The American Journal of Pathology, Vol. 169, No. 5, November 2006

DOI: 10.2353/ajpath.2006.060234

Tumorigenesis and Neoplastic Progression

Fas-Mediated Apoptosis in Cholangiocarcinoma Cells Is Enhanced by 3,3′-Diindolylmethane through Inhibition of AKT Signaling and FLICE-Like Inhibitory Protein

Yabing Chen,* Jianmin Xu,* Nirag Jhala,* Pritish Pawar,* Zeng B. Zhu,* Liping Ma,* Chang-Hyun Byon,* and Jay M. McDonald*†

From the Department of Pathology, University of Alabama at Birmingham; and the Veterans Administration Medical Center,† Birmingham, Alabama*

4Stimulation of Fas-mediated apoptosis has been promoted as a potential therapy for many cancers, including cholangiocarcinoma. We have previously reported that Fas-resistant, but not Fas-sensitive, cholangiocarcinoma cells are tumorigenic in nude mice. The present studies sought to identify molecular targets that promote Fas-mediated apoptosis in cholangiocarcinoma. We found that Fas-resistant cholangiocarcinoma cells exhibited increased constitutive phosphorylation of AKT compared with Fas-sensitive cells. Increased phosphorylation of AKT was also demonstrated in human cholangiocarcinoma tumors and was evident in a mouse xenograft cholangiocarcinoma model. Furthermore, we found that 3,3′-diindolylmethane (DIM), a vegetable autolysis product, promoted Fas-mediated apoptosis of cholangiocarcinoma cells. DIM inhibited phosphorylation of AKT and activation of FLICE-like-inhibitory-protein (FLIP). Inhibition of phosphatidylinositol 3-kinase/AKT decreased FLIP activation and promoted Fas-mediated apoptosis. By contrast, adenovirus-mediated constitutively activated AKT protected cholangiocarcinoma cells from Fas-mediated apoptosis. Decreased activation of extracellular signal-regulated kinase and nuclear factor-κB and increased activation of caspase-3, -8, and -9 were associated with inhibition of AKT and FLIP. These results support AKT and FLIP as potential molecular targets and DIM as a potent compound for cholangiocarcinoma intervention. ***(Am J Pathol 2006, 169:1833–1842; DOI: 10.2353/ajpath.2006.060234)***

Cholangiocarcinoma is a highly malignant neoplasm originating from cholangiocytes of the intra- and extrahepatic biliary system.[1] It is a generally fatal cancer representing 20% of all hepatobiliary malignancies in the United States, a number that is increasing every year.[2] Stimulation of apoptosis has been promoted as a potential therapy for many cancers, including cholangiocarcinoma. A number of molecules have been suggested to regulate apoptosis in cholangiocarcinoma cells, including Mcl-1,[2] cyclooxygenase-2,[3] and the Fas death receptor system.[4] Results from our group and others have suggested that regulation of Fas-mediated apoptosis is a promising therapeutic avenue for cholangiocarcinoma.[4–6] Down-regulation of Fas expression protects cholangiocarcinoma and other tumor cells from Fas-mediated apoptosis.[4,6] In human intrahepatic cholangiocarcinoma, down-regulation of Fas is correlated with increased tumor size and short survival of patients.[6] Consistent with these observations, we have demonstrated that Fas-resistant, but not Fas-sensitive, cholangiocarcinoma cells are tumorigenic in nude mice.[4] In the present studies, we sought to identify molecular targets downstream of the Fas death receptor that promote Fas-mediated apoptosis in cholangiocarcinoma.

Suppression of apoptosis by intracellular survival factors is important in the development of chemoresistance.[7] We have previously reported that the cleaved active form of FLICE-like inhibitor protein (FLIP), a death inhibitor in the Fas-stimulated apoptosis pathway, is increased in Fas-resistant cells.[5] FLIP is an enzymatically inactive homologue of caspase-8, a death mediator in the

Supported by the Veteran's Administration (merit award to J.M.M.).

Accepted for publication August 1, 2006.

A guest editor acted as editor-in-chief for this manuscript. No person at the University of Alabama at Birmingham was involved in the peer review process or final disposition for this article.

Address reprint requests to Yabing Chen, Ph.D., Department of Pathology, University of Alabama at Birmingham, LHRB 511, 1530 3rd Ave. South, Birmingham, AL 35294. E-mail: ybchen@path.uab.edu.

Fas-mediated apoptosis pathway. Recently, FLIP has been shown to divert Fas-mediated death signals into those for cell proliferation in lymphocytes.[8] In addition, up-regulation of FLIP decreased β-cell apoptosis and restored β-cell proliferation.[9] Accordingly, our observation that increased activation of FLIP in Fas-resistant cholangiocarcinoma cells supports a potential link between FLIP and resistance of cells to Fas-mediated apoptosis. Consistently, inhibition of FLIP by an antagonist of calcium/calmodulin-dependent protein kinase II (CaMKII) renders malignant glioma cells more sensitive to Fas-mediated apoptosis.[10]

The protein kinase B/AKT signaling pathways play important roles in regulating apoptosis of cholangiocarcinoma.[11,12] Several studies have suggested that increased constitutive phosphorylation of AKT is associated with increased FLIP and decreased apoptosis.[13–15] However, whether CaMKII and AKT signaling affect FLIP or Fas-mediated apoptosis in cholangiocarcinoma has not been determined. Observations from our group and others have implicated FLIP as a potential candidate target for sensitizing cells to Fas-mediated apoptosis, and antagonists of CaMKII and/or AKT signaling pathway may inhibit FLIP, thus promoting Fas-mediated apoptosis in cholangiocarcinoma cells.

Emerging evidence demonstrates effects of the indole-derivative indole-3-carbinol (I3C) on apoptosis and proliferation of a variety of human cancer cell lines *in vivo* and *in vitro*, including breast, prostate, cervical, and colon cancer.[11,16–18] I3C is a natural vegetable autolysis product from cruciferous vegetables, such as broccoli, Brussels sprouts, cabbage, and cauliflower. The dimeric product 3,3′-diindolylmethane (DIM) is a major component of I3C that has been suggested to be responsible for its biological effects *in vivo*.[19] In prostate cancer cells, inactivation of AKT appears to be responsible for the apoptotic effects of I3C.[18,20] Accordingly, we investigated whether I3C and DIM can enhance Fas-mediated apoptosis in cholangiocarcinoma cells. Results from our studies suggest that AKT and FLIP are potent molecular targets for cholangiocarcinoma prevention and therapy and that DIM may represent a novel nontoxic chemotherapeutic reagent to target these molecules in cholangiocarcinoma.

Materials and Methods

Reagents and Antibodies

I3C and DIM were purchased from LKT Laboratories (St. Paul, MN). All protease inhibitors including AKT inhibitor IV (AKTI), LY 294002, Wortmannin, and calcium/calmodulin-dependent kinase II (CaMKII) antagonist, KN-93, were obtained from Calbiochem (La Jolla, CA). Fas-activating antibody (CH-11) was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-human FLIP antibody was purchased from Alexis Biochemicals (San Diego, CA). Anti-human CaMK II antibody was obtained from BD Biosciences (San Jose, CA). All other antibodies including AKT, phosphorylated-AKT (serine 473), ERK, phosphorylated-ERK (T202/Y204), phosphorylated-IκB, caspase-3, caspase-8, and caspase-9 were purchased from Cell Signaling Technology (Beverly, MA).

Cell Culture

The cholangiocarcinoma cell line Sk-ChA-1 was kindly provided by Dr. A. Knuth (Ludwig Institute for Cancer Research, London, UK). Sk-ChA-1 subpopulations that are sensitive or resistant to Fas-mediated apoptosis were established and characterized as we previously described.[4] Cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), and 10% heat-inactivated fetal bovine serum. All experiments were performed in serum-free Dulbecco's modified Eagle's medium (DMEM) with Hams' nutrient mixture F12 (DMEM/F12; Life Technologies, Inc., Grand Island, NY).

