Wnt-1 Signaling Inhibits Apoptosis by Activating β-Catenin/T Cell Factor–mediated Transcription

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Abstract. Wnt signaling plays a critical role in development and oncogenesis. Although significant progress has been made in understanding the downstream signaling cascade of Wnt signaling, little is known regarding Wnt signaling modification of the cell death machinery. Given that numerous oncogenes transform cells by providing cell survival function, we hypothesized that Wnt signaling may inhibit apoptosis. Here, we report that cells expressing Wnt-1 were resistant to cancer therapy–mediated apoptosis. Wnt-1 signaling inhibited the cytochrome c release and the subsequent caspase-9 activation induced by chemotherapeutic drugs, including both vincristine and vinblastine. Furthermore, we found that Wnt-1–mediated cell survival was dependent on the activation of β-catenin/T cell factor (Tcf) transcription. Inhibition of β-catenin/Tcf transcription by expression of the dominant-negative mutant of Tcf-4 blocked Wnt-1–mediated cell survival and rendered cells sensitive to apoptotic stimuli. These results provide the first demonstration that Wnt-1 inhibits cancer therapy–mediated apoptosis and suggests that Wnt-1 may exhibit its oncogenic potential through a mechanism of anti-apoptosis.

Key words: β-catenin • apoptosis • Wnt signaling • Tcf transcription • cytochrome c

Introduction

The Wnt-1 gene encodes a secreted cell-signaling glycoprotein that plays important roles in embryogenesis and oncogenesis (Nusse and Varmus, 1992; Cadigan and Nusse, 1997; Miller et al., 1999; Peifer and Polakis, 2000; Polakis, 2000). Wnt-1 was originally identified as a protooncogene activated in mammary carcinoma induced by mouse mammary tumor virus (Nusse and Varmus, 1992). Previous studies have demonstrated that Wnt-1 is able to transform several mammary epithelial cell lines, and that transgenic mice expressing Wnt-1 in the mammary gland develop mammary epithelial hyperplasias and adenocarcinomas (Wong et al., 1994; Bradley and Brown, 1995; Shimizu et al., 1997). The Wnt signaling pathway has been intensely studied in a variety of organisms. The activation of β-catenin/T cell factor (Tcf)[1]–mediated transcription by Wnt signal transduction has been found to play a key role in its biological functions (Molenaar et al., 1996; Gat et al., 1998; Orford et al., 1999).

In the absence of Wnt signaling, β-catenin is associated with a cytoplasmic complex containing the adenomatous polyposis coli protein (APC), the glycogen synthase kinase-3β (GSK-3β), and axin (Su et al., 1993; Yost et al., 1996; Hayashi et al., 1997; Sakanaka et al., 1998; Sakanaka and William, 1999). In this complex, GSK-3β constitutively phosphorylates β-catenin at both serine and threonine residues of the NH_2-terminal region (known as GSK-3β consensus sites), which are well conserved within the catenin family of proteins (Yost et al., 1996; Miller et al., 1999). The

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[1]*Abbreviations used in this paper:* Apaf-1, apoptotic protease–activating factor-1; APC, adenomatous polyposis coli; β-gal, β-galactosidase; DN, dominant-negative mutant; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; GSK-3β, glycogen synthase kinase-3β; IAP, inhibitors of apoptosis; IκB, inhibitor of κB; IKK, IκB kinase; JNK, c-Jun NH_2-terminal kinase; Lef, lymphocyte enhancer factor; NF-κB, nuclear factor kappa B; PI, propidium iodide; PI3K, phosphatidyinositol-3 kinase; PS, phosphatidylserine; Tcf, T cell factor; TNF, tumor necrosis factor; VCR, vincristine; VBL, vinblastine.

phosphorylated β-catenin is targeted by β-transducing repeat–containing protein, a component of the SCF (Skp1-Cullin-F-box) ubiquitin ligase complex, resulting in the ubiquitination and the subsequent degradation of β-catenin by the proteasome (Hart et al., 1999; Kitagawa et al., 1999; Liu et al., 1999; Maniatis, 1999; Winston et al., 1999). In the presence of Wnt signaling, a frizzled receptor activates the intracellular protein, dishevelled, to inhibit APC–GSK-3β–axin activity, leading to the accumulation of free cytosolic β-catenin (Barth et al., 1997; Rubinfeld et al., 1997; Polakis, 2000). Subsequently, β-catenin can translocate to the nucleus and form a complex with members of the lymphocyte enhancer factor (Lef)/Tcf family of transcription factors to activate the expression of target genes. The Tcf family members alone have no transcriptional activation functions. β-Catenin provides transactivation domains and can stimulate transcription activation by binding to Tcf proteins (Behrens et al., 1996; Hsu et al., 1998; Roose et al., 1999). Many important genes, including c-myc, cyclin D1, and metalloproteinase, which are involved in oncogenesis, have been identified to be regulated by the activation of β-catenin/Tcf–mediated transcription (He et al., 1998; Crawford et al., 1999b; Kolligs et al., 1999; Shtutman et al., 1999; Tetsu and McCormick, 1999). Although mutations in the Wnt gene have not been found in human cancers, mutations in the downstream components of Wnt signaling, most of which lead to the constitutive activation of β-catenin/Tcf–mediated transcription, have been found to be associated with a variety of human cancers (Kinzler and Vogelstein, 1996; Miller et al., 1999). For example, germline APC mutations can cause hundreds of benign colorectal tumors, some of which develop into cancer. Somatic mutations of the APC gene are associated with >80% of sporadic colorectal adenomas and carcinomas (Kinzler and Vogelstein, 1996). Mutations in the putative GSK-3β phosphorylation sites of β-catenin have been found in many human cancers, such as colorectal cancer, hepatocellular carcinoma, and melanoma (Morin et al., 1997; Rubinfeld et al., 1997; Caca et al., 1999). Most recently, axin mutations have been identified from hepatocellular carcinoma (Satoh et al., 2000).

Apoptosis is characterized by condensation of the nucleus, specific protein degradation, and DNA fragmentation (Chao and Korsmeyer, 1998; Earnshaw et al., 1999; Vaux, 1999). The caspase family of cysteine proteases has been identified as critical components in executing the apoptotic program (Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997; Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998; Vaux, 1999). Studies have demonstrated that mitochondrial damage plays an important function in initiating the cascade of caspase activation in response to stress stimuli, such as chemotherapeutic drugs (Chao and Korsmeyer, 1998; Green and Reed, 1998). Disruption of the outer mitochondrial membrane by apoptotic stimuli results in the release of cytochrome c into the cytosol (Liu et al., 1996). Cytochrome c, along with ATP and apoptotic protease–activating factor-1 (Apaf-1), recruits and processes procaspase-9 (Li et al., 1996; Zou et al., 1997). The active caspase-9 subsequently activates effector caspases, such as caspase-3, that in turn cleave key proteins to induce apoptosis (Liu et al., 1996; Zou et al., 1997). Although it is not known whether Wnt signaling modulates the cell death machinery, interestingly, overexpression of several downstream mediators of Wnt signaling has been found to regulate apoptosis (Morin et al., 1996; He et al., 1999; Orford et al., 1999; Strovel and Sussman, 1999). Using an inducible expression system, Morin et al. found that overexpression of APC in human colorectal cancer cells induced apoptosis. Orford et al. reported that ectopic expression of β-catenin inhibited apoptosis associated with a loss of attachment to extracellular matrix (anoikis) by unknown mechanisms. Paradoxically, overexpression of dishevelled, an active component of Wnt-1 signaling for stabilization of β-catenin, has been found to induce apoptosis (Strovel and Sussman, 1999). In spite of these findings, the molecular mechanisms by which these mediators exert their effect on cell survival are not understood.

