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BCL-2 Functions as an Activator of the AKT Signaling Pathway in Pancreatic Cancer

Melinda M. Mortenson,[1] Joseph G. Galante,[1] Oren Gilad,[2] Michael G. Schlieman,[1] Subbulakshmi Virudachalam,[1] Hsing-Jien Kung,[2] and Richard J. Bold[1]*

[1]Department of Surgery, University of California Davis School of Medicine, Sacramento, California
[2]Department of Biochemistry/Molecular Medicine, University of California Davis School of Medicine, Sacramento, California

Abstract BCL-2 is the prototypic anti-apoptotic protein involved in the regulation of apoptosis. Overexpression of BCL-2 is common in pancreatic cancer and confers resistance to the apoptotic effect of chemo- and radiotherapy. Although these cellular effects of BCL-2 are traditionally related to pathways involving the mitochondrial membrane, we sought to investigate whether BCL-2 is involved in other signaling pathways regulating cell survival and focused on AKT. We examined the effect of overexpression of BCL-2 in the MIA-PaCa-2 human pancreatic cancer cell line on the function and subcellular location of AKT. We observed that the stable subclones of MIA-PaCa-2 overexpressing BCL-2 demonstrated increased activity of AKT as well as IKK (a downstream target of AKT), increasing the transcriptional activity of NF-κB. Using immunoprecipitation techniques, we observed co-immunoprecipitation of AKT and BCL-2. Immunocytochemistry demonstrated co-localization of BCL-2 and AKT, which was abrogated by treatment with HA14-1, a small molecule inhibitor of BH-3-mediated protein interaction by BCL-2. Furthermore, treatment with HA14-1 decreased phosphorylation of AKT and increased sensitivity to the apoptotic effect of the chemotherapeutic agent, paclitaxel. These results demonstrate an additional mechanism of regulation of cell survival mediated by BCL-2, namely through AKT activation, in the MIA-PaCa-2 pancreatic cancer cell line. Therefore, directed inhibition of BCL-2 may alter diverse pathways controlling cell survival and overcome the apoptotic resistance that is the hallmark of pancreatic cancer. J. Cell. Biochem. 102: 1171–1179, 2007.

Key words: BCL-2; AKT; pancreatic cancer

INTRODUCTION

BCL-2, an anti-apoptotic protein overexpressed in a variety of cancers, functions through heterodimerization with pro-apoptotic members of the BCL-2 family (e.g. BAX, BAK, etc.) to prevent mitochondrial pore formation, thus preventing cytochrome c release and initiation of apoptosis [Scorrano and Korsmeyer, 2003]. There is recent evidence, though, that BCL-2 may act through survival pathways other than its function at the mitochondrial membrane. Regula et al. have shown that overexpression of BCL-2 in ventricular myocytes activates the transcriptional function of NF-κB through a raf-1/MEKK-1 pathway [Regula et al., 2002]. Whether this mechanism occurs in other cell types is uncertain, especially as increased expression of BCL-2 *decreased* NF-κB activity in 293 cells, 3T3 and PC12 cells [Grimm et al., 1996; Hour et al., 2000; Song et al., 2004].

Constitutive NF-κB activation has been observed in pancreatic cancer, though which of many potential upstream activating signals mediates this event remains unclear. We and others have demonstrated that the serine/threonine protein kinase AKT is one mechanism of activating NF-κB in pancreatic cancer [Arlt et al., 2003; Fahy et al., 2003; Liptay et al., 2003]. Activation of AKT has been recently demonstrated to be sufficient to promote neoplastic development of pancreatic cancer.

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*Correspondence to: Richard J. Bold, Division of Surgical Oncology, Suite 3010, UC Davis Cancer Center, 4501 X Street, Sacramento, CA 95817.
E-mail: richard.bold@ucdmc.ucdavis.edu

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Stanger et al. generated a pancreas-specific knockout of PTEN, a negative regulator of the AKT pathway; these mice demonstrate high basal AKT activation and develop pancreatic ductal metaplasia progressing to invasive ductal adenocarcinoma [Stanger et al., 2005]. The mechanism of activation of AKT in pancreatic cancer remains unclear and likely multifactorial. Loss of PTEN expression due to promoter methylation mediates activation of AKT in some, but not all pancreatic tumor cell lines [Asano et al., 2004]. Though mutation of K-ras is a common, and perhaps inciting, event in pancreatic cancer, it does not appear to be a significant mediator of AKT activation [Matsumoto et al., 2002].

