Suppression of Bcl-xL Deamidation in Human Hepatocellular Carcinomas

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Abstract

Bcl-xL is an antiapoptotic member of the Bcl-2 family, which inhibits apoptosis initiated by various cellular stresses, and has a pivotal role in the survival of tumor cells. Researchers have previously observed elevated expression of Bcl-xL in some human malignancies. In this study, we present evidence that human Bcl-xL is deamidated at asparagines 52 and 66 and that the rate of Bcl-xL deamidation is significantly lower in hepatocellular carcinomas than in normal or adjacent nontumor liver tissues. Because protein deamidation of Bcl-xL imports a complete “loss of function” of this antiapoptotic molecule, the present study indicates that tumor cells may acquire resistance to apoptosis and a survival advantage by suppressing deamidation as well as by increasing the expression of Bcl-xL. Thus, suppression of Bcl-xL deamidation may play a critical role in the regulation of cell death by apoptosis.

Introduction

Bcl-xL is an antiapoptotic member of the Bcl-2 family, which is located mainly on the outer membrane of mitochondria and inhibits a common pathway of apoptosis, at least in part, by preventing the release of cytochrome c into cytosol (1, 2). We have demonstrated previously (3) that Bcl-xL was expressed in hepatoma cells and that down-regulation of Bcl-xL by antisense oligonucleotide stimulated apoptosis in hepatoma cells in response to cellular stresses, such as serum starvation, p53 activation, and staurosporine treatment. Because down-regulation of endogenously expressed Bcl-xL alone was sufficient for the induction of apoptosis in hepatoma cells by various cellular stresses, Bcl-xL must play an integral role in suppressing apoptosis when hepatoma cells are exposed to proapoptotic conditions. Thus, Bcl-xL that is expressed endogenously in HCCs functions as a pivotal antagonist to apoptosis. However, levels of Bcl-xL expression were higher than those of adjacent nontumor liver tissues or normal livers only in a subset of HCC tissues (3), and therefore overexpression of Bcl-xL alone does not appear to fully account for the increased resistance to apoptosis in HCCs mediated by Bcl-xL. In this study, we show evidence that Bcl-xL is posttranslationally modified by deamidation at asparagines 52 and 66 in its loop domain. We also demonstrate that deamidated Bcl-xL is a major form in normal and nontumor liver tissues, whereas the level of deamidated Bcl-xL is lower than that of unmodified Bcl-xL in the majority of HCC tissues. Bcl-xL deamidation has been shown to produce a complete loss of the antiapoptotic function of Bcl-xL. We propose that suppression of Bcl-xL deamidation may be an important mechanism by which malignant tumors acquire resistance to apoptosis.

Materials and Methods

Human Liver Tissues and Cell Lines. We obtained 20 pairs of HCCs and adjacent nontumor counterparts at the time of surgical resection. Ten normal human liver tissues adjacent to hepatic metastatic tumors of colon cancer were obtained at the time of surgery. We stored all tissues at −80°C until used. Human hepatoma cell lines HepG2 and FOCUS (5) were cultured with DMEM supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO).

Western Immunoblot. We homogenized cells or tissues in a lysis buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonfluoride, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and PBS (pH 7.4). Cell lysates were centrifuged at 10,000 × g for 20 min at 4°C. Protein content in the supernatants was determined by a BCA protein assay kit (Pierce, Rockford, IL). Twenty μg protein/lane was electrophoretically separated on 10−12% SDS-PAGE and transferred to nitrocellulose membranes. We then incubated nitrocellulose membranes with rabbit anti-Bcl-xL polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to detect Bcl-xL. We used horseradish peroxidase-conjugated antirabbit antibody and an enhanced chemiluminescence system (NEN Life Science Products, Boston, MA) to visualize bound primary antibodies. Optical densities of bands were analyzed by using NIH Image Program, Version 1.61.

In Vitro Phosphatase Treatment. We homogenized HepG2 cells treated with vincristine or paclitaxel (150–500 ng/ml) for 18 h or human liver tissues with a lysis buffer [150 mM NaCl, 1% Triton X-100, 50 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonfluoride, and 10 mM Tris-Cl (pH 7.4)]. We centrifuged the cell lysates at 10,000 × g for 20 min at 4°C. Twenty μg of mammalian lysate were incubated with or without 200 units of phosphatase (New England BioLabs, Beverly, MA) at 30°C for 2 h and then subjected to Western analysis.

