An Anti-Wnt-2 Monoclonal Antibody Induces Apoptosis in Malignant Melanoma Cells and Inhibits Tumor Growth

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ABSTRACT

Activation of the Wnt/β-catenin signaling pathway has been associated with human cancers. To test whether Wnt-2 signal is a survival factor in human melanoma cells and thus represents a potential therapeutic target, we investigated the effects of inhibition of Wnt-2 signaling in human melanoma cell lines. We have developed a novel monoclonal antibody against the NH2 terminus of the human Wnt-2 ligand that induces apoptosis in human melanoma cells overexpressing Wnt-2. Whereas incubation of this antibody with normal cells lacking Wnt-2 expression does not induce apoptosis, Wnt-2 signaling blockade by the ligand-binding antibody is confirmed by down-regulation of Dishevelled and β-catenin. Wnt-2 small interfering RNA treatment in these cells yielded similar apoptotic effects and downstream changes. Down-regulation of an inhibitor of apoptosis family protein, survivin, was observed in both the Wnt-2 antibody-treated and small interfering RNA-treated melanoma cell lines, suggesting that the antibody induces apoptosis by inactivating survivin. In an in vivo study, this monoclonal anti-Wnt-2 antibody suppresses tumor growth in a xenograft model. These findings suggest that the anti-Wnt-2 monoclonal antibody may be useful for the treatment of patients with malignant melanoma.

INTRODUCTION

The Wnt protein family plays an important role in embryogenesis and carcinogenesis. The binding of Wnt ligand to its receptor Frizzled activates Wnt canonical signaling pathways (1, 2). The activation of the Wnt/β-catenin 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 T-cell factor transcription factors to make a heterodimeric complex that activates the transcription of Wnt target genes, e.g., c-myc and cyclin D1 (3–6). Wnt/β-catenin signaling is associated with cell survival in various cell types (7–12). Noncanonical pathways, referred to as Wnt signaling pathways that signal independently of β-catenin, may signal through calcium flux, c-Jun NH2-terminal kinase, and G proteins (13).

β-Catenin, adenomatosis polyposis coli (APC), and axin mutations have been found in human cancers, suggesting that constitutive activation of the Wnt canonical pathway contributes to carcinogenesis (4). The human Wnt-2 gene, located on chromosome 7q31.3, is highly expressed in fetal lung and weakly expressed in placenta (14). Wnt-2 has been implicated in mouse mammary tumorigenesis through gene amplification (15) and, more importantly, in human carcinogenesis by up-regulation (16–19). For instance, frequent up-regulation of Wnt-2 has been implicated in mouse mammary tumorigenesis through gene expression in fetal lung and weakly expressed in placenta (14). Wnt-2 has been found in human cancers, suggesting that constitutive activation of the Wnt canonical pathway contributes to carcinogenesis (4). The human Wnt-2 gene, located on chromosome 7q31.3, is highly expressed in fetal lung and weakly expressed in placenta (14).

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The human Wnt-2 gene, located on chromosome 7q31.3, is highly expressed in fetal lung and weakly expressed in placenta (14). Wnt-2 has been implicated in mouse mammary tumorigenesis through gene amplification (15) and, more importantly, in human carcinogenesis by up-regulation (16–19). For instance, frequent up-regulation of Wnt-2 has been reported in human colorectal cancer and gastric cancer (14). It has been reported recently by in situ hybridization that specific Wnt genes are overexpressed in human colon cancer and melanoma, further supporting the role of Wnt-2 overexpression in human carcinogenesis (20, 21).

To test our hypothesis that the Wnt-2 signal is a survival factor that plays a causal role in human melanoma cells, we have evaluated the effect of a specific anti-Wnt-2 monoclonal antibody in several human melanoma cell lines. Treatment of melanoma cells with an anti-Wnt-2 antibody inhibited Wnt signal and induced apoptosis. In an in vivo tumor model, treatment with the anti-Wnt-2 antibody induced apoptosis in the xenograft tumors and inhibited growth of xenograft tumors.

MATERIALS AND METHODS

Cell Culture. Human melanoma cell lines LOX, FEM, and FEMX were a kind gift of Dr. Oystein Fodstad (Norwegian Radium Hospital). The SK-Mel-2 cell line was purchased from American Type Culture Collection (Manassas, VA). These cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Normal human epithelial keratinocytes were obtained from Cambrex (Walkersville, MD) and cultured in keratinocyte basal medium with KGM SingleQuots provided by Cambrex. Primary fresh human cultures were established from small resected portions of metastatic melanoma in patients undergoing pulmonary metastasectomy for treatment of their recurrent disease (Institutional Review Board Approval H8714-15319-04). All cells were cultured at 37°C in a humid incubator with 5% CO2.