Despite the uncertainties of the mechanism(s) of activation, inhibition of AKT activity has been demonstrated to increase the sensitivity to the apoptotic effect of chemotherapy in pancreatic cancer [Ng et al., 2000; Perugini et al., 2001; Shah et al., 2001; Bondar et al., 2002; Fahy et al., 2004; Takeda et al., 2004]. However, the majority of these studies have utilized approaches that cannot be translated to human use, such as the PI-3 kinase inhibitors or dominant negative constructs; current PI-3 kinase inhibitors (e.g. wortmannin and LY294002) are sufficiently toxic in animal studies to prevent attempted translation to clinical use in humans. Therefore the identification of nontraditional, upstream signals mediating the activation of AKT in pancreatic cancer may allow targeted inhibition in human studies. Given the potential role for BCL-2 in altering NF-κB activity and the known pathway from AKT to NF-κB, we developed the hypothesis that overexpression of BCL-2 is a potential mechanism to activate the AKT/NF-κB signaling pathway in pancreatic cancer. Should BCL-2 be found to be a significant stimulator of AKT activity, antisense strategies currently in use in humans [Morris et al., 2002] could be a targeted therapy to inhibit AKT activity in pancreatic cancer.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The MIA-PaCa-2 cell line was obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagles medium (Gibco/BRL; Gaithersburg, MD) with 10% fetal calf serum. All chemical reagents were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise specified. Antibodies to AKT (Cell Signaling, Inc.; Beverly, MA), phospho-AKT (Biosource; Camarillo, CA), BCL-2 (Pharmingen; San Diego, CA), IκBα (Cell Signaling), phospho-IκBα (Cell Signaling), and actin (Santa Cruz; Santa Cruz, CA) were used. HA 14-1, a small molecule inhibitor of BCL-2 [Wang et al., 2000] was obtained from Bio-Mol (Plymouth Meeting, PA). MIA-PaCa-2 cells were cultured in 100-mm dishes and transfected with a pCDNA-3.1 vector (Invitrogen; Carlsbad, CA) containing the BCL-2 cDNA. After 16 h in serum free media, cells were rinsed, and fresh medium was added containing G418 (1000 μg/mL); for another 4–6 weeks the medium was replaced every three days. Outgrowth of the single colonies were harvested and expanded as separate clones. Maintenance culture of individual, stably transfected cell lines used G418 at 600 μg/mL. A control cell line was created under similar conditions using a pCDNA-3.1 vector without the BCL-2 cDNA insert.

Immunoblotting

In brief, cells were harvested, lysed and the soluble protein fraction was separated on a 10% SDS-PAGE. Separated polypeptides were then electrophoretically transferred to 0.2-mm nitrocellulose membranes (Schleicher & Schuell; Keene, NH), probed overnight with primary antibodies and developed using species-specific secondary antisera. Immunoreactive material was detected by the enhanced chemiluminescence technique (Amersham; Piscataway, NJ).

Electrophoretic Mobility Shift Assay (EMSA.)

An oligonucleotide corresponding to the consensus NF-κB binding sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was radiolabeled with [γ-^{32}P]ATP by a T4 kinase reaction for 30 min at room temperature. Unincorporated label was removed by chromatography through a G-25 spin column (Roche Diagnostics Corp.; Indianapolis, IN). Ten μg of nuclear protein extract (Pierce; Rockford IL) was incubated with the radiolabeled NF-κB oligonucleotide for 20 min at room temperature. DNA: protein complexes were separated by electrophoresis through a nondenaturing 4% polyacrylamide gel in 0.5 × TBE at 300 V for 1.0 h. Autoradiographic films were developed following exposure for 18 h (−20°C).