In Vitro Deamidation Reaction and Isoaspartate Analysis. Dr. N. Ino (University of Michigan Medical School, Ann Arbor, MI) generously provided recombinant Bcl-xL protein, which contains entire human Bcl-xL, and NH2-terminal histidine and hemagglutinin epitope (6). We incubated the recombinant protein or tissue lysates (20 μg) with an alkaline buffer [25 mM glycine-NaOH (pH 10.1)] or a neutral buffer [50 mM Tris-Cl (pH 7.2)] for 6 or 16 h at either 4°C or 30°C. The mobility on SDS-PAGE was determined by Western immunoblot or Coomassie Brilliant Blue staining.

The amount of isoaspartate in recombinant proteins was detected by enzyme-magnetic transfer of 3H-labeled methyl groups from S-adenosyl-L-methionine in a reaction catalyzed by protein L-isoaspartyl methyltransferase (ISOQUANT™ isoaspartate detection kit; Promega, Madison, WI; Ref. 7). The result was expressed as isoaspartate residue/protein (mol/mol) using isoaspartate-containing peptide as a reference.

Site-directed Mutagenesis. A Hemagglutinin-tagged human bcl-xL expression plasmid, pcDNA_HABcl-xL, was generously provided by Dr. G. Nunez (University of Michigan Medical School). Plasmids containing mutant bcl-xL encoding alanine 52, alanine 66, and/or alanine 185 instead of asparagine (N52A, N66A, N185A, N52A/N66A, N66A/N185A, N52A/N185A, and N52A/N66A/N185A) were generated from pcDNA_HABcl-xL by site-directed mutagenesis with the QuickChange Site-Directed Mutagenesis System (Stratagene, La Jolla, CA), and verified by sequencing. Focus cells were transfected with each original plasmid or mutant plasmids using Lipofectin reagent (Life Technologies, Inc., Gaithersburg, MD). The cell lysates were prepared 2 days after transfection and subjected to Western analysis as described earlier.
Results

Bcl-xL Migrated as Doublet Bands in Human Liver Tissues.

We investigated the expression of Bcl-xL in 20 surgically obtained human HCC tissues, 20 adjacent nontumor liver tissues, and 10 normal livers. Western blots with antibodies to Bcl-x revealed two major bands in these human liver tissues with apparent molecular masses of 30 and 32 kDa (Fig. 1A). Interestingly, the more slowly migrating Bcl-xL (the 32-kDa band) was expressed at a higher level than that of the fast-migrating Bcl-xL (the 30-kDa band) in normal human liver tissues as well as in the adjacent nontumor counterparts of HCC tissues (Fig. 1A). In contrast, a fast-migrating 30-kDa Bcl-xL band was expressed at a level higher than that of the 32-kDa Bcl-xL band in a majority of HCC tissues. The ratio of absorbance of the 30-kDa band to that of the 32-kDa band (30 kDa/32 kDa) was greater than 1 in 11 of 20 cases of HCCs, 1 of 20 adjacent nontumor liver tissues, and in none of the 10 normal livers (P < 0.01, HCC versus adjacent nontumor tissue or normal liver by Bonferroni’s t test after significant ANOVA; Fig. 1B). Thus, the 30-kDa band is a major form of Bcl-xL in a majority of HCC tissues, whereas the relative amount of 32-kDa Bcl-xL is increased in normal and nontumor liver tissues.

The More Slowly Migrating 32-kDa Bcl-xL Is Not Phosphorylated.

Recent research reported that microtubule-disrupting agents induce phosphorylation of Bcl-xL and that the phosphorylation produces a more slowly migrating Bcl-xL band on the gel electrophoresis (8). Therefore, we sought to determine whether or not the observed 32-kDa band was a phosphorylated form of the Bcl-xL protein. We treated HepG2 hepatoma cells, which endogenously express both the 30- and 32-kDa bands of Bcl-xL, with microtubule-disrupting agents vincristine and paclitaxel. The treatment of HepG2 cells with vincristine or paclitaxel produced a new band on SDS-PAGE at a position of approximately 33 kDa that migrated more slowly than endogenous 30- and 32-kDa Bcl-xL (Fig. 2). This 33-kDa band dissipated completely by in vitro λ-phosphatase treatment before electrophoresis, but the same treatment did not affect the migration patterns of the 30- and 32-kDa bands that were endogenously expressed in HepG2 cells. Notably, the treatment of tissue lysate with λ-phosphatase did not affect the migration pattern of the 30- and 32-kDa Bcl-xL that was endogenously expressed in HCC tissues either. Thus, both the 30- and 32-kDa Bcl-xL observed in human hepatoma cell lines, as well as in human liver tissues, are not phosphorylated, and therefore 32-kDa Bcl-xL differs from the phospho-

formation of a more slowly migrating band is associated with the deamidation of Bcl-xL.