Immunohistochemistry Analysis

Immunohistochemistry analyses were performed to determine the expression of phosphorylated AKT in human cholangiocarcinoma sections and in mouse xenograft tumors derived from human cholangiocarcinoma cells.

Patients

Formalin-fixed, paraffin-embedded tissue sections (5 μm) from patients (six males and four females) with extrahepatic biliary tract carcinoma (extrahepatic cholangiocarcinoma) were studied. Five adjacent nonneoplastic large ducts of these resected cholangiocarcinoma and five nonneoplastic large biliary duct sections obtained during resection of tissues for carcinoma of the head of the pancreas served as controls. Ten sections were analyzed per tissue sample.

Mouse Xenograft Tumors

Experiments were performed as we previously described.[4,5] In brief, 8-week-old athymic (nu/nu) female BALB/c mice (Charles River Laboratories, Wilmington, MA) were used for tumor inoculation. The animal use protocol was approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. In brief, Fas-resistant cholangiocarcinoma cells [5×10^6 cells in 200 μl of phosphate-buffered saline (PBS)/site] were inoculated subcutaneously into the flanks of seven mice. Tumors were examined and measured every week. After 4 weeks, tumors were removed. After determination of the tumor size, these tumors were placed in buffered formalin and embedded in paraffin.

Staining

Slides were deparaffinized, rehydrated, and then heated in 10 mmol/L citrate buffer (pH 6.0) for 40 minutes

using a steamer. The slides were blocked with 10% normal rabbit serum for 30 minutes. After washing in PBS, the slides were incubated with anti-phosphorylated AKT-1 antibody (concentration 1:50; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. Then the secondary biotinylated anti-rabbit IgG was applied for 30 minutes followed by 30 minutes of incubation with a polymer (Envision plus; DAKO, Carpinteria, CA). Slides used as negative controls did not receive incubation with primary antibodies. After rinsing, slides were visualized by diaminobenzidine chromogen solution and counterstained with routine hematoxylin and eosin. Brown staining in greater than 10% of the cells was considered as a positive stain.

Adenovirus Transduction of Cells

Adenovirus-mediated altered expression of AKT was used to determine the direct effect of phosphorylation of AKT in cholangiocarcinoma cells on Fas-mediated apoptosis. Adenovirus encoding constitutively active AKT (Ad-AKT) and dominant-negative AKT (DN-AKT) were kindly provided by Dr. Kenneth Walsh (Boston University School of Medicine).[21] Cholangiocarcinoma cells were grown in six-well plates and infected for 2 hours in DMEM/F12 and 16 hours in serum-containing media with recombinant adenovirus stocks at a multiplicity of infection of 100 pfu/cell and subsequently cultured in growth medium for 48 hours before CH-11 exposure. Apoptosis of cells transduced with Ad-AKT and DN-AKT was determined and compared with cells transduced with control adenovirus.

Assessment of Apoptosis

Fas-mediated apoptosis was induced with Fas-activating antibody (CH-11; Upstate Biotechnology) at concentrations of 250 to 500 ng/ml. The effects of I3C, DIM, CaMK II antagonist KN-93, and pharmacological inhibitors of phosphatidylinositol 3-kinase/AKT signaling, including LY294002, Wortmannin, and AKTI, on Fas-mediated apoptosis were determined by preincubating cells in serum-free DMEM/F12 with selected concentrations of these inhibitors for 30 minutes and subsequentially exposing cells to CH-11 (500 ng/ml) in DMEM/F12 for 24 hours. Annexin V and propidium iodide (PI) staining was performed to assess apoptosis with the use of the Annexin V-fluorescein isothiocyanate apoptosis detection kit (BD Biosciences, Palo Alto, CA) and analyzed by flow cytometry. The percentage of cells that were Annexin V-positive and PI-negative was considered as apoptotic.

Western Blot Analysis

Western blot analysis was performed to determine the intracellular expression of proteins. Cells were washed in ice-cold PBS, and protein extracts were isolated with lysis buffer containing 100 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% sodium dodecyl sulfate, 10% glycerol, 1% Triton X, 5 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L phenylmethyl sulfonyl fluoride, 10 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L β-glycerophosphate, and protease inhibitor cocktail tablets (Roche, Indianapolis, IN).

Concentrations of protein were determined with the Bicinchoninic acid kit (Sigma, St. Louis, MO). Extracted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon P membranes (Millipore, Bedford, MA). Membranes were blocked in Tris-buffered saline containing 5% nonfat milk and 0.1% Tween 20 and incubated with primary antibodies. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Research Diagnostics, Inc., Flanders, NJ) was used to confirm equal protein loading. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) and enhanced chemiluminescence detection reagents (Amersham Biosciences, Piscataway, NJ). The density of bands was analyzed with the use of densitometry and Kodak software (Eastman-Kodak, Rochester, NY).

Determination of Surface Fas Expression

Surface Fas expression was determined to test whether up-regulation of surface Fas expression mediated the apoptotic effect of AKT inhibition. After treatment, cells were washed twice with ice-cold PBS, detached with trypsin and resuspended in 1% bovine serum albumin in PBS. Cells were then stained with phycoerythrin-conjugated anti-Fas antibody (BD Pharmingen, San Diego, CA) or phycoerythrin-conjugated isotope control IgG1 (mouse phycoerythrin-IgG1). After washing twice in PBS plus 1% bovine serum albumin, cells were fixed in 3% paraformaldehyde, washed, and resuspended in PBS plus 1% bovine serum albumin. The expression of surface Fas was analyzed by flow cytometry.

Statistical Analysis

Results are expressed as means $\pm$ SD. Differences between two groups were identified with Student's *t*-test. For multiple groups, one-way analysis of variance and Student-Newman-Keuls tests were used to identify differences. Significance was defined as $P < 0.05$.

Results

Increased Expression of CaMKII and Phosphorylation of AKT in Fas-Resistant Cells

We have previously isolated subpopulations of cholangiocarcinoma cells, SK-ChA-1, and determined that two subpopulations of cholangiocarcinoma cells are sensitive (Fas-S) or resistant (Fas-R) to Fas-mediated apoptosis based in part on their surface expression of Fas.[4] In the present studies, we sought to identify

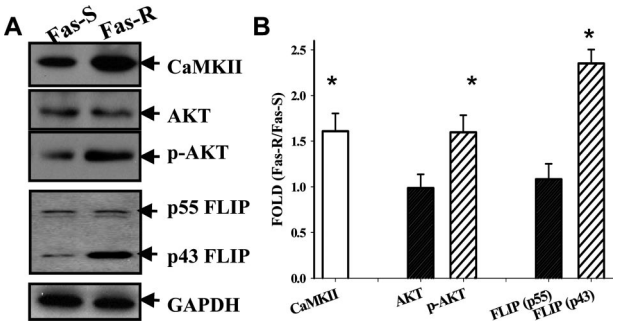


Figure 1. Expression and/or activation of CaMKII, AKT, and FLIP in Fas-resistant and Fas-sensitive cholangiocarcinoma cells. **A:** Western blot analysis of the expression of CaMKII, AKT, pAKT, and FLIP in Fas-resistant (Fas-R) and Fas-sensitive (Fas-S) cells. Representative blot of six independent experiments is shown. **B:** The density of each band was normalized to the density of GAPDH, and comparison of the mean densities of each protein in Fas-resistant cells compared with that in Fas-sensitive cells was calculated as fold changes. Increased expression of CaMKII and increased constitutive phosphorylation of AKT (pAKT) and FLIP (cleaved FLIPp43) were demonstrated in Fas-resistant cells ($n = 6$, $^*P < 0.05$).

