily, is down-regulated in a subset of HCC appearing in the context of
viral hepatitis (12). Whether the expression of other members of the BCL2 family of proteins is similarly altered in HCC remains unclear.

To address this issue, in the present study, we performed a survey of the expression of proteins from the BCL2 family, starting from surgically resected samples. We report the frequent occurrence of a decrease in the expression levels of BAD, a proapoptotic BH3-only member of the BCL2 family, in HCC. We examined the relevance of this decrease and analyzed its therapeutic implications.

Materials and Methods

Samples

Fourteen pairs of samples consisting of HCC/matched nontumor tissue were obtained from surgical resections done at Amiens University Hospital, France, between August 2005 and February 2009. All patients presenting with resectable HCC were included in this study. A detailed pathologic assessment of the tumor as well as nontumor tissues was done. A summary of the histologic observations and the baseline characteristics of the patients are provided in Supplementary Table S1. Sixteen control liver tissue samples were also obtained: the corresponding samples, hereby called “normal,” were obtained from surgical resections done following a diagnosis of hepatic metastasis of colorectal cancer. All samples were kept frozen at −80°C. The corresponding protocol was approved by the Comité de Protection des Personnes 2 Nord-Ouest.

Cell culture and reagents

All cell lines were cultured and characterized as indicated in Supplementary Material. Sorafenib (BAY49-4006) was kindly provided by Bayer Healthcare. ABT-737 and its inactive enantiomer were kindly provided by Abbott. All inhibitors were kept as stocks in DMSO at −20°C.

Western blots

Liver samples (minimal volume of 1 cm³ to prevent sampling artifacts) were homogenized in Laemmli buffer. Complete cell extracts were prepared in radioimmunoprecipitation assay buffer. Proteins were precipitated, loaded on SDS-PAGE, and transferred to nitrocellulose membranes using standard procedures. Antibodies used are described in Supplementary Material. Enhanced chemiluminescence reaction was used for revelation. Western blots were scanned and quantified using the software ImageJ (NIH).

Quantitative PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen) and reverse-transcribed using High Capacity cDNA Reverse Transcription kit and random hexamer (Applied Biosystems). Amplification was done with the TaqMan Universal PCR master Mix on an ABI 7900HT Sequence Detection System (Applied Biosystems) using primers and probe sets for BAD and human β-actin/glyceraldehyde 3-phosphate dehydrogenase (TaqMan Gene Expression Assay, Applied Biosystems).

RNA interference

Silencer select validated siRNA directed against BAD (si1861 and si1862) and Silencer negative control (am4635 and am4637 siRNAs) were purchased from Applied Biosystems. Transfections were done using the siPORT-neoFX reagent (Applied Biosystems) and Opti-MEM transfection medium (Invitrogen), according to the manufacturer’s instructions.

Fluorescence microscopy

Cells grown on glass coverslips were fixed with 3.7% paraformaldehyde, permeabilized with 0.01% Triton X-100, and stained with relevant antibodies. Nuclei were stained with 4′,6-diamidino-2-phenylindole. Coverslips were mounted in Mowiol (Calbiochem) and observed with a Nikon Eclipse TE2000U microscope equipped with a plan APO VC 60X/1.40 objective under oil immersion. Images were processed and superposed with the Photoshop software (Adobe).

Viability assays

Trypan blue exclusion assays were performed by the use of an automated cell counter Countess (Invitrogen), using the manufacturer’s protocol. For clonogenicity assays, Huh7 cells (100 cells per 15-mm diameter dish) were incubated for 10 days under the indicated conditions. Cells were stained with Giemsa, and the number of clones with more than 20 cells is presented.

Statistical analyses

Wilcoxon unpaired test, Pearson r coefficient, and Student’s t test were used as indicated. A value of P < 0.05 was considered as threshold for significance.