Recombinant rat Bcl-xL has been shown to undergo protein deamidation (9). An asparagine that is followed by a glycine is susceptible to deamidation, and human Bcl-xL protein contains three such candidate sites (asparagines 52, 66, and 185; Ref. 10). The difference in electrophoretic mobility between 30- and 32-kDa Bcl-xL may result from deamidation of the protein. Alkaline conditions augment the rate of deamidation, in which asparagines are converted to a mixture of aspartates and isoaspartates (10, 11). We treated HCC tissue lysates with an alkaline buffer, which promotes in vitro asparagine deamidation (11), and then examined the expression of Bcl-xL by Western blot analysis. Treatment of the tissue lysates with the alkaline buffer (pH 10) for 6 h at 30°C, but not at 4°C, resulted in an increase of the 32-kDa band and a decrease of the 30-kDa band (Fig. 3A). Treatment of the tissue lysates with the same alkaline buffer for a longer incubation time (16 h) at the same temperature further increased the intensity of the 32-kDa band and caused a dissipation of the 30-kDa band. Incubation of the same lysates with a neutral buffer (pH 7.4), either at 4°C or 30°C for 16 h, did not affect the mobility of Bcl-xL on SDS-PAGE. Thus, alkaline conditions, which facilitate protein deamidation, appeared to modify 30-kDa Bcl-xL and produce 32-kDa Bcl-xL.

Fig. 1. Bcl-xL migrates as doublet bands of 30 and 32 kDa in human liver tissues. A, Western analysis for Bcl-xL expression in HCC (T), adjacent nontumor liver tissues (NT), and normal liver tissues. A representative blot is shown. Both the 30- and 32-kDa bands were specifically reactive to anti-Bcl-x antibody because they became undetectable in the presence of the immunizing peptide (data not shown). B, the ratio of absorbance of the 30-kDa band to that of the 32-kDa band (30 kDa/32 kDa) in normal livers (Normal), nontumor livers (NT), and HCCs (T).
To confirm the deamidation of Bcl-xL, we investigated the isoaspartate content of the recombinant human Bcl-xL that had been treated with an alkaline buffer under the same experimental conditions, because deamidation converts asparagine residues in polypeptides to a mixture of aspartates and isoaspartates, and the remaining asparagines undergo spontaneous conversion to isoaspartates. The methyl-accepting capacity of the recombinant Bcl-xL for protein isoaspartyl methyl transferase, which correlates with the isoaspartate content of the protein, significantly increased after the alkaline treatment (0.43 ± 0.09 versus 0.04 ± 0.01 mol/mol protein before alkaline treatment; P < 0.01 by Student’s t test). Thus, in vitro alkaline treatment produced deamidation of the Bcl-xL protein and increased its isoaspartate content, a product of asparagine deamidation. In addition, the same alkaline treatment resulted in a modification of recombinant Bcl-xL to migrate more slowly on SDS-PAGE (Fig. 3A). These results further support the idea that the more slowly migrating species is deamidated Bcl-xL protein.

### Bcl-xL Is Deamidated at Asparagines 52 and 66.
We prepared seven mutant bcl-xL constructs (N52A, N66A, N185A, N52A/N66A, N66A/N185A, N52A/N185A, and N52A/N66A/N185A) in which alanine(s) were substituted for asparagine 52, 66, or 185 as described in “Materials and Methods.” Because alanines are not susceptible sites for protein deamidation, substitution of alanines for asparagines would prevent deamidation of Bcl-xL. The mutant bcl-xL expression plasmids were then transfected into FOCUS hepatoma cells. FOCUS cells that were transfected with a wild-type bcl-xL plasmid expressed Bcl-xL as doublet bands (Fig. 4A). On the other hand, a more slowly migrating Bcl-xL band was not detected in FOCUS cells that were transfected with a N52A/N66A or N52A/N66A/N185A plasmid, although FOCUS cells that were transfected with a N52A, N66A, N185A, N66A/N185A, or N52A/N185A plasmid expressed Bcl-xL as doublet bands (Fig. 4A). Thus, substitution of alanines for both asparagines 52 and 66 inhibited the production of the more slowly migrating Bcl-xL, but substitution of alanines for asparagine 52, 66, or 185 alone; both asparagines 66 and 185; or both asparagines 52 and 185 did not inhibit the generation of the more slowly migrating Bcl-xL protein. These results indicate that asparagine 52 and 66 residues are the susceptible sites of protein deamidation and are necessary for the production of the more slowly migrating form of Bcl-xL on SDS-PAGE.