NF-κB Transcriptional Activity

The NF-κB luciferase reporter plasmid contains four tandem copies of the NF-κB consensus sequence fused to a TATA-like promoter upstream of the firefly luciferase gene (Clontech; Palo Alto, CA). Transfection efficiency was normalized by co-transfection with a *Renilla luciferase* reporter, pRL-TK (Promega Corp.). The plasmids (NF-κB, 2.5 μg; pRL-TK, 0.25 μg) were transiently transfected into the cells using lipofectin reagent (Invitrogen). Dual luciferase assays were performed on 20 μL of sample supernatant (Promega, Corp.) using an Analytical Luminescence Laboratory Luminometer (Monolight 2010). Results are expressed as the ratio of firefly/Renilla relative light units (RLUs).

IKK Kinase Assay

Cells were harvested in lysis buffer (20 mM Tris-base, 20 mM NaF, 20 mM β-glycerophosphate, 0.5 mM sodium orthovanadate, 2.5 mM metabisulphite, 5 mM benzamidine, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol, 0.3 M NaCl, 1% Triton-x, and 1% Protease Inhibitor Cocktail) (Sigma). After clarification, equal amounts of cell lysate (250 μg) were immunoprecipitated with IKKγ antibody (generous gift from E. Zandi, Los Angeles, CA) and protein G sepharose beads. A kinase assay was then performed by incubating 5 μCi γ32P-ATP, 1 μg GST-IκBα substrate (gift from E. Zandi), 20 μM ATP and 2 mM DTT in kinase buffer at 30°C for 30 min. After separation of samples on a 10% SDS-PAGE gel and transferring to a nitrocellulose, images were acquired by exposure on phosphoimager using Quantity 1 Imaging Software.

Immunoprecipitation

In brief, exponentially growing cells are lysed in an immunoprecipitation buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP40) on ice for 5 min and clarified by centrifugation (10,000 g, 10 min). hundred μg of total protein used for the immunoprecipitation. Either AKT or BCL-2 was immunoprecipitated using an immobilized monoclonal antibody to either AKT (Calbiochem, San Diego, CA) or BCL-2 (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min at 25°C and isolated using Protein A-agarose. Proteins were then solubilized and immunoblotted as described above.

Immunofluorescence

For fluorescence microscopy, cells were cultured on glass coverslips and fixed in 3.7% paraformaldehyde (5 min at room temperature). Cells were washed in PBS and then solubilized by treatment with 0.5% Triton X-100 for 20 min at room temperature. Blocking of nonspecific binding was achieved by incubation in 3% milk for 15 min at room temperature. Coverslips were incubated with monoclonal antibodies to BCL-2, pAKT and AKT for 2 h at 37°C, washed with PBS, and then incubated (4°C, overnight) with species-specific secondary antibodies (Alexa Fluor^R 555 for pAKT and AKT, Alexa Fluor^R 647 for BCL-2; Invitrogen). Cells were then counterstained for nuclear visualization with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 300 nM; Molecular Probes, Eugene, OR).

RESULTS

Characterization of MIA-PaCa-2 Subclones Overexpressing BCL-2

We isolated and characterized three individual subclones of the MIA-PaCa-2 human pancreatic cancer cell line that overexpress BCL-2 by virtue of stable transfection of the BCL-2 cDNA. All exhibit increased BCL-2 expression compared to the parental cell line (Control) stably transfected with the empty expression vector (pcDNA-3) (Fig. 1A). We initially characterized the activation of the AKT signaling pathway by immunoblotting for the phosphorylated/activated form of AKT. These three clones demonstrated similar levels of total AKT compared to the Control cell line, but increased levels of phosphorylated AKT (pAKT) were observed (Fig. 1A). We further examined a direct substrate of AKT, p70 S6 kinase (phospho-Thr389); more of the phosphorylated form was identified in the BCL-2 overexpressing subclones compared to the Control cell line (Fig. 1A). Immunoblotting for the unrelated kinases Erk1/Erk2 demonstrated that BCL-2 overexpression did not affect activation of this signaling cascade.