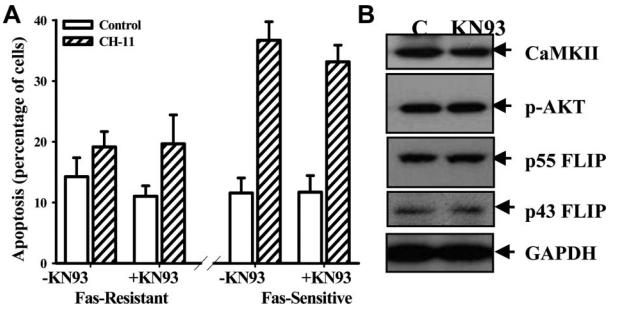


Figure 2. Inhibition of CaMKII on Fas-mediated apoptosis in cholangiocarcinoma cells. **A:** Cholangiocarcinoma cells were preincubated with or without KN-93 (10 μmol/L) for 30 minutes and subsequently exposed to Fas-activating antibody (CH-11, 500 ng/ml) for 24 hours at 37°C. Results shown are from four independent experiments performed in duplicate. **B:** Fas-resistant cells were preincubated without (C) or with KN-93 (10 μmol/L) for 30 minutes and subsequently exposed to Fas-activating antibody (CH-11) for 24 hours. Representative blots for CaMKII, pAKT, and FLIP of four independent experiments are shown.

downstream molecular targets that sensitized cholangiocarcinoma cells to Fas-mediated apoptosis. An increased cleaved active form of FLIP (FLIPp43) has been identified in Fas-resistant cells.[5] Recently, CaMKII and AKT signaling have been demonstrated to affect FLIP, thus regulating Fas-mediated apoptosis.[10–12] Accordingly, we determined the expression and/or activation of CaMKII and AKT in these subclones of cholangiocarcinoma cells. As depicted in Figure 1, A and B, increased expression of CaMKII was evident in Fas-resistant cells (fold increase = 1.6 ± 0.2, $n = 6$; $P < 0.05$ for Fas-R versus Fas-S). Further, increased phosphorylation of AKT and FLIP, as determined by phosphorylated AKT and cleaved FLIPp43, were demonstrated in Fas-resistant cells (fold increases: pAKT = 1.6 ± 0.2 and FLIPp43 = 2.4 ± 0.2, $n = 6$; $P < 0.05$ for Fas-R versus Fas-S). No changes in total AKT and p55FLIP were observed. These results suggested that increased CaMKII and phosphorylation of AKT might be associated with the increase in active FLIPp43 and resistance of cholangiocarcinoma cells to Fas-mediated apoptosis. We therefore investigated whether antagonists of CaMKII and inhibitors of AKT signaling pathway could affect FLIP and sensitize cholangiocarcinoma cells to Fas-mediated apoptosis.

CaMKII Antagonist Does Not Increase Fas-Mediated Apoptosis of Cholangiocarcinoma Cells

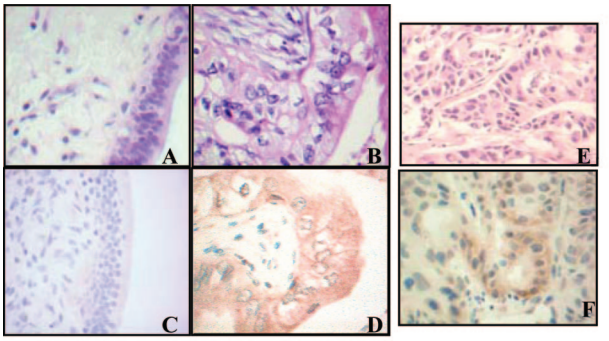
A pharmacological antagonist of CaMKII, KN-93, was used. Preincubation of cells with KN-93 (10 μmol/L) did not induce apoptosis of Fas-resistant cells or Fas-sensitive cells (Figure 2A, controls). Furthermore, preincubation of cells with KN-93 did not affect Fas-mediated apoptosis in Fas-resistant or Fas-sensitive cells (Figure 2A, CH-11). To determine the effect of KN-93 on AKT and FLIP, Western blot analysis was performed with protein extracts from Fas-resistant cholangiocarcinoma cells. Preincubation with KN-93 did not affect the expression of CaMKII. Furthermore, no effects of KN-93 on the phosphorylation of AKT and activation of FLIP were demonstrated (Figure 2B). These results suggest that inhibition of CaMKII is not responsible for sensitizing cholangiocarcinoma cells to Fas-mediated apoptosis.

Increased Phosphorylation in Tumors from Patients with Cholangiocarcinoma

Having demonstrated increased constitutive phosphorylation of AKT in Fas-resistant cholangiocarcinoma cells (Figure 1), we determined the phosphorylation of AKT in tumors from patients with cholangiocarcinoma by immunohistochemical analysis and compared that to adjacent nonneoplastic tissues. Increased phosphorylation of AKT was observed in 5 of 10 cholangiocarcinoma tumors. By contrast, phosphorylation of AKT was not identified in any of the 10 nonneoplastic epithelium samples (Figure 3). As depicted in Figure 3, the nonneoplastic epithelium (A and C) were negative for pAKT, whereas the middle panels (B and D) show a representative cholangiocarcinoma that is positive for pAKT staining. Thus, in this small series, activated AKT is associated with cholangiocarcinoma but not adjacent nonneoplastic bile duct epithelium.

Increased Phosphorylation of AKT in Mouse Xenograft Cholangiocarcinoma Tumors

The expression of pAKT was further determined in mouse xenograft cholangiocarcinoma tumors. Fas-resistant cholangiocarcinoma cells were injected into nude mice and tumor growth was monitored weekly for 4 weeks. Seven tumors with an average diameter of 11.0 ± 1.6 mm ($n = 7$) were examined for pAKT expression. The expression of pAKT was identified in all sections from all seven tumors with mean percentage of positive tumor cells of 27.1 ± 11.1%. No adjacent stromal cells were positive for pAKT staining (Figure 3, E and F).



	pAKT negative	pAKT positive	Total
Non-neoplastic Ducts	10	0	10
Cholangiocarcinoma	5	5	10
Xenograft tumors	0	7	7

Figure 3. Immunohistochemistry of phosphorylated AKT in human biliary ducts and cholangiocarcinoma and mouse xenograft. Immunohistochemical staining was performed with tissues from human cholangiocarcinoma and mouse xenografts. **A:** H&E staining of a nonneoplastic duct. **B:** H&E staining of section from a cholangiocarcinoma demonstrating extrahepatic biliary tract carcinoma with enlarged pleomorphic nuclei showing nuclear membrane irregularity. **C:** Immunohistochemical staining of the benign biliary tract mucosa showing no immunohistochemical expression of pAKT. **D:** Immunohistochemical staining of the extrahepatic biliary tract carcinoma demonstrating expression of pAKT in the cytoplasm. **E:** H&E staining of section from a cholangiocarcinoma mouse xenograft showing tumor cells. **F:** Immunohistochemical staining of the tumor cells and adjacent stromal cells demonstrating expression of pAKT in the tumor cells but not the stromal cells. The table below the figures summarizes the number of samples in each category. Original magnifications, ×400.

