Inhibition of Wnt-1 Signaling Induces Apoptosis in β-Catenin-Deficient Mesothelioma Cells

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ABSTRACT

It is known that Wnt-1 signaling induces apoptosis by activating β-catenin/tcf-mediated transcription. Here, we show that blocking Wnt-1 signaling in β-catenin-deficient mesothelioma cell lines H28 and MS-1 induces apoptotic cell death. Both Wnt-1 small interfering RNA (siRNA) and Dishevelled siRNA induced significant apoptosis in these cell lines. A small molecule inhibitor of c-Jun NH2-terminal kinase inhibited the apoptotic cell killing induced by either Wnt-1 siRNA or Dishevelled siRNA in these cells. Our data suggest that β-catenin-independent noncanonical pathway(s), i.e., Wnt/JNK pathway, may play a role in the apoptotic inhibition caused by Wnt-1 signaling.

INTRODUCTION

Wnt ligand binds directly to its receptor Frizzled (Fz), and this binding activates canonical and noncanonical pathways (1, 2). The activation of the canonical pathway leads to signal transduction through Dishevelled (Dvl) and results in β-catenin stabilization and accumulation in the cytoplasm. β-Catenin translocates into the nucleus and binds to Tcf/Lef transcription factors to make a heterodimeric complex that activates the transcription of Wnt target genes, e.g., c-myc and cyclin D1 (3–6). β-Catenin is a key mediator of the Wnt signal. Noncanonical pathways, referred as Wnt signaling pathways that signal independently of β-catenin, may signal through calcium flux, c-Jun NH2-terminal kinase (JNK), and G proteins (7).

Wnt-1 was originally described as a proto-oncogene in mouse mammary tumor induced by mouse mammary tumor virus (8). Recently, it has been shown that Wnt-1/β-catenin signaling promotes cell survival in various cell types (9–11). For instance, Chen et al. (10) have recently shown that Wnt-1 signaling inhibits apoptosis through β-catenin and that cells expressing Wnt-1 resist apoptosis induction by chemotherapy. In addition, c-Myc was identified as one of the transcriptional targets of β-catenin/Tcf in colorectal cancer cells (5), suggesting that Wnt signaling functions in oncogenesis, in part, is through the growth promoting activity of c-Myc (12). At least two classes of Wnt antagonists have been reported and both classes of molecules prevent ligand-receptor interactions. The first class, including secreted Frizzled-related proteins family and Wnt inhibitory factor-1, binds directly to Wnt ligand. The second class, including Dickkopf family, binds to a low-density lipoprotein-receptor-related protein 6, a subunit of Wnt receptor complex (13). Down-regulation of β-catenin levels by secreted Frizzled-related proteins and Dickkopf-1 sensitize cells to proapoptotic stimuli (14).

To date, β-catenin-independent mechanism(s) have not been linked with the apoptotic inhibition caused by Wnt-1 signaling. We have recently shown evidence of Wnt pathway activation through Dvl overexpression in both human lung cancer and mesothelioma (15, 16). Moreover, we have found that Wnt-1 is expressed in many human cancer cell lines and tissues, and we have also demonstrated that blockade of Wnt-1 signaling [by Wnt-1 small interfering (si)RNA or a Wnt-1-targeted monoclonal antibody] induces apoptosis in some human cancer cells (17). This apoptotic induction should be β-catenin/Tcf-dependent based on the antiapoptosis mechanism proposed for Wnt-1 signaling (10, 12). On the basis of our recent findings, however, we hypothesized that Wnt noncanonical pathway(s) (e.g., Wnt/JNK pathway) may play a role in the apoptosis inhibition caused by Wnt-1 signaling.
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Western analysis of Dishevelled (Dvl)-3 and β-catenin expression in malignant plural mesothelioma cell lines H28 and MS-1 and a non-small cell lung cancer cell line H1703. β-Actin was used as loading control. Cytosolic proteins were prepared and used in these Western blots. TOPFLASH assay of Tcf-dependent transcriptional activity in mesothelioma cell lines H28 and MS-1 and a non-small cell lung cancer cell line H1703. The results are means ± SD (error bars). Experiments were performed in triplicate.

Apoptosis Analysis. Cells were harvested by trypsinization and stained using an Annexin V FITC Apoptosis Detection kit (Oncogene, Cambridge, MA), according to the manufacturer’s protocol. Then stained cells were immediately analyzed by flow cytometry (FACScan; Becton Dickinson, Franklin Lake, NJ). Early apoptotic cells with exposed phosphatidylserine but intact cell membranes bound to Annexin V-FITC but excluded propidium iodide. Cells in necrotic or late apoptotic stages were labeled with both Annexin V-FITC and propidium iodide.