To confirm that asparagine 52 and 66 residues are responsible for modification of Bcl-xL protein by deamidation, we treated the lysates of FOCUS cells that were transfected with wild-type or mutant bcl-xL plasmids at alkaline conditions before Western analysis. Alkaline treatment increased the density of the more slowly migrating Bcl-xL band in FOCUS cells that were transfected with a wild-type bcl-xL plasmid, N52A, N66A, N185A, N66A/N185A, or N52A/N185A, but same alkaline treatment did not increase the expression of the more slowly migrating band at all in FOCUS cells that were transfected with N52A/N66A or N52A/N66A/N185A mutant bcl-xL plasmid (Fig. 4B). These results confirm that substitution of alanines for both asparagines 52 and 66 prevents the generation of the more slowly migrating Bcl-xL band on SDS-PAGE even under the experimental conditions, in which protein deamidation is promoted in vitro by alkaline treatment (Fig. 3B), and therefore we conclude that the asparagine 52 and 66 residues of Bcl-xL are susceptible sites for protein deamidation.

### Discussion
We have reported previously (3) that Bcl-xL plays an important role in inhibiting apoptosis induced by several stress-inducing conditions, such as serum starvation, staurosporine treatment, or p53 activation, and that Bcl-xL is overexpressed in one-third of human HCC tissues. In the present study, we determined that human Bcl-xL was posttranslationally modified by deamidation at asparagine residues and that the deamidated Bcl-xL migrates more slowly than the unmodified protein on SDS-PAGE. To confirm the deamidation of Bcl-xL, we used a recombinant protein of human Bcl-xL and assessed the content of isoaspartate, a product of asparagine deamidation. We found that in vitro alkaline treatment, which promotes protein deamidation, significantly increased the isoaspartate content in recombinant Bcl-xL and caused the protein to migrate more slowly on SDS-PAGE (Fig. 3B). We also established that substitution of alanines for asparagine 52 and 66 prevented the endogenous production of a more slowly migrating band using mutant bcl-xL generated by site-directed mutagenesis (Fig. 4). Deverman et al. (4) recently reported that DNA-damaging agents induced deamidation of Bcl-xL in some tumor cell lines at the same asparagine 52 and 66 residues we have described here. These asparagine residues are located on the unstructured loop domain of Bcl-xL, and they seem to be most susceptible to protein deamidation based on the three-dimensional structure (12, 13). Asparagine deamidation results in the formation of isoaspartate, which can dramatically affect the conformation of a polypeptide and its biological activity (11). Therefore, deamidation of these asparagines imports susceptibility to apoptosis by disrupting the ability of Bcl-xL to block the proapoptotic activity of BH3 domain-only proteins (4).

We have also demonstrated that most, if not all, normal livers and nontumor liver tissues adjacent to HCCs expressed the 32-kDa deamidated form of Bcl-xL at a higher level than that of the 30-kDa unmodified species, whereas the level of deamidated Bcl-xL was lower than that of the unmodified protein in the majority of HCC tissues (Fig. 1A). Asparagine deamidation is a nonenzymatic process of protein modification, which occurs under relatively mild conditions, even in live cells. Because this process can be accelerated in vitro under certain conditions, we examined whether or not long-term storage of liver tissues at −80°C affected the expression and migration patterns of Bcl-xL on SDS-PAGE. In the preliminary experiments, we examined the expression and migration patterns of Bcl-xL in liver tissues that were either freshly isolated from rats or stored for more than 4 years, and we found that they demonstrated similar migration patterns.4 Because the human liver tissues that we used in our study

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4 Unpublished data.
were snap-frozen in liquid nitrogen at the time of surgery and stored at −80°C under the same conditions, the difference in the migration pattern between HCC and adjacent nontumor liver tissues is authentic and is not the result of an experimental artifact. Interestingly, HepG2 and other hepatoma cell lines expressed 30-kDa unmodified Bcl-xL as a major band on SDS-PAGE (Fig. 2 and Ref. 3). In addition, FOCUS cells that were transfected with a wild-type human bcl-xL plasmid also expressed the unmodified Bcl-xL as a major band (Fig. 4B). Thus, deamidation of Bcl-xL protein appears to be suppressed endogenously in human hepatoma cells.

Elevated expression of Bcl-xL has been shown in some other human malignancies such as gastrointestinal adenocarcinomas (14), Kaposi’s sarcoma (15), and multiple myeloma (16). The overexpression of Bcl-xL is considered to be one of the mechanisms by which tumor cells acquire resistance to apoptosis. Because Bcl-xL deamidation is associated with a complete “loss of function” of antiapoptotic Bcl-xL (4), HCCs may acquire a resistance to apoptosis and a survival advantage in vivo by suppressing deamidation of this protein as well by increasing the expression of this molecule. The present study represents the first demonstration that deamidation of endogenous Bcl-xL is suppressed in tumors, raising the possibility that, in addition to increased expression of Bcl-xL, suppression of the deamidation of this molecule may play an important role in malignant tumors becoming resistant to apoptosis.

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References

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