Given that abnormal activation of Wnt-1 signaling is associated with tumor formation and development, here, we directly examined whether Wnt-1 signaling provides cell survival signals that are capable of overcoming chemotherapeutic agent–induced apoptosis. We found that Wnt-1 signaling inhibited chemotherapy-induced apoptosis at the level of the mitochondria, by preventing cytochrome c release and subsequent caspase-9 activation. Furthermore, we found that Wnt-1–mediated β-catenin/Tcf transcription was responsible for providing protection against apoptosis, since the inhibition of β-catenin/Tcf transcription resensitized cells to chemotherapy-induced cell death. This work provides the first proof that activation of the Wnt-1/β-catenin/Tcf signaling would not only provide a cell growth advantage to cancer cells, but would also significantly affect the clinical outcome by inhibiting chemotherapy-induced apoptosis.

Materials and Methods

Cell Culture and Stable Transfection

Rat-1 cells stably expressing Wnt-1 (Rat-1/Wnt-1) and control Rat-1 cells stably expressing β-galactosidase (Rat-1/β-gal) were maintained in DME, supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml), at 37°C under 5% CO_2 and at 95% humidity (Young et al., 1998). For stable transfection, Rat-1/Wnt-1 cells were cotransfected with pcDNA3-flag-DN-Tcf-4, encoding the dominant-negative mutant (DN) of Tcf-4, or control empty vector and pBabe vector, containing a puromycin selectable marker, with Superfect (QIAGEN), according to the manufacturer's protocol (QIAGEN). 2 d after transfection, cells were selected for resistance to puromycin at a concentration of 1.5 μg/ml for 2 wk. The resistant clones were screened for DN-Tcf-4 expression with a monoclonal antibody against the flag epitope after 2 wk of selection. The positive clones were pooled.

Trypan Blue Exclusion, Cell Death ELISA, Annexin V Staining, and LacZ Survival Assay

100,000 cells were plated onto six-well plates the day before stimulation. Cells were treated with vincristine (VCR, 0.5 μg/ml), vinblastine (VBL, 0.5 μg/ml), or tumor necrosis factor (TNF, 10 ng/ml) and cyclohexamide (10 μg/ml). Cell viability was determined by trypan blue exclusion. The supernatant was collected and cell death ELISA was performed as described previously (Wang et al., 1999a,b).

For annexin V staining, 2×10^4 cells were plated on a microscope coverslip in 24-well plates the day before stimulation. 16 h after stimulation, cells were gently washed once with 1× binding buffer, and stained with annexin V conjugated with enhanced green fluorescent protein (EGFP) solution (1:40; CLONTECH Laboratories, Inc.) and propidium iodide (PI, 50 mg/ml, Sigma-Aldrich) for 15 min at room temperature in the dark. After staining, cells were washed twice with 1× binding buffer and fixed in 2% formaldehyde in PBS, pH 7.4, for 20 min. The coverslips were inverted on a drop of Vectashield mounting media (Fisher Scientific) on slides, examined, and photographed under a fluorescence microscope using a filter set for FITC.

For the LacZ survival assay, cells were cotransfected with pcDNA3-LacZ vector and either pcDNA3-Flag-DN-Tcf4 or empty vector with Su-

perfect. 24 h after transfection, cells were treated with VCR or VBL (0.5 μg/ml) for an additional 24 h. The cells were then washed with PBS and fixed with 0.5% gluteraldehyde for 30 min. The cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (GIBCO BRL) for 6 h and blue cells were counted by phase–contrast microscopy. In some experiments, instead of the cotransfection of pcDNA3-LacZ vector, cells were transfected with pCMV-EGFP vector. The GFP-expressing cells were directly examined under a fluorescent microscope. Cell treatments were performed as described above.

Western Blot Analysis

2×10^6 cells were plated in a 100-mm plate the day before stimulation. Cells were treated with VCR or VBL at the concentration of 0.5 μg/ml for the indicated times. The detached and attached cells were collected. Whole cell extracts were prepared with radioimmune precipitation assay buffer containing 1% NP-40, 5% sodium deoxycholate, 1 mM PMSF, 100 mM sodium orthovanadate, and 1:100 protease inhibitors cocktail (Sigma-Aldrich). The proteins were resolved in a 10% SDS-PAGE and transferred to PVDF membrane with a semi-dry transferer apparatus (Bio-Rad Laboratories). The membrane was probed with polyclonal antibodies against phospho-specific c-Jun, Akt, or IκBα protein (Cell Signaling Technology, Inc.) and visualized using an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech), according to the manufacturer's recommendation. For detecting the mitochondrial release of cytochrome c, cytosolic extracts were prepared and examined as described previously (Wang et al., 1999a).

In Vitro Caspase-3 and Caspase-9 Activity Assay

Cells were treated as described above for Western blot. The detached and attached cells were collected, washed with PBS, and lysed in 200 μl ice cold hypotonic lysis buffer provided by the manufacturers (R&D Systems; CLONTECH Laboratories, Inc.; Promega). The cell extracts were centrifuged and supernatants were collected. 200–300 μg of protein extracts were incubated in reaction buffer containing LEHD-pNA (colorimetric caspase-9 substrate; R&D Systems) or Ac-Asp-Glu-Val-Asp (DEVD)-pNA (colormetric caspase-3 substrate; CLONTECH Laboratories, Inc.; Promega) at 37°C for 2–3 h. The samples were analyzed with a plate reader by measuring the OD at 405 nm.

β-Catenin/Tcf Luciferase Assay

The pTopflash and pFopflash constructs contain a luciferase reporter under control of consensus Tcf-binding sites and mutated Tcf-binding sites, respectively (Kolligs et al., 1999). Cells were cotransfected with pTopflash or pFopflash luciferase reporters and the related expression vectors using Superfect. pRL-TK *Renilla* luciferase reporter was cotransfected in each case to normalize for transfection efficiency. Luciferase assays were performed using a dual luciferase system (Promega).