TOPFLASH Assay. Cells were plated in 6-well plates. The TOPFLASH or FOPFLASH reporter plasmid was transfected transiently into cells as described previously (16). Tcf-mediated gene transcription was determined by the ratio of pTOPFLASH:pFOPFLASH luciferase activity, and each was normalized to luciferase activities of the pRL-TK reporter (cotransfected internal control). All experiments were performed in triplicate.

RESULTS AND DISCUSSION

To test our hypothesis that β-catenin is not essential for Wnt-1-induced apoptosis inhibition, the mesothelioma cell line H28, containing a homozygous deletion of the β-catenin gene, was chosen for this study (19). A non-small cell lung cancer cell line H1703 (16) and another mesothelioma cell line MS-1 (20) were also used for control experiments. All these cell lines were found to express Wnt-1 protein by Western blot analysis. The homozygous deletion of β-catenin in H28 cell line was confirmed by Southern blot analysis, and lack of β-catenin expression was validated using Northern and Western blot analysis. We checked their protein expression of cytosolic β-catenin and Dvl-3, and we found no cytosolic β-catenin protein in both H28 and MS-1 cells (Fig. 1A). In a TOP/FOP analysis, lower Tcf/lef
transcription activity level was detected in both H28 and MS-1 cells when compared with H1703 cells. As expected, reexpression of β-catenin in H28 cell line was able to restore the Tcf/lef transcription activity (Fig. 1B).

To assess the role of Wnt-1 in the H28 cells lacking β-catenin, Wnt-1 siRNA and anti-Wnt-1 monoclonal antibody were used to treat the cells. Interestingly, Wnt-1 siRNA was able to kill β-catenin-deficient H28 cells (Fig. 2A), and this cell killing was largely because of apoptosis induction (Fig. 2B). The blockade of Wnt-1 signaling was confirmed by checking the expression of Wnt-1 signaling mediators (Fig. 2C). Wnt-1 is down-regulated by Wnt-1 siRNA, and a Wnt intracellular signaling molecule, Dvl-3, is down-regulated in the H28 cells treated with Wnt-1 siRNA. An inhibitor of apoptosis, survivin, was down-regulated and cytochrome c was up-regulated after the blockade of Wnt-1 signaling. MS-1 cell line lacking cytosolic β-catenin showed similar apoptotic and downstream effect after treatment with Wnt-1 siRNA. Moreover, similar results were seen when we treated both H28 and MS-1 cells with an anti-Wnt-1 monoclonal antibody (Fig. 2D–F; Ref. 17). We have found that C57Wnt-1 cells expresses higher level of surviving when compared with the control C57mv7 cells (Fig. 2G), suggesting that Wnt-1 signaling may induce survivin expression in C57MG cells. A non-small cell lung cancer cell line H1703, which is known to have β-catenin/lef-mediated transcription activity, was used as a control. Both Wnt-1 siRNA and the anti-Wnt-1 monoclonal antibody induced apoptotic cell killing in H1703 cells. Furthermore, we have demonstrated recently that Wnt-1 siRNA induces apoptosis in a breast cancer cell line MCF-7 which also contains wild-type β-catenin (17). These data suggest that a β-catenin-independent mechanism(s) is responsible for the apoptosis induced by blocking Wnt-1 signaling. To our knowledge, this is the first proof that β-catenin is not essential for Wnt-1-induced apoptotic inhibition. It was demonstrated that Wnt-1/β-catenin/lef signaling inhibited apoptosis by preventing cytochrome c release and subse-

Fig. 3. Dishevelled (Dvl) siRNA induces apoptosis in H28 and MS-1 cells. A. 0.5% Crystal Violet staining of cell lines H28, MS-1, and H1703 after transfection of nonsilencing control siRNA or Dvl siRNA (100 nM) (~5–6 days after transfection). Concentration of both control and Dvl siRNA used is 100 nM. B, apoptosis analysis by flow cytometry (Annexin V staining) after Dvl siRNA transfection in cell lines H28, MS-1, and H1703 (100 nM for ~4–5 days). The results (percentage of apoptotic cells) are the means ± SD (error bars). Experiments were performed in triplicate, and a total of 20,000 cells was analyzed in each individual experiment. C. Western analysis after Dvl siRNA transfection in cell lines H28, MS-1, and H1703 (100 nM for ~4–5 days). Nonsilencing siRNA was used as control. β-Actin served as loading control. Cytosolic proteins were prepared and used in these Western blots.