Monoclonal Antibody and Immunoprecipitation. The anti-Wnt-2 mouse monoclonal antibody (IgG1) was custom-made at Rockland Inc. (Gilbertsville, PA). The antigen used to raise this monocolonal antibody was a synthetic peptide corresponding to amino acids 49–63 of human Wnt-2. The antigen was chosen bioinformatically based on its hydrophilicity, antigenicity, accessibility, sequence homology, and vicinity to the NH2-terminal region. The peptide sequence was also searched to ensure its specificity to Wnt-2. The test bleed was screened twice by ELISA using the peptide, and the parental clones and the subclones were screened by Western blot analysis.

The monoclonal antibody was affinity purified by using protein G and kept at −80°C. Seize X Mammalian Immunoprecipitation Kit (Pierce Biotechnology, Rockford, IL) was used to precipitate Wnt-2 protein from cell line extracts according to the manufacturer’s protocol and followed by Western blotting. The same monoclonal antibody was used to detect the Wnt-2 protein on the Western blots.

Antibody Incubation. Cells were plated in 6-well plates or 10-cm dishes 1 day before experiments. Then, normal media were replaced with media containing antibodies at various concentrations, and the cells were incubated at 37°C in a humid incubator with 5% CO2. At various time points, the cells were collected using standard protocols for further analysis. Control antibody was mouse IgG1 MOPC21 from Sigma-Aldrich Co. (St. Louis, MO).

RNA Interference. Cells were plated into a 6-well plate with fresh media without antibiotics 24 h before testing. The ion-exchange high-performance liquid chromatography-purified small interfering RNAs [siRNAs (Wnt-2 siRNA and nonsilencing siRNA control; >97% pure)] were purchased from Qiagen-Quagen (Germantown, MD). The siRNA specific for human Wnt-2 is derived from the mRNA sequence (5’-GAAATGGGAAGCGCCAAGC-3’). The lyophilized siRNAs were dissolved in annealing buffer, reheated to 95°C for 1 min, and then incubated for 1 h at 37°C, following the protocol described by Elbashir et al.
Western Blotting. Western blotting was performed as described previously (23). Anti-Wnt-2, anti-Dvl3, and anti-survivin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-3 antibody was from Oncogene (Cambridge, MA). Anti-β-actin antibody was obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-β-catenin antibody was purchased from Transduction Laboratories (Lexington, KY). Anti-cytochrome c antibody was from BD Biosciences (San Diego, CA). To detect alteration of β-catenin and cytochrome c, cytosolic extracts were prepared.

cDNA Expression. Human RNA master blot and Atlas human cancer profiling array II (Clontech Laboratories, Inc., Palo Alto, CA) were hybridized to a [32P]dCTP-labeled human Wnt-2 cDNA probe. Total RNA from various cancer cell lines was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. To analyze differential gene expression after anti-Wnt-2 monoclonal antibody treatment in melanoma cells, the Human Cancer Signal Transduction PathwayFinder (SuperArray Bio-science, Frederick, MD) was used, following the manufacturer’s protocol. The hybridized membranes were exposed to X-ray film for 3 days. The density of the signals was determined by Imagequant software (Molecular Dynamics, Sunnyvale, CA).

Apoptosis Assay. Cells were harvested and stained using an annexin V-FITC apoptosis detection kit (Oncogene), according to the manufacturer’s protocol. Stained cells were analyzed immediately 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 not to propidium iodide. Cells in necrotic or late apoptotic stages were labeled with both annexin V-FITC and propidium iodide. Terminal deoxynucleotidyl transferase-mediated nick end labeling staining of tumor tissue samples harvested from in vivo experiments was performed using the ApopTag Peroxidase in Situ Oligo Ligation Apoptosis Detection Kit (Chemicon International, Temecula, CA) according to the manufacturer’s protocol.