Results

Wnt-1 Signaling Inhibits Apoptosis

To study the molecular mechanisms by which Wnt-1 regulates cell growth and transformation, we previously established Rat-1 cell lines stably expressing Wnt-1 gene (Rat-1/Wnt-1) (Young et al., 1998). This system was used because the Wnt-1 protein has proven difficult to isolate or express in soluble or biologically active form, and, in fact, most studies have ectopically expressed the Wnt-1 gene to study its downstream signaling events (Miller et al., 1999). In this system, Rat-1/Wnt-1 cells adopt an elongated and refractile appearance and proliferate in serum-independent conditions. Activation of the Wnt-1 signal leads to a constitutive upregulation of cytosolic β-catenin,

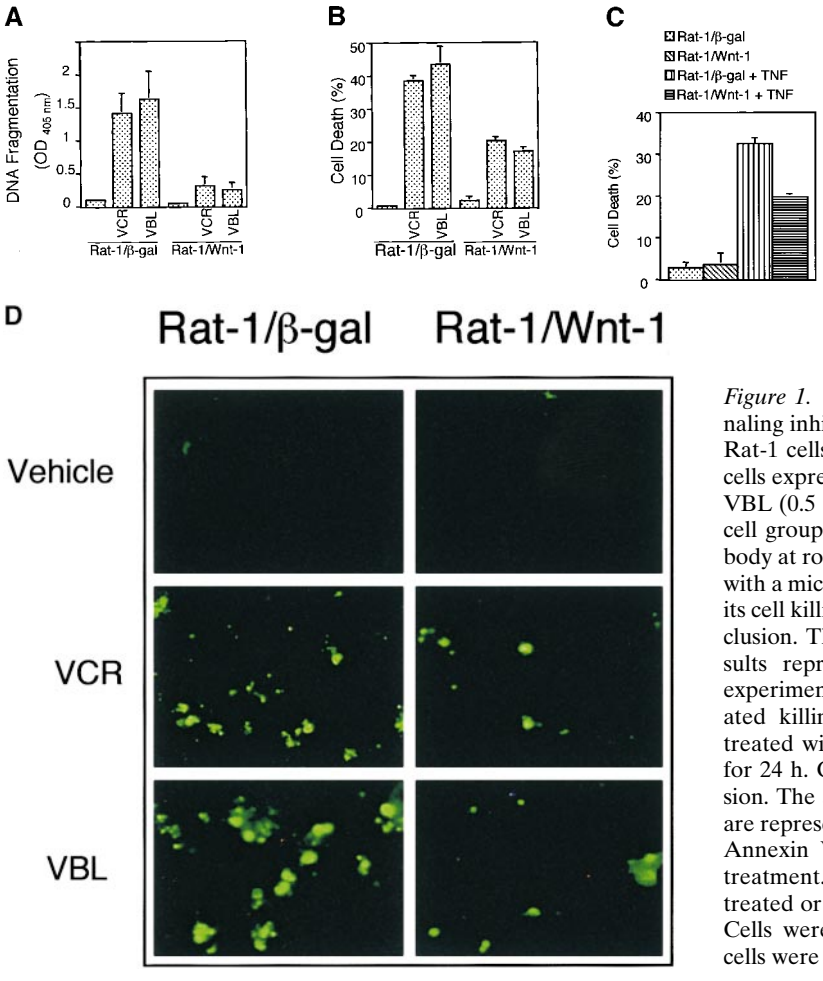

Figure 1. Wnt-1 signaling inhibits apoptosis. (A) Wnt-1 signaling inhibits DNA fragmentation induced by VCR or VBL. Rat-1 cells expressing Wnt-1 (Rat-1/Wnt-1) or control Rat-1 cells expressing β-gal (Rat-1/β-gal) were treated with VCR or VBL (0.5 μg/ml) for 24 h. 20 μl of cell supernatant from each cell group were incubated with anti-histone and -DNA antibody at room temperature for 2 h. The reaction was measured with a microplate reader at 405 nm. (B) Wnt-1 signaling inhibits cell killing. Cell viability was determined by trypan blue exclusion. The assays were performed in triplicate, and the results represent the mean value from three independent experiments. (C) Wnt-1 signaling partially inhibits TNF-mediated killing. Rat-1/Wnt-1 cells and Rat-1/β-gal cells were treated with TNF (10 ng/ml) plus cyclohexamide (10 μg/ml) for 24 h. Cell viability was determined by trypan blue exclusion. The assays were performed in triplicate, and the results are represented as one of three independent experiments. (D) Annexin V staining of apoptotic cells after VCR or VBL treatment. Rat-1/Wnt-1 cells or Rat-1/β-gal cells were untreated or treated with VCR or VBL (0.5 μg/ml) for 14–16 h. Cells were stained with EGFP-annexin (1:40) and PI. The cells were examined by fluorescence microscopy.

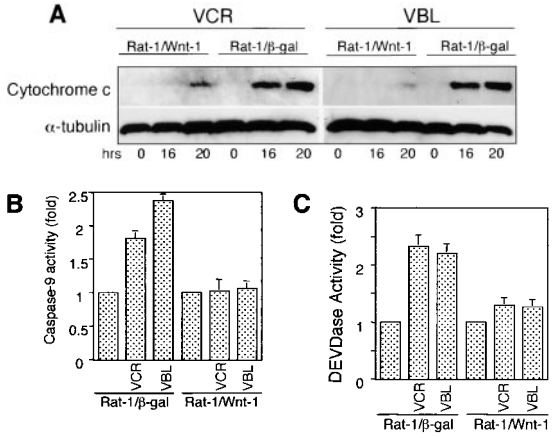

Figure 2. Wnt-1 signaling inhibits VCR- and VBL-mediated cytochrome c release and subsequent caspase-9 activation. (A) Wnt-1 signaling blocked the cytochrome c release. Both Rat-1/Wnt-1 and Rat-1/β-gal cells were treated with VCR or VBL for the indicated times. Cells were fractionated, as described previously, and cytosolic proteins were resolved on a 15% SDS-PAGE and transferred to PVDF membrane with a semi-dry transfer unit. The membrane was probed with a monoclonal antibody against cytochrome c, and the signal was visualized with enhanced chemiluminescence. For internal controls, the blots were stripped and reprobed with monoclonal antibody against α-tubulin (1:2,000). (B) Wnt-1 signaling inhibits caspase-9 activation. Cells were treated with VCR or VBL (0.5 μg/ml) for 16 h. The detached and attached cells were harvested and whole cell extracts were prepared. 200-μg aliquots of protein extracts were incubated with caspase-9 substrate LEHD-pNA (100 μM) for 2–3 h at 37°C. The reaction was measured with a plate reader at 405 nm. The results represent the average values from the two independent experiments. (C) Wnt-1 signaling inhibits caspase-3 activation. Whole cell protein extracts were prepared as described in the legend to B. 200-μg aliquots of protein extracts were incubated with DEVD-pNA substrate for 3 h at 37°C, and the reaction was recorded at 405 nm.

whereas membrane-associated β-catenin remains unaffected. Consequently, there is a constitutive activation of β-catenin/Tcf transcription in Rat-1/Wnt-1 cells, but not in control cells Rat-1/β-gal (Young et al., 1998). Therefore, this well-characterized cell culture model system was used to explore whether Wnt-1 signaling regulates apoptosis.