BCL-2 Overexpression Increases NF-κB Activity

We then more carefully characterized the activation of the AKT signaling pathway with specific reference to the activation of NF-κB

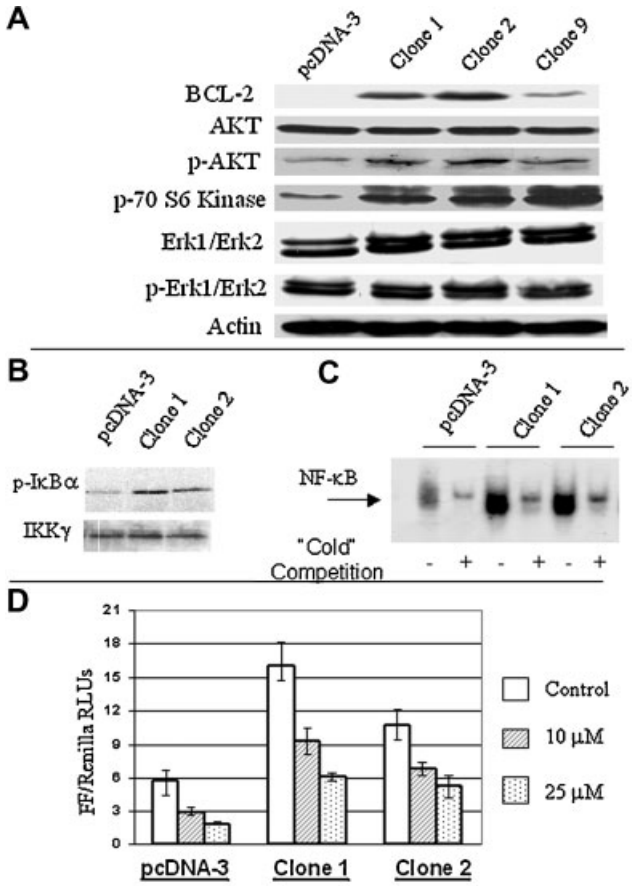

Fig. 1. Overexpression of BCL-2 protein activates the AKT → NF-κB signaling pathway. **A**: Individual stable clones of the MIA-PaCa-2 cell line stably transfected with either an empty eukaryotic expression vector (pcDNA-3) or the BCL-2 cDNA (Clones 1, 2 and 9) were characterized for expression of BCL-2, AKT, phospho-AKT, phospho-p70 S6 Kinase, Erk1/Erk2, phospho-Erk1/Erk2 and actin. **B**: IKK kinase activity from the indicated cell lines follwoing immunoprecipitation of IKKγ and incubation with γ^{32}P-ATP providing the substrate of GST-IκBα separation of samples by 10% SDS-PAGE and autoradiography. Immunoblotting for IKKγ was performed to demonstrate equivalence of input kinase. **C**: EMSA assay for NF-κB nuclear binding activity in indicated cell in the absence or presence of 100-fold molar excess unlabeled (''Cold'') oligonucleotide. **D**: Luciferase assay using a NF-κB consensus sequence-driven luciferase construct in the absence or presence of indicated doses of the PI3 kinase inhibitor LY294002; data shown is luciferase relative light units (RLU) normalized to co-transfected Renilla luciferase.

pathway in these BCL-2 overexpressing subclones. IκB kinase (IKK) is a direct substrate of AKT; phosphorylation of IKK induces activation, which then phosphorylates IκB, causing dissociation from NF-κB and subsequent nuclear translocation to initiate gene transcription [Romashkova and Makarov, 1999; Verma and Stephenson, 1997]. We first examined IKK kinase activity, which was elevated in two BCL-2 overexpressing subclones compared to Control (Fig. 1B). To determine whether the increased IKK activity altered NF-κB activity, we performed both physical (EMSA) and functional (luciferase) assays. The EMSA demonstrated increased nuclear NF-κB binding to a consensus NF-κB sequence in these clones compared to the control (Fig. 1C). We then examined NF-κB transcriptional activity using a NF-κB-driven luciferase reporter. The BCL-2 overexpressing clones demonstrated increased NF-κB activity compared to the control cell line (Fig. 1D). PI-3 kinase, an upstream activator of AKT, was inhibited with LY294002 to determine whether the NF-κB activity was regulated by pathways other than the BCL-2-mediated AKT activation. A dose-dependent inhibition of NF-κB activity by the PI-3 kinase inhibitor LY294002 was observed (Fig. 1D). These data indicate that BCL-2 increases NF-κB activity, though regulation by PI-3 kinase remains intact suggesting

that BCL-2 serves as a co-activator for the NF-κB signal transduction pathway.