Results

A subset of HCCs exhibit a dramatic reduction in BAD expression

To better understand how the survival of HCC cells is regulated, we analyzed the expression of the proteins of the BCL2 family in 14 tumor samples and matched nontransformed tissue surrounding the tumors (hereby called “nontumoral tissue”; Fig. 1A). We noticed large variations in the expression levels of some of the members of the BCL2 family. Setting a 3-fold change in expression as threshold, we noted an alteration in the expression levels of the proteins BAD, BID, BNIP3, BAX and BCLXL in more than one tumor (Table 1). The most frequent alteration that we observed was a strong reduction in the expression of BAD, a proapoptotic BH3-only member of the BCL2 family (Table 1): in 50.0% of the tumors examined (n = 7), BAD protein levels sharply decreased compared with nontumoral tissue. A reduction in the expression of two other proapoptotic proteins, BID and BAX, was also noticed, although at a lower frequency.
(Table 1). In contrast, BNIP3, another BH3-only protein of the BCL2 family, was frequently overexpressed (Table 1). None of the other proteins of the BCL2 family showed altered levels of expression: the proteins were expressed at stable levels (e.g., BAK or BIM), were found overexpressed in a single tumor (BCL2), or could not be detected at the protein level in samples obtained from normal liver or HCC.

To evaluate the changes in the expression of the main members of the BCL2 family of proteins in a semiquantitative fashion, a densitometric analysis of the signal measured for each protein was done. The corresponding results are presented in Fig. 1B (results are given in arbitrary units). Median intensity for BAD was 46.9 (95% confidence interval, 34.3–59.0) in normal livers versus 2.5 (95% confidence interval, 1–13.4) in tumors (P < 0.005, Wilcoxon test). Nontumoral tissues also exhibited reduced expression levels of BAD: 16.1 (95% confidence interval, 6.9–48.4) compared with normal liver, although this reduction did not reach statistical significance compared with normal liver samples. This approach confirmed the occurrence of important variations in BAD expression in HCC, a finding that had, to our knowledge, hitherto not been reported.

BAD is an unstable protein in HCC cells

To examine how the expression of BAD is regulated in HCC, we compared BAD mRNA levels in tumors and nontumoral tissues from three patients exhibiting strongly reduced expression levels of BAD: we found no reduction in the levels of BAD mRNA in these tumors by quantitative PCR (Fig. 2A), suggesting that posttranscriptional

![FIGURE 1. Variations in the expression levels of proteins of the BCL2 family in HCC compared with nontumoral tissue. A, three representative tumors (T) presenting with a decrease in their expression of BAD compared with the corresponding nontumoral (NT) tissues. The indicated markers were analyzed by immunoblotting. B, semiquantitative analysis of the expression of the main proteins of the BCL2 family in HCC. Quantitation was done as indicated in the text, and matched NT/T samples were compared with surgical samples from normal liver (N). Results are expressed in arbitrary units (A.U.) and presented as box plots. *, P < 0.005 compared with normal tissue; #, P < 0.005 compared with nontumoral tissue.](image-url)
regulation mainly accounts for the disappearance of the BAD protein in HCC. Based on this observation, we decided to examine the stability of BAD in a panel of HCC cells. We treated four human hepatoma cell lines, PRL/PRF5, Hep3B, Huh7, and HepG2, either with chloroquine (100 μmol/L), a weak base that prevents lysosomal proteolysis, or the proteasome inhibitor MG132 (5 μmol/L). In all HCC cell lines, a 6-hour treatment with MG132 resulted in a near 3-fold increase in the expression of BAD, indicating that the basal half-life of BAD in HCC cells is around 1 hour (Fig. 2B; Supplementary Fig. S1). In contrast, protein levels of BCLXL, taken as control, remained stable (Supplementary Fig. S1). Therefore, in HCC cells, BAD is a short-lived protein that encounters continuous proteasomal degradation.