Fig. 4. The small molecule c-Jun NH2-terminal kinase (JNK) inhibitor (SP600125) inhibits apoptosis induced by Wnt-1 siRNA, anti-Wnt-1 monoclonal antibody, or Dishevelled (Dvl) siRNA in H28 and MS-1 cells. A. 0.5% Crystal Violet staining of mesothelioma cell line H28 and NSCLC cell line H460 (expressing β-catenin) after transfection of nonsilencing control siRNA, Dvl siRNA, or Wnt-1 siRNA (~5–6 days after transfection) in the presence (bottom wells) and absence (top wells) of the JNK inhibitor SP600125 (10 μM). Concentration of all siRNA used is 100 nM. B–E, morphologies of H28 cells after the treatment with control monoclonal antibody alone, anti-Wnt-1 monoclonal antibody alone, control monoclonal antibody plus the JNK inhibitor SP600125, and anti-Wnt-1 monoclonal antibody plus SP600125, respectively. Antibody concentration used is 10 μg/ml, and SP600125 concentration used is 10 μM. F, apoptosis analysis by flow cytometry (Annexin V staining) in the presence and absence of the JNK inhibitor SP600125 (10 μM) after siRNA transfection (100 nM for ~4–5 days) or monoclonal antibody treatment (10 μg/ml for 3–4 days) in cell lines H28 and MS-1. The results (percentage of apoptotic cells) are the means ± SD (error bars). Experiments were performed in triplicate, and a total of 20,000 cells was analyzed in each individual experiment. G, Western analysis in the presence and absence of the JNK inhibitor SP600125 (10 μM) after siRNA transfection (100 nM for ~4–5 days) or monoclonal antibody treatment (10 μg/ml for 3–4 days) in cell lines H28 and MS-1. β-Actin served as loading control. Cytosolic proteins were prepared and used in these Western blots. H, Western analysis of JNK protein expression in H28 cells after the treatment with control monoclonal antibody alone, anti-Wnt-1 monoclonal antibody alone, control monoclonal antibody plus JNK siRNA, and anti-Wnt-1 monoclonal antibody plus JNK siRNA, respectively. Antibody concentration used is 10 μg/ml, and JNK siRNA used is 100 nM. I, Western analysis of JNK protein expression in H28 cells after the treatment with control monoclonal antibody alone or anti-Wnt-1 monoclonal antibody. β-Actin served as loading control.

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quent caspase activation; however, β-catenin/Tcf transcription is required for this Wnt-1-mediated cell survival (10, 12).

The cytoplasmic Dvl family proteins are membrane-proximal signal intermediates in the Wnt signal pathway. Dvl interacts with Fz receptors and mediates Wnt-1 signaling in both canonical and noncanonical pathways. Dvl proteins consist of DIX, PDZ, and DEP domains. The COOH-terminal DEP domain has been connected with JNK pathway (21). More recently, the PDZ domain, which is involved in both canonical and noncanonical pathways, was found to bind directly to the COOH-terminal region of Fz (22).

To determine whether this β-catenin-independent apoptosis inhibition involves the Wnt signaling molecule Dvl, Dvl siRNA was used to treat H28 cell line (16). Dvl siRNA induced dramatic apoptosis in the β-catenin-deficient H28 cells (Fig. 3, A and B). Similar apoptotic effect was also seen in MS-1 cells that lack cytoplasmic β-catenin (Fig. 3, A and B). After Dvl siRNA treatment, Dvl-3 was confirmed to be down-regulated in all three cell lines by Western blot analysis. An inhibitor of apoptosis, survivin, was down-regulated in both H28 and MS-1 cells treated with Dvl siRNA (Fig. 3C). Our earlier data indicates that Dvl-specific siRNA treatment in H1703 decreased Dvl and β-catenin expression, resulting in reduction of Tcf-dependent transcriptional activity and growth inhibition (16). The physiological significance of down-regulation of Dvl-3 induced by Wnt-1 siRNA is the inhibition of both canonical and noncanonical Wnt pathways, therefore the downstream effects.

In a recent study, we have shown that Dvl-3 overexpression is consistent with higher expression of Wnt-1 in lung cancers, implicating that Wnt-1 may up-regulate Dvl-3 in these tumors (16). Dvl siRNA does not appear to affect expression levels of Dvl-1 and Dvl-2 because the expression levels for both Dvl-1 and Dvl-2 are minimally expressed in the samples tested (16). Interestingly, JNK was found to be up-regulated in these cells after blocking Wnt-1 signaling (Fig. 4). We have also examined the links between Dvl and JNK. In one experiment, we transfected Dvl into two cell lines LARK1A and H513, and the induced Dvl overexpression induced Tcf-dependent transcription activity and then did Western blot analysis using an antiactive JNK antibody. Indeed, we noticed JNK inactivation in both cell lines after expressing exogenous Dvl when compared with the same cell lines transfected with empty vector. In another test, the level of active JNK was reduced in the C57MG and H28 cells after a 24-h treatment with Wnt-1 conditioned medium. Taken together, these findings suggest that Dvl may be required for the apoptotic inhibition induced by Wnt-1 signaling and JNK may be up-regulated by blocking Dvl.