Interaction of BCL-2 and AKT

Given our observations that increasing BCL-2 levels activated a signaling pathway involving AKT, we determined whether there was any interaction between these two proteins using immunoprecipitation techniques. Following immunoprecipitation of AKT in MIA-PaCa-2 as well as two of the BCL-2 overexpressing clones, BCL-2 could be identified in the immunoprecipitated complex (Fig. 2A). Conversely, BCL-2 was immunoprecipitated from MIA-PaCa-2 cells and AKT was identified within the immunoprecipitated complex (Fig. 2A). These data demonstrate that AKT and BCL-2 form a complex through some protein:protein interaction. We then used immunofluorescence techniques to confirm colocalization of these two proteins, examine the subcellular distribution of these two proteins, and detect any effect of BCL-2 overexpression. In MIA-PaCa-2, we noted a granular cytoplasmic distribution of BCL-2 while a diffuse cytoplasmic distribution of AKT was observed (Fig. 2B). Overlaying the images demonstrated colocalization of BCL-2 and AKT in the cytoplasm, though some BCL-2 did not co-localize with AKT and remained in a granular cytoplasmic pattern (Fig. 2B). Overexpression of BCL-2 did not affect the immunofluorescent staining pattern for either BCL-2 (other than increased amounts) or AKT (data not shown).

BCL-2 Overexpression Alters Subcellular Localization of AKT

We then examined the effect of BCL-2 overexpression on AKT activation and subcellular distribution. In MIA-PaCa-2, we noted a primarily cytoplasmic distribution of pAKT, though a very faint nuclear pattern was observed; however a discrete punctate nuclear punctate pattern was observed more significantly in the BCL-2 overexpressing subclone (Fig. 3, top panel). Merging the immunofluorescent data demonstrating co-localization of BCL-2 and pAKT, though not all BCL-2 co-localized with pAKT (Fig. 3, bottom panel). The nuclear fraction of pAKT (increased due to BCL-2 overexpression) did not colocalize with BCL-2 (Fig. 3, inset). These data indicate that BCL-2 facilitated the nuclear translocation of pAKT

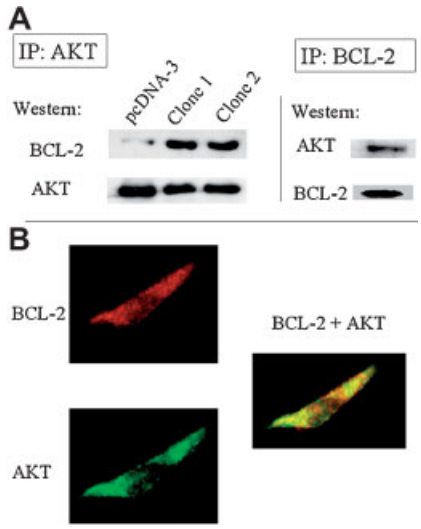

Fig. 2. BCL-2 interacts with AKT. **A**: Immunoprecipitation of AKT in the indicated clones of MIA-PaCa-2 and immunoblotting for BCL-2 or AKT. **B**: Immunoprecipitation of BCL-2 and immunoblotting for AKT or BCL-2. Imunocytochemistry for AKT (green) or BCL-2 (red) and merged images in MIA-PaCa-2 cells with observation of colocalization of BCL-2 with AKT demonstrated by yellow fluorescence in the merged image.

but was not directly involved in the nuclear shuttling of pAKT.

Effect of Inhibiting BH-3-Mediated BCL-2 Binding and AKT Signalling

HA14-1 is a small molecular inhibitor that prevents interaction of BCL-2 with other