Sorafenib, a multikinase inhibitor, activates BAD in HCC cells

Among the BH3-only family of protein, BAD is characterized by its proapoptotic effect achieved through the neutralization of antiapoptotic proteins such as BCLXL (13). This aspect of BAD function is under a stringent negative regulation by phosphorylation: Upon trophic factor signaling, prosurvival kinase pathways such as the RAF-MEK-ERK cascade and PKA and PKB kinases negatively regulate the apoptotic potency of BAD (13-19). On this basis, we decided to examine how BAD expression

<table>
<thead>
<tr>
<th>Name</th>
<th>Variation</th>
<th>% Tumors (n)</th>
</tr>
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<tbody>
<tr>
<td>BAD</td>
<td>Decrease</td>
<td>50.0% (7)</td>
</tr>
<tr>
<td>BNIP3</td>
<td>Increase</td>
<td>50.0% (7)</td>
</tr>
<tr>
<td>BID</td>
<td>Decrease</td>
<td>35.7% (5)</td>
</tr>
<tr>
<td>BCLXL</td>
<td>Increase</td>
<td>28.6% (4)</td>
</tr>
<tr>
<td>BAX</td>
<td>Decrease</td>
<td>21.4% (3)</td>
</tr>
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NOTE: Variations are ranked according to their frequency in the present study. We only report those that were found in more than one tumor.
might regulate the survival of HCC cells. Sorafenib, a multi-
kinase inhibitor, is at the moment the sole pharmaceutical
therapy that is effective against HCC (20, 21), and it exerts
targeted proapoptotic effects on HCC cells (22). We anticipated
that a part of the cytotoxic effects of sorafenib might come from its activation of BAD. To examine this possibility, we analyzed BAD expression levels and phosphorylation on Ser75, an important residue targeted by the RAF-MEK-ERK kinase pathway, a target of sorafenib (17-19), in HCC cells. We found that sorafenib increases BAD expression and prevents its inhibitory phosphorylation on Ser75 in Huh7 cells (Fig. 3A). The regulatory effect of sorafenib on BAD expression levels was at least partially accounted for by tran-
scriptional mechanisms because increased levels of BAD mRNA were detected under these conditions (Fig. 3B). Because the RAF kinases are a target of sorafenib, we decided to examine the contribution of MEK inhibition to the effect of sorafenib on BAD. The chemical inhibitor U0126 and the bacterial toxin lethal factor purified from Bacillus anthracis were applied on HCC cells at doses blocking ERK phosphorylation (Supplementary Fig. S2). However, none of these strategies resulted in an increase in BAD expression in HCC cells (Supplementary Fig. S2), suggesting that the inhibition of MEK alone cannot account for the effects of sorafenib on BAD in HCC. We concluded that sorafenib activates BAD in HCC cells by regulating its expression levels and preventing its inhibition by phosphorylation.

BAD determines the sensitivity of HCC cells to the
cytotoxic effects of sorafenib

To examine the role of BAD in the toxicity of sorafenib
in HCC, we decided to either overexpress or reduce the expres-
sion of this protein. Sorafenib-induced cytotoxicity in HCC cells was estimated by measuring chromatin condensation and trypan blue uptake, and by the use of a clonogenicity assay. Using these assays, we consistently observed a reduction in the viability of Huh7 cells exposed to sorafenib at concentrations that block intracellular phosphor-
ylations (Supplementary Fig. S3). Stable cell lines derived
from Huh7 cells overexpressing BAD were found to exhibit a striking 5-fold increase in their sensitivity to sorafenib-induced cytotoxicity measured by determining the mean of the percentage of cells with condensed chromatin (P < 0.05, Student’s t test; Fig. 4A). Simultaneously, their clone-forming ability was strikingly reduced upon sorafenib exposure, even at the lowest doses examined (Fig. 4B). Conversely, an 80% reduction in the expression of BAD that was achieved using a siRNA knockdown strategy produced a 40% decrease in the number of cells with condensed chromatin after sorafenib treatment (P < 0.05, Student’s t test; Fig. 4C). These results suggest that BAD plays an important role in sorafenib cytotoxicity in the Huh7 human HCC cell line. To extend this conclusion to various other HCC cell lines, we tested the existence of a possible correlation between the expression levels of BAD and the cytotoxicity of sorafenib on our panel of HCC cells in cultures (Fig. 4D). Interestingly, a strong corre-
lation was found between the levels of expression of BAD in the four cell lines examined and sorafenib-induced cyto-
toxicity measured by the percentage of trypan blue uptake (Pearson r = 0.954). The cell lines with the highest levels of expression of BAD (PRL/PRF5 and Hep3B) were found approximately two times more sensitive to sorafenib than those with lower expression levels (Huh7 and HepG2) in terms of cytotoxicity (Fig. 4D). No such correlation could be established with the other members of the BCL2 family such as MCL1, BCLXL, or BIM (data not shown). Collectively, these results suggest that BAD is an important effec-
tor of sorafenib-induced cytotoxicity in HCC cells.