VCR and VBL act by inhibiting microtubule function, and they are widely used in cancer chemotherapy (Stone and Chambers, 2000). These compounds were used individually to treat Rat-1/Wnt-1 and Rat-1/β-gal cells. To compare cell death, DNA fragmentation and histone release from cell culture supernatant were measured with cell death ELISA. As shown in Fig. 1 A, significantly greater DNA fragmentation (over threefold) occurred in Rat-1/β-gal cells than in Rat-1/Wnt-1 cells after VCR or VBL treatment for 24 h. Trypan blue exclusion analysis found that treatment with VCR or VBL caused 40–50% cell death of Rat-1/β-gal cells, but ~20% of Rat-1/Wnt-1 cells (Fig. 1 B). Wnt-1–mediated protection against VCR or VBL killing was sustained for 48 h (data not shown). Note that because many dead cells were rapidly lysed, the percent of cell death in Rat-1/β-gal cells was underscored. Wnt-1–mediated cell survival was not only specific to chemotherapeutic agents, since Rat-1/Wnt-1 cells were also found to be resistant to apoptosis by TNF (Fig. 1 C) and serum deprivation (data not shown).

To determine whether Wnt-1–mediated survival was due to the modification of apoptosis, the cells were also stained with EGFP-conjugate annexin V in conjunction with PI after VCR or VBL treatment for 14 to 16 h. Changes in the plasma membrane of the cell surface are one of the earliest features of cells undergoing apoptosis. After initiating apoptosis, phosphatidylserine (PS) is translocated from the inner face of the plasma membrane to the cell surface (Salvesen and Dixit, 1997; Cryns and Yuan, 1998; Green and Reed, 1998). Because annexin V has a high affinity for PS and can bind to cells with exposed PS, it has been used to detect the early stage of apoptosis (Wang et al., 1998, 1999a). As shown in Fig. 1 D, compared with numerous annexin V positive–stained cells in the Rat-1/β-gal group, only limited staining was detected in the Rat-1/Wnt-1 group after VCR or VBL stimulation. Annexin V–positive cells failed to take up PI (data not shown), indicating cell death via apoptosis, but not necrosis. Together, these results demonstrated that Wnt-1 signaling provided protection against chemotherapeutic agent–induced apoptosis.

Wnt-1 Signaling Blocks the Mitochondrial Release of Cytochrome C and the Subsequent Caspase-9 Activation

Next, we examined the molecular mechanism by which Wnt-1 signal transduction inhibits VCR- or VBL-mediated apoptosis. To initiate apoptosis, stress inducers such as chemotherapeutic agents damage mitochondria by unknown mechanisms, leading to the release of cytochrome c from mitochondria to the cytosol (Soengas et al., 1999; Wang et al., 1999a; Tournier et al., 2000). Cytochrome c interacts with Apaf-1, caspase-9, and ATP leading to the formation of an oligomeric multiprotein complex, the apoptosome. Oligomerization of Apaf-1 leads to activation of caspase-9 (Li et al., 1996; Zou et al., 1997). Biochemical studies have demonstrated that the cytochrome c–mediated Apaf-1 apoptosome plays an essential role in the cancer therapy–mediated apoptosis (Kuida et al., 1998; Yoshida et al., 1998; Soengas et al., 1999). Bcl-2 family proteins have been found to inhibit chemotherapeutic agent–mediated apoptosis by preventing cytochrome c release from mitochondria (Kluck et al., 1997; Yang et al., 1997). To determine whether Wnt-1 inhibits VCR- or VBL-induced cytochrome c release, cytosolic fractions were extracted as described previously (Wang et al., 1998, 1999a). As shown in Fig. 2 A, the release of cytochrome c from mitochondria was suppressed in the Rat-1/Wnt-1 cells, but not in the Rat-1/β-gal cells. Since cytochrome c is required for the activation of procaspase-9, we measured caspase-9 activity using LEHD as a substrate. As shown in Fig. 2 B, caspase-9 activity was significantly inhibited in the Rat-1/Wnt-1 cells, after the addition of either VCR or VBL, compared with Rat-1/β-gal cells. Since activated caspase-9 amplifies the apoptotic program by cleaving and activating downstream executive caspases, such as caspase-3 (Cryns and Yuan, 1998), we also determined whether caspase-3 activity was inhibited in Rat-1/Wnt-1 cells. Consistent with above results, significantly lower DEVDase activity was present in the Rat-1/Wnt-1 cells

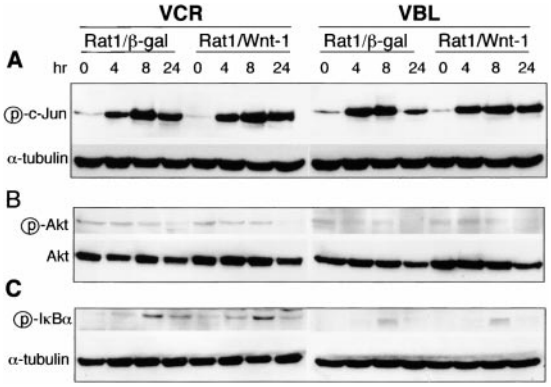

Figure 3. Wnt-1 signaling does not modulate the activation of JNK, Akt kinase, and IKK. Both Rat-1/Wnt-1 and Rat-1/β-gal cells were treated with VCR or VBL (0.5 μg/ml) for the indicated times, and whole cell extracts were subsequently prepared. 50 μg of proteins were resolved on a 10% SDS-PAGE gel. The blots were incubated with polyclonal antibodies against phospho-specific c-Jun (A), Akt (B), or IκBα protein (C) at 4°C overnight, respectively. For internal controls, the blots were stripped and reprobed with monoclonal antibody against α-tubulin (1:2,000) or polyclonal antibody against Akt (1:1,000).

compared with in the Rat-1/β-gal cells after VCR or VBL stimulation (Fig. 2 C). These results demonstrated that Wnt-1 suppressed apoptosis by preventing the release of cytochrome c from mitochondria and the subsequent activation of Apaf-1–caspase-9 apoptosome.

Pro-Apoptotic JNK (c-Jun NH_2-terminal Kinase) Stress Signaling Pathway and Anti-Apoptotic Akt Signaling Pathways Are Not Involved in the Wnt-1–mediated Survival

JNK is a member of the stress-activated group of mitogen-activated protein kinases that are activated by multiple stress inducers. Growing evidence indicates that the activation of the JNK pathway plays a critical role in chemotherapeutic agent–induced apoptosis (Xia et al., 1995; Tournier et al., 2000). As microtubule inhibitors, both VCR and VBL have been found to be potent activators of the JNK pathway (Stone and Chambers, 2000). Interestingly, a downstream mediator of Wnt-1 signaling, dishevelled, has been shown to activate the JNK pathway (Boutro et al., 1998; Li et al., 1999). Thus, we examined whether Wnt-1 signaling modulates the JNK pathway. As shown Fig. 3 A, the kinetics of JNK activation, induced by both VCR and VBL, were almost identical in Rat-1/Wnt-1 and Rat-1/β-gal cells. Although JNK activation was involved in the VCR- or VBL-mediated apoptosis, our results indicated that Wnt-1–mediated prosurvival was not dependent on the modification of JNK pathway in this system.