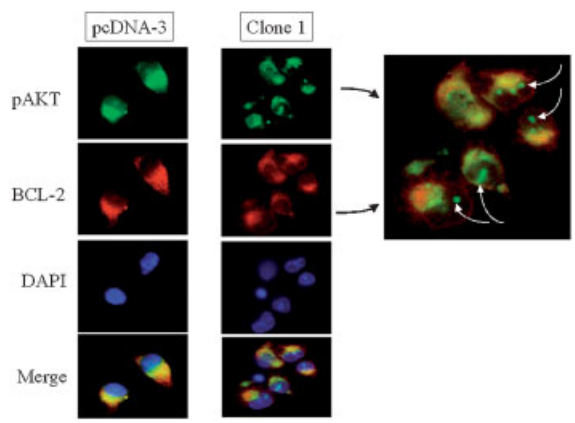

Fig. 3. Increased BCL-2 induces nuclear translocation of AKT. **A**: Immunocytochemistry in control (pcDNA-3) or BCL-2 stably transfected (Clone 1) MIA-PaCa-2 cells for BCL-2 (red) or pAKT (green) and nucleus visualized by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; blue). Bottom row is merged image with cytoplasmic BCL-2 and pAKT co-localized (yellow staining). Inset of the BCL-2 overexpressing Clone 1 demonstrating magnified view of combined BCL-2 and pAKT immunocytochemistry (in the absence of DAPI counterstaining) demonstrating punctate nuclear pAKT (arrows) not colocalized with BCL-2.

proteins by interfering with the BH-3 domain of BCL-2 (Wang et al., 2000). After treatment of the BCL-2 overexpressing MIA-PaCa-2 Clone 1 with HA14-1, we noted a time-dependent decrease in the phosphorylated forms of both AKT and IκB without effect on total AKT or IκB levels (Fig. 4A). These data suggest that disrupting BH-3 mediated binding of BCL-2 inhibits activity of AKT and NF-κB. To determine whether HA14-1 has any effect on the BCL-2:AKT interaction we previously identified, similar immunoprecipitation studies were performed. Once again, the BCL-2 overexpressing MIA-PaCa-2 Clone 1 was treated with HA14-1 and subsequently total protein lysate was immunoprecipitated for BCL-2 and immunoblotted for either AKT or BCL-2 (Fig. 4B). We noted that HA14-1 treatment reduced the amount of AKT in the BCL-2 bound complex. Lastly, immunofluorescence studies demonstrated that treatment of these cells with HA14-1 decreased pAKT content and nearly eliminated the nuclear content of pAKT (Fig. 4C). We also noted that a decrease in the association of BCL-2 with pAKT, further suggesting that HA14-1 inhibited the interaction of BCL-2 with AKT, presumably through the BH-3 domain of BCL-2.

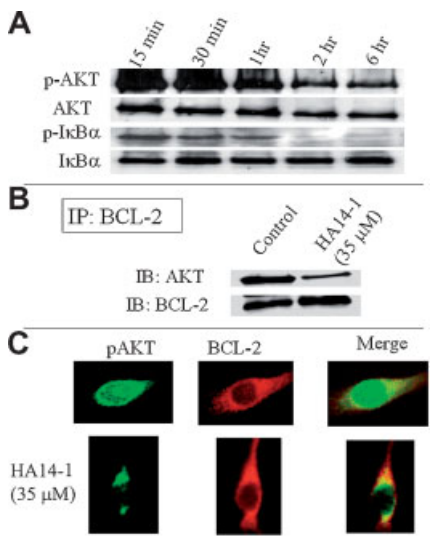

Fig. 4. Inhibition of BCL-2 blocks AKT signaling. **A**: Immunoblotting of MIA-PaCa-2 cells for phospho-AKT, AKT, phospho-IκBα and IκBα following the indicated treatment time with HA14-1 (35 μM). **B**: MIA-PaCa-2 treated in the absence or presence of (35 μM; 6 hrs) and then immunoprecipitated for BCL-2 with immunoblotting for AKT or BCL-2. **C**: Immunocytochemistry in MIA-PaCa-2 cells for BCL-2 (red) or pAKT (green) and merged images in the absence or presence of (35 μM; 6 hrs).