The compound ABT-737 potentiates the cytotoxic
effects of sorafenib in HCC cells

Because the proteins of the BCL2 family play a cru-
Celsius role in the response of cancer cells to a variety of
chemotherapeutic agents, pharmaceutical strategies have

![FIGURE 3. Sorafenib activates BAD in HCC. A, sorafenib modulates the expression of BAD and its activation in HCC cells. Huh7 cells were exposed to the indicated concentrations of sorafenib for 18 h, and were analyzed for their expression of BAD and its phosphorylated Ser75 form by immunoblotting. Results shown are representative of three independent experiments. B, quantitative analysis of BAD mRNA levels in HCC cells treated with sorafenib. Huh7 cells were treated for 18 h with sorafenib, and mRNA levels of BAD were analyzed by quantitative PCR. Results are normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA (results are average from two experiments).](image-url)
attempted to directly interfere with the function of these proteins (23). ABT-737 is a recently developed compound that binds to and inhibits the antiapoptotic proteins of the BCL2 family with a specificity mimicking BAD (23, 24). Based on our previous observations indicating that BAD is a key player in the regulation of HCC cell survival, we reasoned that ABT-737 might be a potent adjunct to sorafenib to promote its cytotoxicity in HCC cells. We tested the cytotoxic effects of ABT-737 applied at various concentrations on HCC cells (Supplementary Fig. S4). To investigate the possible synergy between ABT-737 and sorafenib, we applied ABT-737 or its inactive enantiomer at subtoxic concentrations (5 μmol/L). When Huh7 cells were simultaneously exposed to ABT-737 and sorafenib,
we observed a strong synergy between these two compounds in terms of apoptosis induction, estimated by condensed chromatin formation under microscopic examination (Fig. 5A), a clonogenicity assay (Fig. 5B), or with caspase-3 cleavage followed by immunoblotting (Fig. 5C). Similar results were obtained with HepG2 cells (Supplementary Fig. S5A and B), whereas various cell lines of nonliver origin were much less sensitive to sorafenib and ABT-737 (Supplementary Fig. S5C). The combined cytotoxic effect of ABT-737 and sorafenib on HCC cells clearly resulted from the mitochondrial action of these drugs: cytochrome c was released from the mitochondria of Huh7 cells as early as 3 hours after the exposure to ABT-737/sorafenib, but not after a single application of ABT-737 or sorafenib alone, as shown by immunoblotting (Fig. 5D) or immunofluorescence (Fig. 6). We concluded that ABT-737 potentiates the induction of apoptosis by sorafenib in HCC cells.

Molecular basis for the synergistic effect between ABT-737 and sorafenib on HCC cells

To explore the mechanism that accounts for the synergistic effect of ABT-737 and sorafenib on HCC cells, we analyzed the expression of the various members of the BCL2 family of proteins in the panel of HCC cell lines (Fig. 7). Cellular extracts were prepared from Huh7, HepG2, Hep3B, and PRL/PRF5 cell lines exposed for 3 hours to sorafenib and/or ABT-737. We noted that sorafenib altered the expression levels of some proapoptotic as well as antiapoptotic members of the BCL2 family. However, a strong increase in the expression levels of BAD was the most striking effect detected in the four cell lines treated with ABT-737 (Fig. 7). A reduction in the expression of the prosurvival protein MCL1 combined with an increase in the expression of the proapoptotic BIM were also found in the Huh7 cell line, in accordance with the previous report by Liu et al. (22), pointing to MCL1 as a target for sorafenib. However, the expression levels of MCL1 and BIM were stable over this short period in the other three cell lines (Fig. 7). We concluded that the sensitizing action of ABT-737 toward sorafenib correlates with the ability of this compound to increase the expression and apoptotic potency of BAD. Finally, we examined the effect of sorafenib and ABT-737 on the activation of the proapoptotic protein BAX, a core component of the mitochondrial membrane permeabilization machinery (5). An increase in BAX immunoreactivity toward the monoclonal antibody 6A7, raised against
the amino-terminus of BAX, was found in Huh7 cells treated simultaneously with sorafenib and ABT-737 for 3 hours, showing that BAX was readily activated under these conditions (Supplementary Fig. S6). We concluded that the experimental modulation of BCL2 expression and activation afforded by ABT-737 constitutes a promising strategy for the potentiation of apoptosis induction of HCC cells and that BAD is a possible therapeutic target for this type of cancer.