I3C and DIM Affect Fas-Mediated Apoptosis in Cholangiocarcinoma Cells

Inhibition of AKT in prostate cancer has been postulated to mediate the effect of I3C on Fas-mediated apoptosis.[18,20] I3C and DIM are anti-cancer reagents that have been demonstrated to affect the growth and apoptosis of a variety of tumor cells.[16–18,22] However, the role of I3C and DIM in Fas-mediated apoptosis in cholangiocarcinoma cells has not been determined. Thus, we examined whether I3C and DIM affect Fas-mediated apoptosis in cholangiocarcinoma cells and whether AKT signaling mediates the effect of these indole derivatives. We found that preincubation of cholangiocarcinoma cells with DIM enhanced Fas-mediated apoptosis in both Fas-sensitive (Figure 4A, DIM + CH-11 = 51.8 ± 1.5%; CH-11 = 32.6 ± 2.5%, $n = 3$; $P < 0.001$) and Fas-resistant cells (Figure 4A, DIM + CH-11 = 43.1 ± 0.8%; CH-11 = 18.3 ± 2.9%, $n = 3$; $P < 0.001$) as determined by flow cytometry after Annexin V and PI staining. The effect of DIM on Fas-mediated apoptosis was dose-dependent, whereas I3C, at the concentrations of 12.5 to 150 nmol/L, had no effect on Fas-mediated apoptosis (Figure 4B, $n = 3$). We further determined the involvement of CaMKII and AKT signaling in Fas-mediated apoptosis enhanced by DIM. The expression of CaMKII was not affected by DIM and I3C (Figure 4C), whereas inhibition of pAKT by DIM (30 μmol/L), but not I3C (100 μmol/L), was demonstrated by Western blot analysis (Figure 4C, $n = 3$).

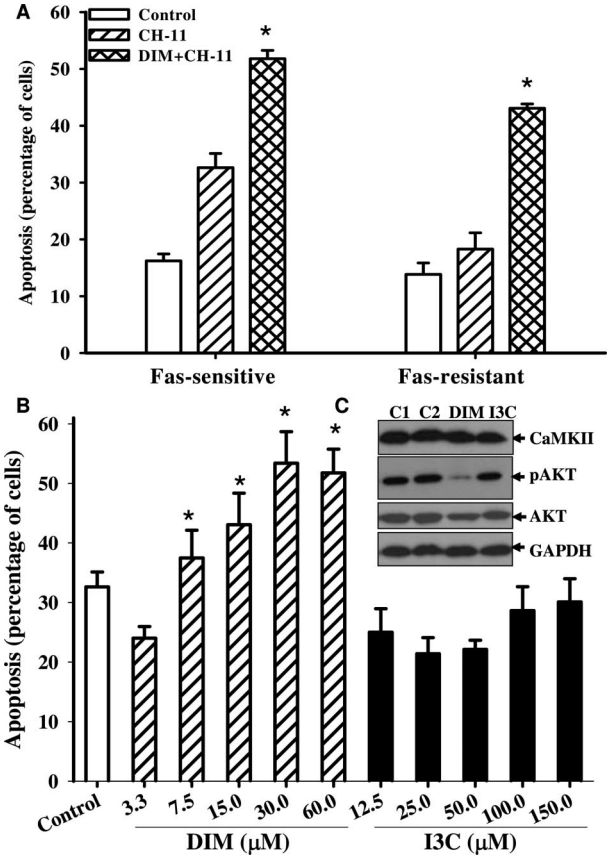


Figure 4. Effect of I3C and DIM on Fas-mediated apoptosis in cholangiocarcinoma cells. **A:** DIM enhanced Fas-mediated apoptosis in both Fas-resistant cells and Fas-sensitive cells. Cholangiocarcinoma cells were incubated with I3C (100 μmol/L) and its dimeric product DIM (25 μmol/L) for 30 minutes and subsequently exposed to Fas-activating antibody (CH-11, 500 ng/ml) for 24 hours. Results shown are of three independent experiments performed in duplicate (*$P < 0.001$, CH-11 versus DIM + CH-11). **B:** Cells were exposed to various concentrations of DIM and I3C for 30 minutes and subsequently exposed to CH-11 for 24 hours ($n = 3$, *$P < 0.05$ compared with control). **C:** Inhibition of pAKT by DIM (30 μmol/L), but not I3C (100 μmol/L), was demonstrated by Western blotting. A representative blot of three independent experiments each performed in duplicate is shown.

Inhibitors of Phosphatidylinositol 3-Kinase/Akt Signaling Enhance Fas-Mediated Apoptosis in Cholangiocarcinoma Cells

Having identified that inhibition of pAKT by DIM was associated with DIM-enhanced Fas-mediated apoptosis in cholangiocarcinoma cells (Figure 4, A and B), we determined whether direct inhibition of AKT signaling via its inhibitors promoted Fas-mediated apoptosis. Pharmacological inhibitors of phosphatidylinositol 3-kinase (PI3-K)/Akt signaling, including AKTI (500 nmol/L), LY294002 (50 μmol/L), and Wortmannin (25 μmol/L), were used. We found that preincubation of AKTI, LY294, and Wortmannin did not induce apoptosis in Fas-sensitive cells (Figure 5A, $n = 6$) or Fas-resistant cells (Figure 5B, $n = 3$) but sensitized both Fas-sensitive (Figure 5A) and Fas-resistant cells (Figure 5B) to Fas-mediated apoptosis. The effects of these inhibitors on Fas-mediated apoptosis were concentration-dependent (Figure 5, C and D).