Effect of HA14-1 Treatment on Cellular Effect of the Chemotherapy Paclitaxel

We and others have demonstrated that overexpression of BCL-2 induces resistance to the apoptotic effect of diverse chemotherapies in pancreatic cancer [Bold et al., 1999; Shi et al., 2002; Giovannetti et al., 2006]. Investigation into potential mechanisms to overcome this apoptotic resistance had included targeted inhibition of the AKT/NF-κB pathway; inhibition of either AKT or NF-κB has been shown to sensitize pancreatic cancer cell lines to the apoptotic effect of standard chemotherapeutic agents [Fahy et al., 2003; Fujioka et al., 2003]. As HA14-1 treatment inhibits AKT activity and NF-κB function, we sought to determine whether HA14-1 treatment alters apoptotic sensitivity to chemotherapy, specifically paclitaxel. Treatment of MIA-PaCa-2 cells with paclitaxel decreased cell viability by a modest amount (Fig. 5A); in a dose-dependent fashion, treatment with HA14-1 further decreased cell viability but without effect when administered in the absence of paclitaxel. We then examined the cells using FACS analysis to specifically evaluate the effect on cell cycle distribution given the primary effect of paclitaxel on inducing a G_2/M arrest. We noted HA14-1 had minimal effects on the cell cycle distribution, though simultaneously treatment with paclitaxel increased the blunted the G_2/M arrest of paclitaxel and increased the sub-G_O fraction (apoptotic cells) from 19.8% to 31.3% (Fig. 5B).

DISCUSSION

We demonstrate that overexpression of BCL-2 in human pancreatic cancer cells activates a signaling pathway from AKT to NF-κB-mediated transcriptional activation; furthermore, BCL-2 interacts with AKT and indirectly facilitates nuclear translocation of pAKT. The ability of BCL-2 to alter NF-κB function has been previously demonstrated; however in some models NF-κB activity is increased following overexpression of BCL-2 [de Moissac et al., 1998; Regula et al., 2002] while in other models NF-κB activity is decreased [Grimm et al., 1996; Hour et al., 2000; Song et al., 2004]. de Moissac et al. have shown that in ventricular myocytes, BCL-2 overexpression increases IKK activity, increases I-κB phosphorylation, and NF-κB nuclear translocation and transcriptional activation [de Moissac et al., 1998]. Our data

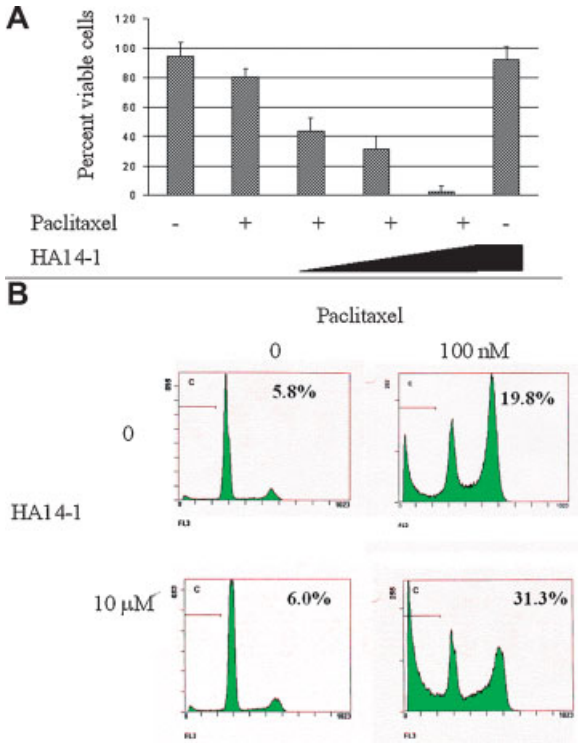

Fig. 5. Inhibition of BCL-2 increases apoptotic sensitivity to paclitaxel. **A**: Effect of increasing doses of HA14-1 (10–50 μM) on the effect of paclitaxel (100 nM; 24 hrs) on cellular viability of MIA-PaCa-2 cells. **B**: Effect of paclitaxel (100 nM), HA14-1 (10 μM) or the combination (24 h treatment) and cell cycle distribution detected by FACS analysis; induction of apoptosis (sub-G_0 fraction) is quantitated and noted in upper corner of each result.

similarly demonstrates in pancreatic cancer that BCL-2 overexpression increases IKK activity, which may be an additional pathway by which NF-κB is constitutively activated in this disease. While we and others have noted a relationship between activation of AKT and high levels of BCL-2, we had concluded that the BCL-2 was downstream in the signaling pathway from AKT; our data now suggests that BCL-2 may also function upstream of AKT signaling.