Akt, also referred to as PKB (protein kinase B), is a serine/threonine protein kinase that has been shown to inhibit apoptosis in response to multiple growth factors and mitogens (Datta et al., 2000; Madrid et al., 2000). Thus, we also compared whether there were any differences in Akt activity after VCR or VBL stimulation in both cell lines. As shown in Fig. 3 B, Rat-1/Wnt-1 and Rat-1/β-gal cells displayed similar levels of basal phorsphorylation activity that decreased with similar kinetics after VCR or VBL

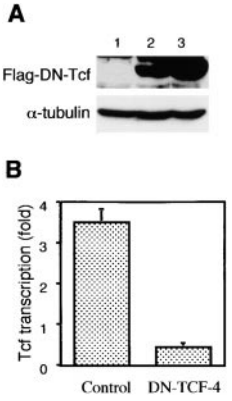

Figure 4. Inhibition of constitutive activation of β-catenin/Tcf transcription by DN-Tcf. (A) Establishment of stable Rat-1/Wnt-1 transfectants expressing the dominant-negative mutant of Tcf-4. Rat-1/Wnt-1 cells were cotransfected with pcDNA3-Flag-DN-Tcf vector or control empty vector and pBabe vector containing puromycin selectable marker and selected with puromycin (1 μg/ml) for 2 wk. About 80 resistant clones were screened with monoclonal antibody against flag epitope. The five positive clones were acquired and pooled together as Rat-1/Wnt-1/DN-Tcf cells. Lane 1 represents the stable control clones expressing puromycin-resistant marker (Rat-1/Wnt-1/P); lane 2 and 3 represent the stable Rat-1/Wnt-1/DN-Tcf expressing flag-DN-Tcf. For internal controls, the blots were stripped and reprobed with monoclonal antibody against α-tubulin (1:2,000). (B) Inhibition of β-catenin/Tcf transcription by DN-Tcf. Rat-1/Wnt-1/DN-Tcf cells and Rat-1/Wnt-1/P cells were transfected with either pTopflash or pFopflash luciferase reporter plasmid. The pRL-TK *Renilla* luciferase reporter was cotransfected to normalize for transfection efficiency. Luciferase activity was measured 1 d after transfection. The fold activation was determined by comparing with pTopflash activity with pFopflash luciferase activity. Transcriptional activation values represent triplicate samples that were counted and averaged.

treatment. The results also were consistent with a previous report that stress stimuli can inhibit Akt activity (Zundel and Giaccia, 1998). Finally, we also examined whether Wnt-1 signaling regulates another anti-apoptotic mediator, namely, the transcription factor NF-κB (nuclear factor κB), which has been found to play an important role in inhibition of TNF- or chemotherapy-induced apoptosis (Wang et al., 1996, 1998, 1999a,b; Madrid et al., 2000). Classic NF-κB is described as the p50–p65 heterodimer that is typically found sequestered in the cytoplasm by the IκB group of inhibitory proteins. The nuclear translocation of NF-κB occurs rapidly after the IκB kinase (IKK)–mediated phosphorylation and subsequent degradation of IκB. Thus, using a polyclonal antibody against phospho-specific IκBα, we assessed IKK activity in Rat-1/Wnt-1 cells and Rat-1/β-gal cells after VCR or VBL stimulation. As shown in Fig. 3 C, the kinetics of IKK activation was similar in both cell lines. Additionally, use of an NF-κB–responsive promoter construct in transfections of both cell lines showed no difference in NF-κB transcriptional activity after VCR or VBL treatment (Chen, S., and C.-Y. Wang, unpublished observation). Together, these data argue that the anti-apoptotic function of Wnt-1 is not mediated through its modulation of either JNK, Akt, or NF-κB activity.

Wnt-1–mediated Cell Survival Is Dependent on the Activation of β-Catenin/Tcf–mediated Transcription

Rat-1/Wnt-1 cells were previously shown to induce the constitutive activation of β-catenin/Tcf–mediated transcription by substantially increasing the level of cytosolic β-catenin (Young et al., 1998). Thus, we determined whether Wnt-1–mediated cell survival was dependent on β-catenin/Tcf transcription. Previous studies have demon-