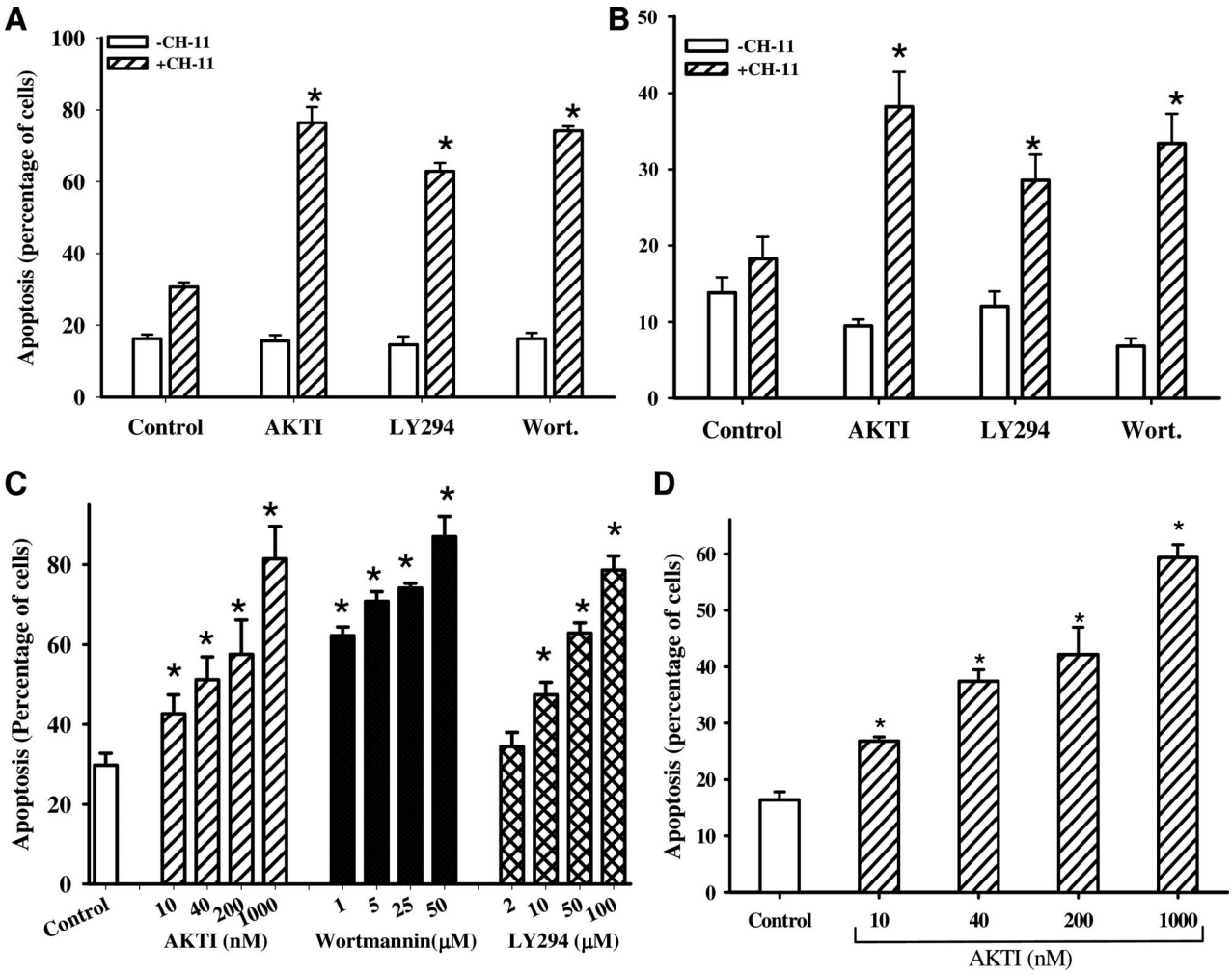


Figure 5. Effect of phosphatidylinositol 3-kinase/Akt signaling on Fas-mediated apoptosis in cholangiocarcinoma cells. Fas-sensitive (**A**, $n = 6$) and Fas-resistant (**B**, $n = 3$) cells were preincubated with inhibitors of PI 3-kinase/Akt signaling: AKTI (500 nmol/L), LY294002 (50 μmol/L), and Wortmannin (25 μmol/L) for 30 minutes and subsequently exposed to CH-11 (500 ng/ml) for 24 hours (*$P < 0.05$ for each inhibitor compared with CH-11 alone). **C:** Fas-sensitive cells were exposed to AKTI, Wortmannin, and LY294002 at the concentrations indicated for 30 minutes and subsequently exposed to CH-11 (500 ng/ml) for 24 hours ($n = 3$, *$P < 0.05$ compared with control). **D:** Fas-resistant cells were exposed to AKTI at 0 (control), 10, 40, 200, and 1000 nmol/L for 30 minutes and subsequently exposed to CH-11 (500 ng/ml) for 24 hours ($n = 4$, *$P < 0.05$ for AKTI + CH-11 compared with CH-11 only).

Adenovirus Encoding Dominant-Negative and Constitutively Activated AKT Regulates Fas-Mediated Apoptosis

The direct effects of AKT inhibition or activation on Fas-mediated apoptosis were further characterized with constitutively activated and dominant-negative AKT viruses. Transduction of the dominant-negative AKT (DN-AKT) and constitutively activated AKT (Ac-AKT) viruses did not affect apoptosis of cells compared with controls (Figure 6A, −CH-11 group). Consistent with the results from the use of AKT pharmacological inhibitors, Fas-sensitive cholangiocarcinoma cells infected with DN-AKT adenovirus were more sensitive to Fas-mediated apoptosis (Figure 6A, +CH-11 group). By contrast, Ac-AKT adenovirus partially protected Fas-mediated apoptosis (Figure 6A). Inhibition of pAKT, p55FLIP, p43FLIP, and pIκB was exhibited in cells infected with DN-AKT adenovirus, whereas induction of pAKT, p43FLIP, pERK, and pIκB was exhibited in cells infected with Ac-AKT (Figure 6, B and C; $n = 3$, *$P < 0.05$).

AKT Inhibition Does Not Affect Cell Surface Expression of Fas

We have previously demonstrated that the sensitivity to Fas-mediated apoptosis of the subpopulations of cholangiocarcinoma cells was correlated with the cell surface expression of Fas. Induction of cell surface expression of Fas appears to sensitize Fas-resistant cells to Fas-mediated apoptosis. Accordingly, one apparent mechanism by which DIM and AKT inhibitors could enhance Fas-mediated apoptosis would be by increasing cell surface expression of Fas. We determined the effects of CaMKII antagonist and inhibitors of PI-3K/AKT signaling pathways on cell surface Fas expression. Cell surface expression of Fas was analyzed after the cells were preincubated with control condi-

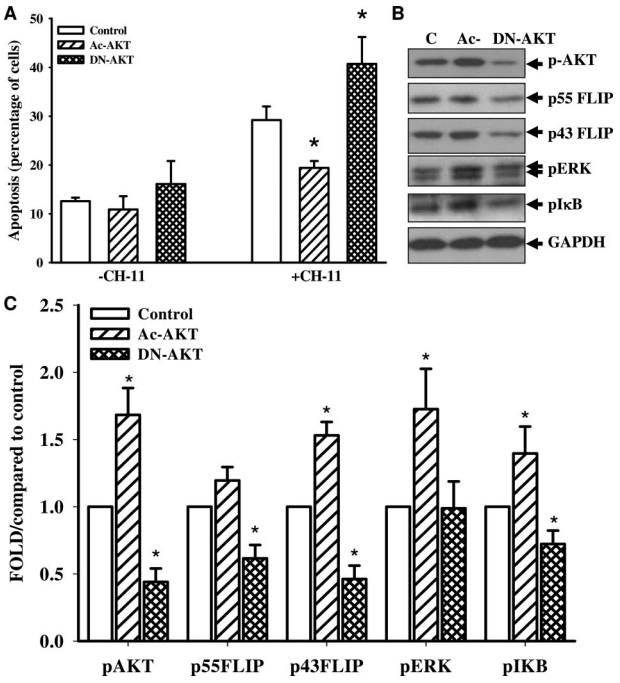


Figure 6. Effect of adenovirus encoding dominant-negative (DN-AKT) and constitutively activated AKT (Ac-AKT) on Fas-mediated apoptosis. **A:** Cholangiocarcinoma cells at 50% confluence were exposed to control adenovirus or DN-AKT or Ac-AKT at 100 pfu/cell in serum-free DMEM/F12 media for 2 hours, and then DMEM containing 20% FBS was added to cells. Viral suspension was removed 16 hours after infection, and cells were grown in fresh growth media for 24 hours and subsequentially exposed to CH-11 (500 ng/ml) for 24 hours. DN-Akt adenovirus promoted, whereas constitutively activated Akt adenovirus protected, Fas-mediated apoptosis ($n = 3$, $^*P < 0.05$). **B:** Western blot analyses of the expression of pAKT, p55FLIP, p43FLIP, and pIκB in cells with adenovirus-mediated altered expression of AKT. Representative blots of three independent experiments are shown. **C:** The density of each band was normalized to the density of GAPDH, and comparison of the mean densities of each protein in Ac-AKT or DN-Akt cells compared with the control cells was calculated as fold changes ($n = 3$, $^*P < 0.05$).

tion (DMEM/F12) or DMEM/F12 with KN-93 (10 μmol/L), AKTI (500 nmol/L), LY294002 (50 μmol/L), Wortmannin (25 μmol/L), DIM (30 μmol/L), and I3C (100 μmol/L) for 30 minutes. No significant effect of these inhibitors on cell surface expression of Fas was observed in either Fas-sensitive or Fas-resistant cholangiocarcinoma cells ($n = 3$, data not shown).

DIM and AKT Inhibitors Decrease Activation of FLIP, ERK, and NF-κB

Previous studies have suggested that AKT signaling regulates apoptosis through FLIP. We have demonstrated that Fas-resistant cholangiocarcinoma cells exhibit increased levels of the cleaved active form of FLIPp43. Accordingly, the effects of DIM and AKT inhibitors on FLIP were determined. Inhibition of pAKT, but not total AKT expression, by DIM, LY294002, and Wortmannin was confirmed in cholangiocarcinoma cells pretreated with these inhibitors (Figure 7, A and C). Furthermore, inhibition of FLIP, particularly the cleaved active FLIPp43 was evident in cells pretreated with AKTI, LY294002, Wortmannin, and DIM (Figure 7, A and C).