To date, there is limited data that couples BCL-2 to AKT, either physically or functionally. Kim et al. have reported the only functional link; antisense-mediated reduction of BCL-2 levels in gastric cancer reduced pAKT levels, though no mechanism was investigated [Kim et al., 2004]. The physical interaction of BCL-2 and AKT remains unclear. Four domains of BCL-2 (BH-1, BH-2, BH-3 and BH-4) have been described that are involved in homo- and heterodimerization with other BCL-2 family members [Hanada et al., 1995], though AKT lacks any of these. Conversely, the primary regulatory domain in AKT is the pleckstrin homology (PH) domain, though BCL-2 lacks this motif. The oncogene TCL1 and the heat shock protein hsp90 are among the best described co-activators of AKT for which the binding domains of AKT have been characterized. TCL1 binds to the PH domain of AKT and increases kinase activity [Laine et al., 2000]; hsp90 binds adjacent to the catalytic domain and appears to function as a scaffold for substrate recruitment [Sato et al., 2000]. We believe that our data are the first to demonstrate that BCL-2 functions as a coactivator for AKT, though the specifics of the domain interaction remain unclear.

Although we used a model of overexpression of BCL-2 in the MIA-PaCa-2 human pancreatic cancer cells, the interaction and cellular consequences of BCL-2:AKT interaction was also demonstrated in non-transfected cells. Physical studies demonstrated an interaction of BCL-2 and AKT in non-transfected MIA-PaCa-2 cells and treatment with HA14-1 to inhibit BH-3 mediated binding of BCL-2 reduced the interaction of these proteins, decreased AKT phosphorylation and sensitized cells to the apoptotic effect of chemotherapy. High levels of expression of BCL-2 are common in pancreatic cancer and have been linked to both mutation of p53 and constitutive activation of NF-κB [Sinicrope et al., 1996; Fujioka et al., 2003; Fahy et al., 2005]. Furthermore, we have previously demonstrated that among pancreatic cancer cells, MIA-PaCa-2 has low levels of BCL-2 [Bold et al., 1999]; therefore overexpression of BCL-2 by cDNA transfection allows investigation of the biochemical and cellular consequences observed in pancreatic tumors with high BCL-2 levels.

We also demonstrate nuclear translocation of pAKT induced by overexpression of BCL-2. Interestingly, TCL1 has also been shown to not only activate AKT, but also mediate nuclear translocation [Pekarsky et al., 2000]. Unlike BCL-2, however, TCL1 can shuttle between the cytoplasm and the nucleus and was found to co-localize with nuclear AKT. Nuclear translocation of activated AKT has been observed following growth factor stimulation of MC3T3 and PC-12 cells, though the biologic consequence of this remains unclear [Borgatti et al., 2000; Borgatti et al., 2003]. It is well established that the erbB family of transmembrane tyrosine kinase

receptors signals through AKT [Zhou et al., 2000]. The monoclonal antibody to erbB2, trastuzamab, has become standard treatment for metastatic breast cancer. In a recent clinical trial of neoadjuvant trastuzamab in breast cancer, a decrease in nuclear pAKT, but not cytoplasmic pAKT, was observed in tumor samples following trastuzamab treatment; interestingly, those tumors in which reduction of nuclear pAKT after trastuzamab occurred were more likely to demonstrate a cytotoxic response to trastuzumab therapy [Mohsin et al., 2005]. Therefore, inhibition of nuclear, but not cytoplasmic, AKT activity may be more important in cytotoxic cancer therapy. If so, targeted disruption of the BCL-2 mediated activation and nuclear translocation of AKT may be a specific approach to this signaling event in cancer.

In summary, we have identified a nontraditional pathway of AKT activation. Therapies directed at inhibiting BCL-2 provide an alternative to PI-3 kinase inhibition that may restore apoptotic sensitivity in pancreatic cancer. We believe that targeting this survival pathway is unlikely to have any significant clinical benefit in pancreatic cancer; we and others have demonstrated that the primary benefit of AKT inhibition *in vitro* and *in vivo* is not induction of apoptosis but instead restoration of apoptotic sensitivity to traditional chemotherapeutic agents [Ng et al., 2000; Arlt et al., 2003; Fahy et al., 2003]. Given the clinical development of BCL-2 antisense or inhibitor strategies, our data suggests that these may provide the greatest clinical benefit in pancreatic cancer when combined with standard chemotherapy.

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