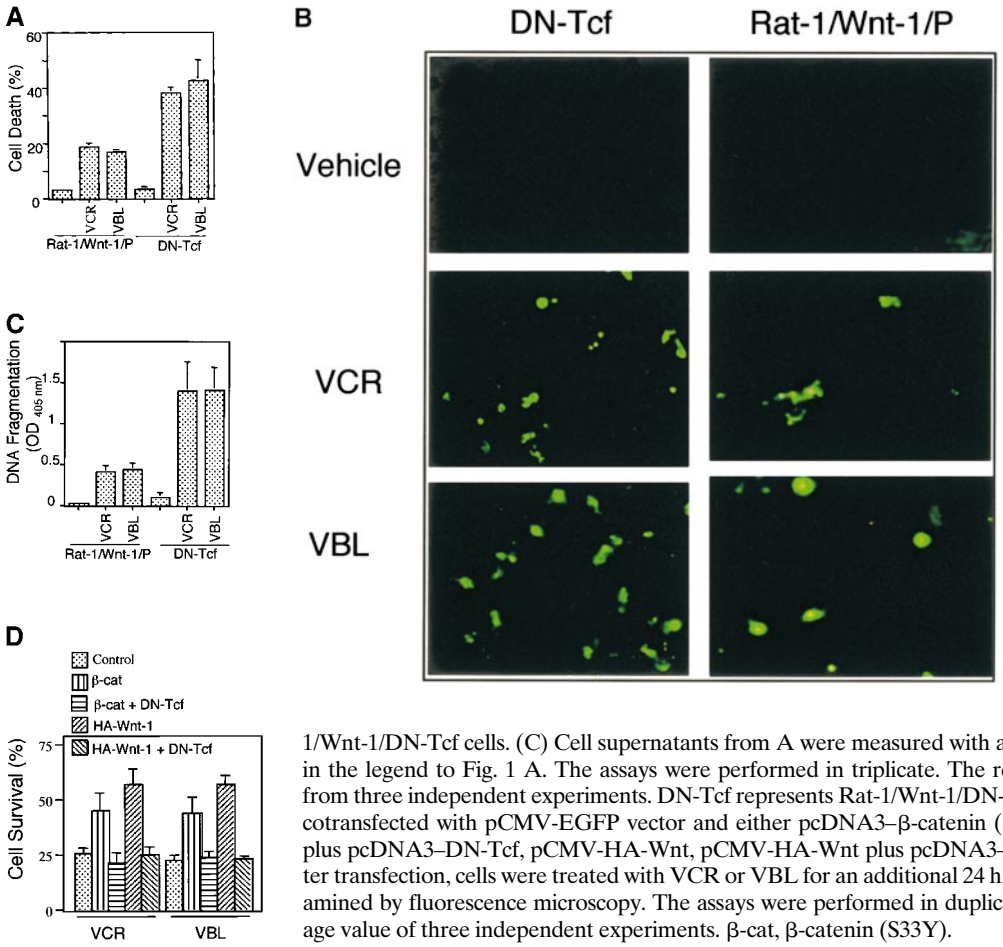

Figure 5. Wnt-1–mediated anti-apoptosis is dependent on the activation of β-catenin/Tcf transcription. (A) Inhibition of Tcf transcription renders cells sensitive to VCR- or VBL-mediated killing. Both Rat-1/Wnt-1/DN-Tcf cells and Rat-1/Wnt-1/P cells were treated with VCR or VBL (0.5 μg/ml) for 24 h. The detached and attached cells were harvested and cell viability was determined with trypan blue exclusion. DN-Tcf represents Rat-1/Wnt-1/DN-Tcf cells. (B) Annexin V staining of apoptotic cells after VCR or VBL treatment. Rat-1/Wnt-1/DN-Tcf cells or Rat-1/β-gal/P cells were untreated or treated with VCR or VBL (0.5 μg/ml) for 14–16 h. Cells were stained with EGFP-annexin (1:40) and PI. The cells were examined by fluorescence microscopy. DN-Tcf represents Rat-1/Wnt-1/DN-Tcf cells. (C) Cell supernatants from A were measured with a cell death ELISA kit, as described in the legend to Fig. 1 A. The assays were performed in triplicate. The results represent the average values from three independent experiments. DN-Tcf represents Rat-1/Wnt-1/DN-Tcf cells. (D) Rat-1/β-gal cells were cotransfected with pCMV-EGFP vector and either pcDNA3–β-catenin (S33Y), pcDNA3–β-catenin (S33Y) plus pcDNA3–DN-Tcf, pCMV-HA-Wnt, pCMV-HA-Wnt plus pcDNA3–DN-Tcf, or control vector. 24 h after transfection, cells were treated with VCR or VBL for an additional 24 h. The GFP-expressing cells were examined by fluorescence microscopy. The assays were performed in duplicate. The results represent the average value of three independent experiments. β-cat, β-catenin (S33Y).

strated that the Tcf NH_2 terminus is required for binding to β-catenin and that Tcf mutant proteins lacking NH_2-terminal sequences retain DNA-binding activity, but function in a dominant-negative fashion (Behrens et al., 1996; Korinek et al., 1997; Kolligs et al., 1999; Prieve and Waterman, 1999). To inhibit β-catenin/Tcf–mediated transcription, Rat-1/Wnt-1 cells were stably transfected with a dominant-negative mutant of the Tcf-4 construct or an empty vector as a control. Five positive clones expressing DN-Tcf were identified and pooled as Rat-1/Wnt-1/DN-Tcf cells according to the level of protein expression (Fig. 4 A, lanes 2 and 3). The puromycin-resistant clones, which expressed empty vector, were pooled and designed as control cells Rat-1/Wnt-1/P cells (Fig. 4 A, lane 1). Interestingly, Rat-1/Wnt-1/DN-Tcf cells exhibited morphologies and growth characteristics similar to those of Rat-1/β-gal cells, indicating DN-Tcf functionally blocked Wnt-1 signal transduction (data not shown). To confirm that DN-Tcf inhibited Wnt-1–mediated constitutive activation of β-catenin/Tcf transcription, Rat-1/Wnt-1/DN-Tcf cells and Rat-1/β-gal/P cells were transfected with pTopflash or pFopflash luciferase reporter. The pTopflash and pFopflash constructs contain the luciferase reporter either under the control of consensus Tcf-binding sites or mutated Tcf-binding sites, respectively (Korinek et al., 1997; He et al., 1998; Kolligs et al., 2000; Xu et al., 2000). As shown in Fig. 4 B, the β-catenin/Tcf transcription activity was also suppressed in Rat-1/Wnt-1/DN-Tcf cells. We then determined whether inhibition of β-catenin/Tcf–mediated transcription rendered cells sensitive to VCR- or VBL-mediated killing. As shown in Fig. 5 A, nearly 50% of Rat-1/Wnt-1/DN-Tcf cells, compared with only 20% of Rat-1/Wnt-1/P cells, were killed after VCR and VBL stimulation. In addition, the increase in both annexin V and DNA fragmentation indicated that restoration of cell death by the expression of DN-Tcf was dependent on the apoptotic program (Fig. 5, B and C). Taken together, these results indicated that Wnt-1–mediated anti-apoptosis was dependent on the β-catenin/Tcf–mediated transcription.

Because Wnt signaling promotes the accumulation of β-catenin, we also determined whether the overexpression of stable β-catenin containing a missense mutation of tyrosine for serine at codon 33 (S33Y) directly inhibited VCR- or VBL-mediated apoptosis by the transient transfection. As shown in Fig. 5 D, the overexpresion of β-catenin (S33Y), but not control vector, in Rat-1/β-gal cells significantly inhibited VCR- or VBL-mediated killing and coexpression of DN-Tcf blocked β-catenin–mediated survival. Interestingly, though coexpression of DN-Tcf also blocked Wnt-mediated survival, overexpression of Wnt-1 by the transient transfection more potently inhibited VCR- or VBL-mediated killing than that of β-catenin, indicating that Wnt-1 may activate other signaling pathways or molecules to enhance β-catenin/Tcf–mediated anti-apoptotic function (Fig. 5 D).

If Wnt-1–mediated β-catenin/Tcf transcription suppressed the mitochondrial release of cytochrome c induced by VCR or VBL, the inhibition of Tcf transcription

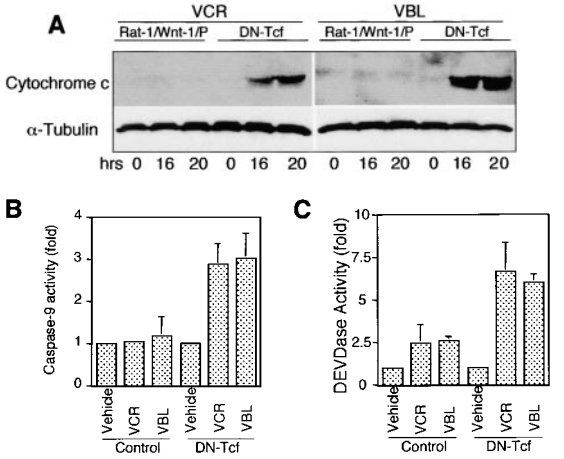

Figure 6. Inhibition of Tcf transcription renders cell sensitive to VCR- or VBL-mediated cytochrome c release. (A) Both Rat-1/Wnt-1/P and DN-Tcf cells were treated with VCR or VBL (0.5 μg/ml) for the indicated times. Cytosolic proteins were detected with a monoclonal antibody against cytochrome c, as described in the legend to Fig. 3 A. (B and C) Inhibition of Tcf transcription renders cells sensitive to VCR- or VBL-mediated caspase activation. Cells were treated with VCR or VBL (0.5 μg/ml) for 16 h. The detached and attached cells were harvested and whole cell proteins were extracted according to the manufacturer's instruction. 200 μg of proteins were incubated with LEHD-pNA substrate and 200 μg proteins were incubated with DEVD-pNA substrate. The results represent the average value from two independent experiments. DN-Tcf represents Rat-1/Wnt-1/DN-Tcf.

should restore the apoptotic potential of these chemotherapeutic agents. As predicted, both VCR and VBL potently induced cytochrome c release from mitochondria to the cytosol in Rat-1/Wnt-1/DN-Tcf cells, but not in Rat-1/Wnt-1/P cells (Fig. 6 A). Consistent with cytochrome c function, there was higher activity of caspase-9 and caspase-3 in Rat-1/Wnt-1/DN-Tcf cells than in Rat-1/Wnt-1/P cells after VCR or VBL stimulation (Fig. 6, B and C). In conclusion, these results confirmed that the Wnt-1–mediated β-catenin/Tcf transcription inhibited apoptosis by preventing the release of cytochrome c from mitochondria and the subsequent activation of caspase-9.