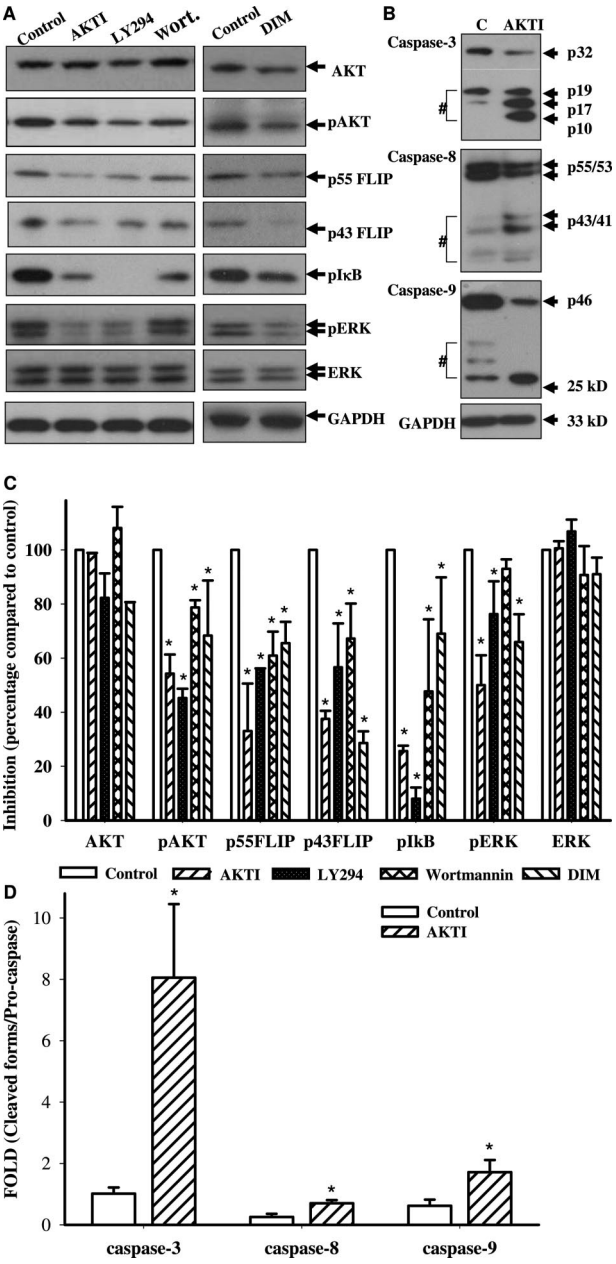


Figure 7. Signaling pathways affected by inhibitors of PI 3-k/Akt signaling and DIM. **A:** Effects of inhibitors of PI 3-k/Akt signaling and DIM on the expression and/or activation of ERK and NF-κB signaling pathways. Cholangiocarcinoma cells (Fas-resistant) were exposed to PI 3-k/Akt signaling inhibitors: AKTI (500 nmol/L), LY294002 (50 μmol/L), Wortmannin (25 μmol/L), and DIM (25 μmol/L) for 30 minutes and then CH-11, 500 ng/ml for 24 hours. Representative blot of four independent analyses determining AKT, pAKT, FLIP, pIκB, ERK, and pERK is shown ($n = 4$). The expression of GAPDH was used as control for equivalent loading. **B:** Effects of AKT inhibition on activation of caspase-3, -8, and -9. Western blot analysis was performed to determine the expression of full-length and cleaved forms of caspase-3, -8, and -9 in cells with or without preincubation with AKTI (500 nmol/L) before exposure to CH-11. Enhanced cleavage/activation of caspase-3, -8, and -9 was demonstrated in cells exposed to AKTI + CH-11 compared with those in cells exposed to CH-11 alone (# depicts the cleaved/activated forms of each caspases). Representative blot of four independent experiments is shown. **C:** The density of each band was normalized to the density of GAPDH, and comparison of the mean densities of each protein in AKTI-, LY294-, Wortmannin-, or DIM-treated cells compared with the control cells was calculated as fold changes ($n = 4$, $P < 0.05$). **D:** Comparison of the density of the total cleaved forms of each caspases (#) to the intact form (caspase-3 = p32, caspase-8 = p55/53, and caspase-9 = p46) was calculated as fold changes.

FLIP has been demonstrated to divert death signaling to cell proliferation via activation of extracellular signal-regulated kinase (ERK) and nuclear factor (NF)-κB signaling pathways in lymphocytes.[8,23] We further determined whether FLIP signals through NF-κB and ERK. The expression of total ERK was not affected by AKT inhibition (Figure 7, A and C). However, AKTI, LY294002, Wortmannin, and DIM significantly decreased the phosphorylated form of AKT (pAKT). In addition, inhibition of the phosphorylated form of IκB, a mediator in the NF-κB pathway, was demonstrated, indicating inhibition of NF-κB signaling by these inhibitors (Figure 7, A and C). Similar results were observed in cholangiocarcinoma cells infected with adenoviruses with Ac-AKT or DN-AKT (Figure 6, B and C). Taken together, these results demonstrate that inhibition of activation of FLIP, ERK, and NF-κB signaling pathways is associated with increased apoptosis induced by AKT inhibition.

AKT Inhibition Increases Activation of Caspase-3, -8, and -9

Activation of caspase-3 and -8 mediates Fas-induced apoptosis in cholangiocarcinoma cells.[4,5] Further, AKT has been demonstrated to modulate caspase-9 activation.[24] Accordingly, Western blot analyses were performed to determine the cleavage/activation of caspase-3, -8, and -9. As depicted in Figure 7, B and D, increased activation of caspase-3, -8, and -9 was demonstrated in cells preincubated with AKTI, as evident by increased presence of the cleaved form of these caspases. These results suggest that increased activation of caspase-3, -8, and -9 after AKT inhibition enhanced Fas-mediated apoptosis.

Discussion

Suppression of apoptosis by intracellular survival factors is important in the development of chemoresistance. In the present studies, we determined the expression of several prosurvival molecules in cholangiocarcinoma cells so as to identify potential molecular targets that may sensitize these cells to Fas-mediated apoptosis. We demonstrated that increased phosphorylation of AKT was associated with resistance of cholangiocarcinoma cells to Fas-mediated apoptosis. Phosphorylation of AKT was also identified in cholangiocarcinoma xenografts in mouse and cholangiocarcinoma in patients. These results are consistent with previous reports that AKT signaling plays an important role in regulating cholangiocarcinoma proliferation and apoptosis.[11,12] In addition, these results are in agreement with several previous observations that activation of AKT is an important regulator of Fas sensitivity in tumor cells[25,26] and that inhibition of AKT sensitizes cells to Fas-mediated apoptosis in cancer cells.[27,28] Interestingly, inhibition of CaMKII did not sensitize cholangiocarcinoma cells to Fas-mediated apoptosis, which disagrees with previous studies that inhibition of CaMKII inhibits the expression of FLIP and sensitizes glioma cells to Fas-mediated apoptosis.[10] Such a discrepancy may be explained by higher concentrations of the CaMKII inhibitor KN-93 (100 versus 10 μmol/L in our studies) and longer exposure time (24 hours versus 30 minutes) used in the latter studies.

We further explored the effect of two dietary indole derivatives, I3C and DIM, on phosphorylation of AKT and Fas-mediated apoptosis. Similar to the observation in breast cancer cells,[16] we found that DIM enhanced Fas-mediated apoptosis in cholangiocarcinoma cells and inhibition of AKT activation appeared to contribute to the proapoptotic effects of DIM in cholangiocarcinoma cells. However, we found no effect of I3C on Fas-mediated apoptosis. I3C has been shown to sensitize TRAIL-induced apoptosis in LNCaP prostate cancer cells[29] and induce apoptosis in a variety of hormone-related cancer cells, including breast cancer cells,[16,30] prostate cancers,[18,31] and other reproductive cancers such as endometrial cancer.[32] Cholangiocarcinoma is a nonhormone-related cancer; the effect of I3C on these cells may be different from those on hormone-related cancer cells.[16,18,30–32] Furthermore, in the present studies, cells were exposed to I3C for a short period of time (30 minutes) before exposure to Fas-activating antibody (CH-11), whereas in the studies of Howells and colleagues,[30] breast cancer cells were exposed to I3C at 250 μmol/L for 72 hours or several days. In addition, *in vivo* studies with rodents have suggested that DIM, the major acid condensation product of I3C, may mediate the physiological effects of dietary I3C.[19,31] This is in agreement with our results demonstrating that DIM, but not I3C, enhanced Fas-mediated apoptosis in cholangiocarcinoma cells.