To date, the role of JNK pathway in Wnt signaling is still unclear (7). It has been shown that Dvl induces JNK activity through DEP domain in planar cell polarity pathway in vertebrate cells (21). However, several recent studies indicated that Wnt-5a, a JNK activator involved in planar cell polarity pathway, serves as an antagonist of Wnt canonical pathway mediated by Wnt-1 class signals (23, 24); therefore, Wnt-5a may function as a tumor suppressor. Given the complexity of Wnt canonical and noncanonical pathways, it is conceivable that Wnt-1 class and Wnt5a class may recruit different set of positive/negative regulators, including Dvl-binding proteins. Several Dvl-binding proteins may directly or indirectly induce effects that consistent with our current findings. Firstly, a Wnt signaling-negative regulator Axin can induce JNK activity and β-catenin down-regulation (25, 26). Secondly, an antagonist of Wnt signaling, Naked, was shown to interact with Dvl and negatively regulate β-catenin but positively regulate JNK-mediated planar cell polarity pathway (27, 28). Thirdly, a Dvl-associated kinase and positive regulator of Wnt signaling, Par-1, activates the canonical pathway but inhibits the JNK-mediated planar cell polarity pathway (29). In this study, activation of JNK was not noticed in most of the cancer cells naturally expressing both Wnt-1 and Dvl-3. JNK activation was only seen in these cells after treatment with siRNA for either Wnt-1 or Dvl-3.

To further examine the possible involvement of JNK in Wnt-1-induced apoptosis inhibition, a selective JNK-1, JNK-2, JNK-3 inhibitor, SP600125 (30), was used to treat the H28 cells combined with Wnt-1 siRNA, anti-Wnt-1 monoclonal antibody, or Dvl siRNA. Recently, the JNK inhibitor has been shown to inhibit chemotherapy-induced apoptosis, at least in part, through the inhibition of caspase-3 activity (31). In this study, this small molecule inhibitor of JNK inhibits significantly the apoptotic cell killing induced by Wnt-1 siRNA, anti-Wnt-1 monoclonal antibody, or Dvl siRNA (Fig. 4). In the β-catenin-deficient H28 and MS-1 cells, the JNK inhibitor showed limited toxicity in cultured cells and reduced dramatically the apoptotic cell death induced by either Wnt-1 siRNA or Dvl siRNA (Fig. 4, A–F). In a non-small cell lung cancer cell line H460 with intact β-catenin, the JNK inhibitor failed to inhibit apoptotic cell death induced by Wnt-1 siRNA (Fig. 4A). These data suggest that the apoptosis induction in the H28 and MS-1 cells lacking β-catenin may require activated JNK pathway, and the results also suggest that both canonical and noncanonical pathways may be involved in the apoptosis induced by blockade of Wnt-1 signaling in H460 cells. The inhibition of activated JNK-1 was confirmed by checking the activated form of JNK-1 (Fig. 4G). By Western blot analysis, it was found that the clevaed form of caspase-3 was reduced and survivin was up-regulated when treatment with the JNK inhibitor was combined with Wnt-1 siRNA, anti-Wnt-1 antibody, or Dvl siRNA in both H28 and MS-1 cells (Fig. 4G). These data suggest that caspase-3 and survivin may be involved in the induction of apoptosis occurs as a result of blocking Wnt-1 signaling. Furthermore, JNK siRNA was used to test if it can show similar effect as SP600125. Our results indicate that JNK siRNA was also able to inhibit the apoptosis induced by an anti-Wnt-1 antibody in H28 cells (Fig. 4H). In addition, we have found that Wnt-2 siRNA also induces apoptosis in H28 cells (data not shown), suggesting that this effect may be specific for more than one Wnt-1 class proteins (e.g., Wnt-1, Wnt-2). Lastly, we have shown that the inhibition of Wnt-1 signaling by an anti-Wnt-1 antibody does not affect protein level of JNK in H28 cells (Fig. 4I).

Activation of JNK is essential for apoptosis induced by multiple stress inducers, including tumor growth factor α (32, 33). To date, little is known about the connection of Dvl and apoptosis in a JNK-dependent manner. This study suggests that Dvl inactivates the JNK-dependent apoptotic pathway in some cancer cell lines. Blockade of Wnt-1 signaling induces programmed cell death, at least in part, through Dvl/JNK-dependent pathway.

REFERENCES


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