**Discussion**

Reduced sensitivity to apoptosis is a pivotal event in tumor progression (3). A decrease in the susceptibility to apoptosis can theoretically be achieved either by overexpression of antiapoptotic members of the BCL2 family or downregulation of their proapoptotic counterparts. Here, we report a new mechanism capable of preventing apoptosis in HCC cells, involving a reduction in the expression of BAD, a proapoptotic protein of the BCL2 family. Our findings are consistent with the emerging view that the BH3-only proteins are key regulators of apoptosis, whose expression is often altered in tumor cells (10). Recently, BID, another member of the subfamily of BH3-only proteins, was shown to be downregulated in a subset of HCC occurring in the context of viral hepatitis (12). Based on our results, we suggest that the alteration of the expression of selected BH3-only proteins is an important event in a subset of HCC. Because our study is based on a small population of resected HCC, larger cohorts will be required to precisely estimate the frequency of each molecular anomaly and relate them to clinical parameters.

In this study, we explored the role of BAD in cell death, taking into account that BAD was reported to exert an...
important regulatory role in this process in normal liver cells (25). We observed that sorafenib, a kinase inhibitor that is currently the sole medical treatment with shown efficacy on HCC, partially exerts its proapoptotic effects on HCC cells through BAD. Sorafenib increases the expression of BAD and at the same time prevents its inhibitory phosphorylation, thereby sensitizing HCC cells to apoptosis. Although sorafenib is essentially characterized as an inhibitor of the RAF kinases, we found that the sole inhibition of the RAF-MEK-ERK pathway cannot account for the effect of sorafenib on BAD. Chemical and enzymatic inhibitors of the MEK kinases applied on HCC cells did not reproduce the effect of sorafenib on BAD and did not exert a significant cytotoxic effect on HCC cells (data not shown). Interestingly, several kinases and phosphatases regulate BAD in a tiered fashion, and BAD probably serves as a signaling node, integrating essential pathways to regulate the apoptotic program (13, 18). The identification of the kinase(s) that regulate BAD and are the target(s) of sorafenib in HCC is therefore a complex task that will require further investigations. In addition, more studies will be required to examine the functional consequences of the reduction in expression of BAD that we report in HCC. Beyond apoptosis regulation, BAD was recently reported to modulate the cell cycle, autophagy, or glucose metabolism in different cell types (13). Whereas our observations indicate that BAD is a key player in the regulation of apoptosis in HCC cells, further studies examining the function of BAD in liver carcinogenesis are clearly required.

How could our findings help improve the current handling of HCC? Although sorafenib is effective for the treatment of HCC, patients respond in different ways to this...
treatment, and there are no good parameters that can be used to predict which patients will benefit the most from this treatment (26, 27). Assuming that the death of tumor cells is important to achieve HCC regression, we propose that the exploration of BAD expression within the tumor cells might help identify these patients. A more remote application of our findings might be the improvement of therapeutic schemes based on sorafenib. Strategies based on the interference with proteins of the BCL2 family have recently emerged as promising adjuncts to oncogenic kinase inhibitors for the treatment of different cancers (28). Our observations on ABT-737 constitute, to our knowledge, the first report on the use of a BCL2 inhibitor in HCC. Our results suggest that interference with proteins of the BCL2 family might offer clinical benefits in these tumors. Future therapeutic strategies aiming to restore the function of BAD in the cancer cell might therefore help improve the treatment of HCC.

Disclosure of Potential Conflicts of Interest

J-C. Barbare: consultant, Bayer France. Other authors have reported no conflict of interest.

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