We found that AKT inhibition did not affect surface expression of Fas, indicating that AKT inhibition renders the cells more sensitive to Fas-mediated apoptosis through mechanisms other than up-regulation of cell surface expression of Fas, a mechanism that has been shown previously to increase Fas-mediated apoptosis in cholangiocarcinoma cells.[5] PI3-K inhibitors have been shown to enhance the susceptibility to Fas-mediated apoptosis in HL-60 cells through down-regulation of FLIP, a Fas-signaling inhibitor.[33] Inhibition of FLIP in cholangiocarcinoma increases Fas-mediated apoptosis.[6] In the present studies, we demonstrated that the expression of p43FLIP (the cleaved active form of FLIP) was increased in the Fas-resistant cholangiocarcinoma cells (Figure 1, A and B), which is consistent with emerging evidence that the expression of FLIP is increased in cancer cells.[34–36] Up-regulation of FLIP presumably protects cancer cells from Fas-mediated apoptosis as determined in glioma cells[10] and HeLa cells.[37] By contrast, down-regulation of FLIP in a variety of cancer cells presumably sensitizes these cancer cells to Fas-mediated apoptosis.[10,38–40] Results from all these studies point to FLIP as an important regulator of Fas-mediated apoptosis. Accordingly, increased activation of FLIP in Fas-resistance cholangiocarcinoma cells appears to contribute, at least in part, to the resistance of these cells to Fas-mediated apoptosis via diverting death signaling to those for cell survival.

FLIP has also been demonstrated to promote activation of NF-κB and ERK that promote proliferation of lymphocytes.[8,23] Activation of AKT has been reported to increase NF-κB-mediated cell survival.[41–43] Activation of NF-κB involves phosphorylation of an inhibitory protein of NF-κB, IκB, the phosphorylation of which induces its ubiquitination and degradation and allows the translocation of NF-κB into the nucleus to activate gene transcription.[44] Increased phosphorylation of IκB and NF-κB activation is induced by AKT activation in breast cancer cells.[18,43] By contrast, inhibition of IκBα phosphorylation and activation of NF-κB and AKT has been observed in DIM-treated breast and prostate cancer cells.[18,31] Consistent with these reports in hormone-related cancers, we found that inhibition of AKT by DIM and AKTI inhibited the activation of NF-κB, as determined by decreased expression of phosphorylated-κB in cholangiocarcinoma cells.

Another signaling pathway that mediates the effect of FLIP activation is activation of ERK signaling.[8,34] We demonstrated that decreased activation of ERK, as determined by the expression of phosphorylated ERK, was present in cells pretreated with the inhibitors of AKT or DIM. Such results are consistent with previous observations that DIM inhibits ERK in breast cancer cells[45] and that activation of ERK, associated with activation of AKT, protects cells from Fas-mediated apoptosis.[46] In addition, we found increased activation of caspase-3, -8, and -9 in cells pretreated with the AKT inhibitor. We have previously identified that activation of caspase-3 and -8 are responsible for Fas-mediated apoptosis in cholangiocarcinoma cells.[5] AKT has been reported to phosphorylate and inactivate caspase-9.[24] Activation of caspase-9 and caspase-3 has been associated with DIM/I3C-induced apoptosis in human breast cancer cells and osteosarcoma cells.[47] Accordingly, increased activation of these proapoptosis proteases in cholangiocarcinoma cells increased sensitivity of these cells to Fas-mediated apoptosis.

In summary, we have found that inhibition of phosphorylation of AKT enhanced Fas-mediated apoptosis, which appeared to be mediated through a mechanism involving inhibition of the activation of FLIP and its downstream signaling pathways including ERK and NF-κB. DIM, a natural digestive product of vegetable component I3C, was an effective sensitizer to Fas-mediated apoptosis in cholangiocarcinoma cells via inhibition of AKT and FLIP activation. Results from the present studies may provide important insights into the development of novel therapeutic strategies for cholangiocarcinoma.

Acknowledgment

We thank Dr. Kenneth Walsh (Boston University, School of Medicine) for AKT adenoviruses.

References

1. Ahrendt SA, Nakeeb A, Pitt HA: Cholangiocarcinoma. Clin Liver Dis 2001, 5:191–218
2. Taniai M, Grambihler A, Higuchi H, Werneburg N, Bronk SF, Farrugia DJ, Kaufmann SH, Gores GJ: Mcl-1 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in human cholangiocarcinoma cells. Cancer Res 2004, 64:3517–3524
3. Nzeako UC, Guicciardi ME, Yoon JH, Bronk SF, Gores GJ: COX-2 inhibits Fas-mediated apoptosis in cholangiocarcinoma cells. Hepatology 2002, 35:552–559
4. Pan G, Vickers SM, Pickens A, Phillips JO, Ying W, Thompson JA, Siegal GP, McDonald JM: Apoptosis and tumorigenesis in human cholangiocarcinoma cells. Involvement of Fas/APO-1 (CD95) and calmodulin. Am J Pathol 1999, 155:193–203
5. Ahn EY, Pan G, Vickers SM, McDonald JM: IFN-gamma upregulates apoptosis-related molecules and enhances Fas-mediated apoptosis in human cholangiocarcinoma. Int J Cancer 2002, 100:445–451
6. Que FG, Phan VA, Phan VH, Celli A, Batts K, LaRusso NF, Gores GJ: Cholangiocarcinomas express Fas ligand and disable the Fas receptor. Hepatology 1999, 30:1398–1404
7. Yuan ZQ, Feldman RI, Sussman GE, Coppola D, Nicosia SV, Cheng JQ: AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: implication of AKT2 in chemoresistance. J Biol Chem 2003, 278:23432–23440
8. Kataoka T, Budd RC, Holler N, Thome M, Martinon F, Irmler M, Burns K, Hahne M, Kennedy N, Kovacsovics M, Tschopp J: The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. Curr Biol 2000, 10:640–648
9. Maedler K, Fontana A, Ris F, Sergeev P, Toso C, Oberholzer J, Lehmann R, Bachmann F, Tasinato A, Spinas GA, Halban PA, Donath MY: FLIP switches Fas-mediated glucose signaling in human pancreatic beta cells from apoptosis to cell replication. Proc Natl Acad Sci USA 2002, 99:8236–8241
10. Yang BF, Xiao C, Roa WH, Krammer PH, Hao C: Calcium/calmodulin-dependent protein kinase II regulation of c-FLIP expression and phosphorylation in modulation of Fas-mediated signaling in malignant glioma cells. J Biol Chem 2003, 278:7043–7050
11. Wu T, Leng J, Han C, Demetris AJ: The cyclooxygenase-2 inhibitor celecoxib blocks phosphorylation of Akt and induces apoptosis in human cholangiocarcinoma cells. Mol Cancer Ther 2004, 3:299–307
12. Sirica AE: Cholangiocarcinoma: molecular targeting strategies for chemoprevention and therapy. Hepatology 2005, 41:5–15
13. Thakkar H, Chen X, Tyan F, Gim S, Robinson H, Lee C, Pandey SK, Nwokorie C, Onwudiwe N, Srivastava RK: Pro-survival function of Akt/protein kinase B in prostate cancer cells. Relationship with TRAIL resistance. J Biol Chem 2001, 276:38361–38369
14. Jo M, Kim TH, Seol DW, Esplen JE, Dorko K, Billiar TR, Strom SC: Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nat Med 2000, 6:564–567
15. Panka DJ, Mano T, Suhara T, Walsh K, Mier JW: Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. J Biol Chem 2001, 276:6893–6896
16. Nachshon-Kedmi M, Yannai S, Fares FA: Induction of apoptosis in human prostate cancer cell line, PC3, by 3,3′-diindolylmethane through the mitochondrial pathway. Br J Cancer 2004, 91:1358–1363
17. Bonnesen C, Eggleston IM, Hayes JD: Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. Cancer Res 2001, 61:6120–6130
18. Rahman KW, Sarkar FH: Inhibition of nuclear translocation of nuclear factor-{kappa}B contributes to 3,3′-diindolylmethane-induced apoptosis in breast cancer cells. Cancer Res 2005, 65:364–371
19. Grose KR, Bjeldanes LF: Oligomerization of indole-3-carbinol in aqueous acid. Chem Res Toxicol 1992, 5:188–193
20. Chinni SR, Sarkar FH: Akt inactivation is a key event in indole-3-carbinol-induced apoptosis in PC-3 cells. Clin Cancer Res 2002, 8:1228–1236
21. Ouchi N, Kobayashi H, Kihara S, Kumada M, Sato K, Inoue T, Funahashi T, Walsh K: Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells. J Biol Chem 2004, 279:1304–1309
22. Chinni SR, Li Y, Upadhyay S, Koppolu PK, Sarkar FH: Indole-3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells. Oncogene 2001, 20:2927–2936
23. Mitsiades N, Mitsiades CS, Poulaki V, Anderson KC, Treon SP: Intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human multiple myeloma cells. Blood 2002, 99:2162–2171

24. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC: Regulation of cell death protease caspase-9 by phosphorylation. Science 1998, 282:1318–1321
25. Oehme I, Bosser S, Zornig M: Agonists of an ecdysone-inducible mammalian expression system inhibit Fas ligand- and TRAIL-induced apoptosis in the human colon carcinoma cell line RKO. Cell Death Differ 2006, 13:189–201
26. Osaki M, Kase S, Adachi K, Takeda A, Hashimoto K, Ito H: Inhibition of the PI3K-Akt signaling pathway enhances the sensitivity of Fas-mediated apoptosis in human gastric carcinoma cell line, MKN-45. J Cancer Res Clin Oncol 2004, 130:8–14
27. Honjo S, Osaki M, Ardyanto TD, Hiramatsu T, Maeta N, Ito H: COX-2 inhibitor, NS398, enhances Fas-mediated apoptosis via modulation of the PTEN-Akt pathway in human gastric carcinoma cell lines. DNA Cell Biol 2005, 24:141–147
28. Bai J, Sui J, Demirjian A, Vollmer Jr CM, Marasco W, Callery MP: Predominant Bcl-XL knockdown disables antiapoptotic mechanisms: tumor necrosis factor-related apoptosis-inducing ligand-based triple chemotherapy overcomes chemoresistance in pancreatic cancer cells in vitro. Cancer Res 2005, 65:2344–2352
29. Jeon KI, Rih JK, Kim HJ, Lee YJ, Cho CH, Goldberg ID, Rosen EM, Bae I: Pretreatment of indole-3-carbinol augments TRAIL-induced apoptosis in a prostate cancer cell line, LNCaP. FEBS Lett 2003, 544:246–251
30. Howells LM, Gallacher-Horley B, Houghton CE, Manson MM, Hudson EA: Indole-3-carbinol inhibits protein kinase B/Akt and induces apoptosis in the human breast tumor cell line MDA MB468 but not in the nontumorigenic HBL100 line. Mol Cancer Ther 2002, 1:1161–1172
31. Aggarwal BB, Ichikawa H: Molecular targets and anticancer potential of indole-3-carbinol and its derivatives. Cell Cycle 2005, 4:1201–1215
32. Kojima T, Tanaka T, Mori H: Chemoprevention of spontaneous endometrial cancer in female Donryu rats by dietary indole-3-carbinol. Cancer Res 1994, 54:1446–1449
33. Kondo G, Iwase M, Watanabe H, Uchida M, Takaoka S, Ohashi M, Ito D, Nagumo M: Enhancement of susceptibility to Fas-mediated apoptosis in HL-60 cells through down-regulation of cellular FLICE-inhibitory protein by phosphatidylinositol 3-kinase inhibitors. Oncol Rep 2005, 14:1215–1222
34. Wang W, Prince CZ, Mou Y, Pollman MJ: Notch3 signaling in vascular smooth muscle cells induces c-FLIP expression via ERK/MAPK activation. Resistance to Fas ligand-induced apoptosis. J Biol Chem 2002, 277:21723–21729
35. Wajant H: Targeting the FLICE inhibitory protein (FLIP) in cancer therapy. Mol Interv 2003, 3:124–127
36. Lee SH, Kim HS, Kim SY, Lee YS, Park WS, Kim SH, Lee JY, Yoo NJ: Increased expression of FLIP, an inhibitor of Fas-mediated apoptosis, in stomach cancer. APMIS 2003, 111:309–314
37. Kamarajan P, Sun NK, Chao CC: Up-regulation of FLIP in cisplatin-selected HeLa cells causes cross-resistance to CD95/Fas death signalling. Biochem J 2003, 376:253–260
38. Song JH, Song DK, Herlyn M, Petruk KC, Hao C: Cisplatin down-regulation of cellular Fas-associated death domain-like interleukin-1beta-converting enzyme-like inhibitory proteins to restore tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human melanoma cells. Clin Cancer Res 2003, 9:4255–4266
39. Tran SE, Meinander A, Holmstrom TH, Rivero-Muller A, Heiskanen KM, Linnau EK, Courtney MJ, Mosser DD, Sistonen L, Eriksson JE: Heat stress downregulates FLIP and sensitizes cells to Fas receptor-mediated apoptosis. Cell Death Differ 2003, 10:1137–1147
40. Watanabe K, Okamoto K, Yonehara S: Sensitization of osteosarcoma cells to death receptor-mediated apoptosis by HDAC inhibitors through downregulation of cellular FLIP. Cell Death Differ 2005, 12:10–18
41. Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin Jr AS, Mayo MW: Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. Mol Cell Biol 2000, 20:1626–1638
42. Burow ME, Weldon CB, Melnik LI, Duong BN, Collins-Burow BM, Beckman BS, McLachlan JA: PI3-K/AKT regulation of NF-kappaB signaling events in suppression of TNF-induced apoptosis. Biochem Biophys Res Commun 2000, 271:342–345
43. Sizemore N, Leung S, Stark GR: Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. Mol Cell Biol 1999, 19:4798–4805
44. Chen Lf, Fischle W, Verdin E, Greene WC: Duration of nuclear NF-kappaB action regulated by reversible acetylation. Science 2001, 293:1653–1657
45. Contractor R, Samudio IJ, Estrov Z, Harris D, McCubrey JA, Safe SH, Andreeff M, Konopleva M: A novel ring-substituted diindolylmethane,1,1-bis[3′-(5-methoxyindolyl)]-1-(p-t-butylphenyl) methane, inhibits extracellular signal-regulated kinase activation and induces apoptosis in acute myelogenous leukemia. Cancer Res 2005, 65:2890–2898
46. Flaherty DM, Hinde SL, Monick MM, Powers LS, Bradford MA, Yarovinsky T, Hunninghake GW: Adenovirus vectors activate survival pathways in lung epithelial cells. Am J Physiol 2004, 287:L393–L401
47. Ge X, Yannai S, Rennert G, Gruener N, Fares FA: 3,3′-Diindolylmethane induces apoptosis in human cancer cells. Biochem Biophys Res Commun 1996, 228:153–158