Inhibition of β-Catenin/Tcf–mediated Transcription Potentiates Apoptosis in Human Colorectal Cancer Cells

Abnormal activation of β-catenin/Tcf–mediated transcription has been widely found in human cancers, including colorectal carcinomas. The well-characterized human colorectal cancer cell line SW480 has been found to contain a constitutively high level of β-catenin/Tcf–mediated transcriptional activity due to the inactivation of the APC tumor suppressor gene (Munemitsu et al., 1995; Korinek et al., 1997; He et al., 1998). The expression of DN-Tcf construct inhibited the constitutive activation of β-catenin/Tcf transcription in SW480 cells (Fig. 7 A). Using this cell line, we examined whether inhibition of endogenous β-catenin/Tcf transcription potentiated VCR- or VBL-mediated apoptosis by the transient transfection assay. As shown in Fig. 7 B, though the expression of DN-Tcf alone didn't affect cell survival, VCR- and VBL-mediated killing was significantly enhanced by the expression of DN-Tcf vector,

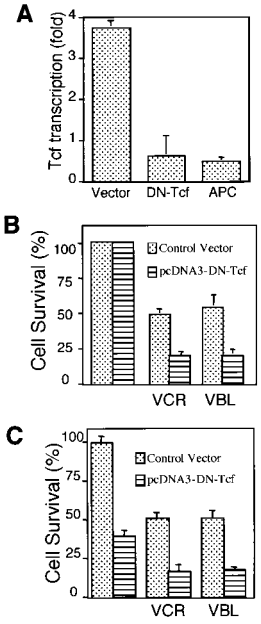

Figure 7. Inhibition of constitutive activation of Tcf transcription render cell sensitive to VCR- or VBL-mediated apoptosis. (A) The expression of DN-Tcf inhibits the constitutive activation of β-catenin/Tcf transcription. SW480 cells were cotransfected with pTopflash or pFopflash luciferase report plasmid and pcDNA3-flag-DN-Tcf, pcDNA-APC vector, or control empty vector with Superfect. PRL-TK *Renilla* luciferase reporter was cotransfected in each case to normalize for transfection efficiency. 24 h after transfection, luciferase assays were performed using a dual luciferase system. The fold activation was determined by comparing pTopflash activity with pFopflash luciferase activity. Each group of assays was performed in triplicate and results represent from one of three independent experiments. (B) Inhibition of constitutive β-catenin/Tcf transcription renders cells sensitive to VCR- or VBL-mediated killing. SW480 cells were cotransfected with pcDNA3-LacZ and pcDNA3-flag-DN-Tcf vector or control vector for 24 h. After transfection, cells were treated with VCR or VBL (0.5 μg/ml) for an additional 24 h. Cells were fixed with 0.5% gluteraldehyde and incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (0.5 mg/ml) for 6 h. The blue-positive cells were counted from three fields in each well. The assays were performed in triplicate. The results represent the average value of two independent experiments. (C) Inhibition of β-catenin/Tcf transcription induces spontaneous apoptosis in HT29 cells. HT29 cells were cotransfected with pCMV-EGFP and either pcDAN3-DN-Tcf or control vector. Cell treatments were performed as described in the legend to B. The GFP-expressing cells were counted by fluorescence microscopy. The assays were performed in duplicate. The results represent the average value of three independent experiments.

but not empty vector, as determining the LacZ-positive cells (blue cells). Morin et al. (1996) had reported that the expression of APC in a human colorectal cell line HT-29 induced apoptosis by unknown mechanisms, using an inducible expression system. Subsequent studies by several groups demonstrated that induction of the expression of APC in that cell line reduced the level of β-catenin and inhibited the β-catenin/Tcf–mediated transcription (Korinek et al., 1997; Kolligs et al., 2000). Thus, we also used this cell line to examine whether the inhibition of β-catenin/Tcf modulated VCR- or VBL-mediated killing. Interestingly, in the absence of VCR or VBL stimulation, ~60% of cells were killed after the transfection of DN-Tcf compared with that of control vector (Fig. 7 C). The dead cells were rounded up and detached from plates with apoptotic characteristics, including condensed chromatin and fragmented nuclei (data not shown). In the presence of VCR or VBL stimulation, only 20% of cells transfected with DN-Tcf compared with 50% of cells transfected with control vector were alive (Fig. 7 C). Taken together, our findings demonstrated that the inhibition of endogenous β-catenin/Tcf–mediated transcription in human colorectal cells potentiated VCR- or VBL-mediated apoptosis and/or induced spontaneous apoptosis.

Discussion

Wnt proteins represent a growing family of secreted signaling molecules that are expressed in diverse tissues and have been shown to regulate cell proliferation, growth, and differentiation (Cadigan and Nusse, 1997; Miller et al., 1999; Peifer and Polakis, 2000). Importantly, the deregulation of the Wnt-1 signaling pathway has been linked with tumorigenesis, presumably by promoting cell growth and proliferation. Here, we demonstrated that Wnt-1, a fundamental member of the Wnt family proteins, potently inhibited chemotherapy-mediated apoptosis. Given that many oncogenes transform cells by providing cell survival function, our results also indicate that Wnt-1 may transform cells via an anti-apoptotic mechanism, in addition to promoting cell growth and proliferation. Interestingly, c-myc, a potent oncogene that promotes cell cycle progression, is a β-catenin/Tcf transcriptional target (He et al., 1998). However, studies have demonstrated that c-myc–mediated transformation requires other survival signals to prevent apoptosis (Miller et al., 1999; Soengas et al., 1999). Our results indicate that Wnt-1–mediated β-catenin/Tcf transcription is also an important survival pathway. It not only plays a role in the promotion of cell proliferation and cell cycle progression, but also may provide an important survival function to facilitate cell transformation.

The downstream mediators of Wnt-1 signal pathway include dishevelled, β-catenin, APC, GSK-3β, and axin (Miller et al., 1999; Polakis, 2000; Salic et al., 2000). Overexpression of APC has been shown to induce apoptosis in the human colorectal cancer cell line HT-29 by unknown mechanisms (Morin et al., 1996). Using this cell line, we found that the inhibition of endogenous β-catenin/Tcf–mediated transcription also triggered cell suicide program. Given the fact that overexpression of APC has been shown to destabilize β-catenin and inhibit β-catenin/Tcf–mediated transcription (Korinek et al., 1997; Kolligs et al., 2000), our findings suggest that APC-induced apoptosis was through the inhibition of β-catenin/Tcf–mediated transcription. Wnt signaling activates dishevelled to inhibit GSK-3β activity, resulting in the stabilization of β-catenin and the activation of β-catenin/Tcf–mediated transcription. Thus, β-catenin stabilization, as well as the formation of a complex with Tcf transcription factors, is likely to play a critical role in Wnt-mediated anti-apoptosis. Indeed, overexpression of β-catenin can overcome anoikis and promote cell cycle progression (Orford et al., 1999). Consistent with that study, our results demonstrated that overexpression of β-catenin also inhibited chemotherapeutic drug–mediated apoptosis, though it was relatively weaker than that of Wnt-1. However, the dominant-negative mutant of Tcf rendered cell sensitive to apoptotic stimuli, which indicated that Wnt-1–mediated anti-apoptosis was dependent on β-catenin/Tcf–mediated transcription. In contrast, Hetman et al. (2000) found that the expression of β-catenin could not protect cortical neurons from apoptosis induced by trophic withdrawal. This difference may be due to both a cell type and apoptotic stimuli. Additionally, in that cell model system, GSK-3β activation might be a dominant pro-apoptotic signal that could not be inhibited by β-catenin. During the preparation of this manuscript, it is reported that Wnt signaling was involved in B-lymphocyte survival and proliferation (Reya et al., 2000), and that Wnt-1 promoted cell survival in the absence of serum (Bournat et al., 2000). Our results, presented here, significantly extend these findings and provide the first demonstration that Wnt-1 signaling is associated with chemoresistance. Furthermore, we have elucidated the molecular mechanism by which Wnt-1 activation inhibits apoptosis. Our results demonstrated that Wnt-1–mediated β-catenin/Tcf transcription blocked chemotherapy-mediated cytochrome c release and subsequent caspase-9 activation. However, our results cannot rule out the possibility that Wnt-1–mediated β-catenin/Tcf transcription also plays a role in other steps of caspase activation cascade. Recently, Steinhusen et al. (2000) have demonstrated that β-catenin is cleaved by caspase-3 during staurosporine-induced apoptosis and that cleaved β-catenin reduced its transactivation potential. Although we could not find that β-catenin was cleaved in our cell model system, it suggests that some apoptotic stimuli may antigonize Wnt-mediated survival signaling by cleaving β-catenin.

Overexperssion of dishevelled, which is known to positively mediate β-catenin stabilization in the Wnt-1 signaling pathway, has also been shown to induce apoptosis (Strovel and Sussman, 1999). Although apparently inconsistent with our current findings, genetic and biochemical studies have demonstrated that dishevelled also functions to activate the JNK pathway, in addition to transducing Wnt signals for the stabilization of β-catenin. The activation of JNK has been found to play a critical role in initiating apoptosis (Xia et al., 1995; Tournier et al., 2000). Thus, overexpression of dishevelled may abnormally activate the JNK pro-apoptotic signaling that can suppress the β-catenin/Tcf–mediated survival function, leading cells down the apoptotic pathway. Finally, in our model system, JNK was not activated by Wnt-1 signaling, which indicates that dishevelled primarily mediated the Wnt-1 pro-survival functions under physiological conditions. Interestingly, GSK-3β has also been found to have both pro- and anti-apoptotic functions (Pap and Cooper, 1998; Hetman et al., 2000; Hoeflich et al., 2000). Inhibition of GSK-3β by phosphatidyinositol-3 kinase (PI3K)/Akt promotes cell survival and activation of GSK-3β by trophic withdrawal and induces apoptosis (Pap and Cooper, 1998; Hetman et al., 2000). Our results also indicate that GSK-3β is a pro-apoptotic molecule. Paradoxically, GSK-3β activation has been found to play an anti-apoptotic role in TNF-mediated apoptosis by activation of NF-κB. However, how GSK-3β stimulates NF-κB transcription is unknown. According to studies by Ding et al. (2000) and others (Yost et al., 1996; Yuan et al., 1999; Hetman et al., 2000), it is likely that GSK-3β may exist in several signaling complexes. One is in the APC–axin–conductin complex that is in specific response to Wnt signaling; one is regulated by PI3K/Akt, but not Wnt signaling; and one is responsible for TNF-induced NF-κB activation. The inhibition of GSK-3β in the APC–axin–conductin complex by Wnt or in the PI3K/Akt-responsive complex by growth factors may lead to cell survival, whereas the inhibition of a fraction of GSK-3β, leading to inactivation of NF-κB, may render cells sensitive to TNF killing.

Several important genes, including c-myc and cyclin D1, have been found to be regulated by Wnt (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999). Currently, it is unknown whether Wnt-1 signaling regulates the expression of anti-apoptotic genes. Since Bcl-2 family

proteins have been found to play roles in the mitochondria and inhibit chemotherapy-mediated apoptosis (Chao and Korsmeyer, 1998; Green and Reed, 1998), we have applied gene array analysis to examine whether Wnt-1–mediated β-catenin/Tcf transcription regulates the expression of Bcl-2 family proteins, including Bcl-2, Bcl-xL, Bax, Bad, and Bik. No differences have been found in the expression of these genes between Rat-1/Wnt-1 cells and Rat-1/β-gal cells, suggesting that Bcl-2 family members are not direct transcription targets of Wnt-1 signaling. However, we cannot rule out the possibility that Wnt-1 signaling or Wnt-1–mediated β-catenin/Tcf transcription may posttranslationally modify these proteins. We also performed Western blot analysis to examine another group of anti-apoptotic molecules, namely inhibitors of apoptosis (IAP), including c-IAP1, c-IAP2, and X-linked IAP (Crawford et al., 1999a; Vaux, 1999; Verhagen et al., 2000; Chen, S., and C.-Y. Wang, unpublished observation). Again, no differences were found between Rat-1/Wnt-1 and Rat-1/β-gal cells. Reya et al. (2000) reported that the fas gene is negatively regulated by Lef-1 transcription. However, in our model system, Fas was not involved in the VCR- or VBL-mediated apoptosis (data not shown). Thus, these results indicate that Wnt-1–mediated β-catenin/Tcf transcription may regulate other anti-apoptosis molecules yet to be identified.

Considering the fact that the constitutive activation of β-catenin/Tcf transcription has been found in a variety of human cancers, our results have important implications for human cancer therapy. The constitutive activation of β-catenin/Tcf transcription, at least, has a dual function, though it may have other functions as well. One is to promote cell cycle progression and cell proliferation, as described previously (Miller et al., 1999; Polakis, 2000). Another is to provide cell survival function. Therefore, in the future, the application of a gene therapy approach or the development of small molecules to inhibit β-catenin/Tcf–mediated transcription may improve the efficacy of chemotherapy by enhancing apoptosis in cancer cells.

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