Tumor Growth in Nude Mice. Groups of eight female nude mice, 5–6 weeks old, received s.c. injection with 1 × 107 LOX cells in the dorsal area in a volume of 100 μl. Three days later, the tumors were uniformly formed, and the animals then received i.p. injection twice weekly with monoclonal anti-Wnt-2 antibody, a control monoclonal antibody, or PBS buffer in a volume of 100 μl as well. Both the anti-Wnt-2 monoclonal antibody and the control antibody were injected at a dose of 250 μg. Tumor size was determined at weekly intervals, and tumor volume was calculated using width (x) and length (y) with the formula V = 0.5xy². Where x < y.

Statistical Analysis. The data shown represent mean values ± SD. The unpaired t test was used to compare different treatments and cell lines.

RESULTS

Monoclonal Anti-Wnt-2 Antibody Precipitates Wnt-2 Proteins in Cell Extracts. We first studied Wnt-2 mRNA expression in normal human tissues using cDNA dot blots. In normal human organs, Wnt-2 mRNA was highly expressed in the placenta and fetal lung and weakly expressed in adult lung (Fig. 1A). Minimal or no expression was observed in all other normal human tissues in the human RNA master blot containing 50 human tissues (Fig. 1A). Using a commercially available anti-Wnt-2 antibody raised against a peptide mapping near the NH2 terminus of human Wnt-2 (Santa Cruz Biotechnology), we found evidence of Wnt-2 protein overexpression in four human melanoma cell lines (LOX, FEM, FEMX, and SK-Mel-2) when compared with normal human epithelial keratinocytes. Wnt-2 protein expression was found in all four melanoma cell lines tested and primary cultures freshly made from metastatic specimens resected from two patients with melanoma (Fig. 1B). By contrast, Wnt-2 expression was not observed in normal human epithelial keratinocytes.

Next, we developed a monoclonal antibody against a Wnt-2 NH2-terminal peptide (see Materials and Methods*). To test whether the anti-Wnt-2 monoclonal antibody could bind specifically to the native form of Wnt-2 protein in cultured cells, we performed immunoprecipitation with this monoclonal antibody alone or after preincubation with blocking peptide (30-fold over the antibody) in cell extracts from two cell lines (Fig. 1C). In control C57Wnt-2 and LOX human melanoma cells, Wnt-2 protein was precipitated by the monoclonal anti-Wnt-2 antibody. When the anti-Wnt-2 monoclonal antibody was preincubated with blocking peptide, its ability to precipitate Wnt-2

Fig. 1. Wnt-2 expression in human normal and tumor tissues and cancer cell lines. A, expression of Wnt-2 mRNA in normal human tissues. Left panel, normal human RNA master blot (Clontech Laboratories, Inc.) was hybridized to a [32P]dCTP-labeled human Wnt-2 cDNA probe. Wnt-2 mRNA was highly expressed in placenta and fetal lung and weakly expressed in normal lung, P, placenta; FL, fetal lung; NL, normal lung. Right panel, quantitative estimate of the relative expression of Wnt-2 mRNA in normal human tissues. The blot was carefully normalized using eight housekeeping numbers. B, Western analysis of Wnt-2 expression in melanoma cell lines (LOX, FEMX, FEM, and SK-Mel-2). Normal human epithelial keratinocytes (NHKEK) were used as normal control. Case 1 and Case 2 represent two primary cultures freshly made from two patients with melanoma. Whole cell proteins were isolated. C, immunoprecipitation of Wnt-2 protein in cell lines with the anti-Wnt-2 monoclonal antibody. Seize X Mammalian Immunoprecipitation Kit (Pierce Biotechnology) was used to precipitate Wnt-2 protein from cell lysate according to the manufacturer’s protocol and followed by Western blotting. The same monoclonal antibody was used to detect the Wnt-2 protein on the Western blots of LOX cells and C57Wnt-2 cells, which served as a positive control. Lanes 1 and 2, immunoprecipitation performed with monoclonal antibody alone and monoclonal antibody blocked by preincubation with blocking peptide (30-fold over the antibody) with C57Wnt-2 cell lysate; Lanes 3 and 4, immunoprecipitation performed with monoclonal antibody alone and monoclonal antibody blocked by preincubation with blocking peptide with LOX cell lysate. The position of Wnt-2 protein is indicated by an arrow at 34 kDa. D, expression of Wnt-2 in matched noncancerous skin and melanoma tissues. N, noncancerous skin; M, melanoma tumor. The dot blot was hybridized to [32P]dCTP-labeled human Wnt-2 cDNA probe.
protein was blocked in both cell lines. Wnt-2 protein was not precipitated by the monoclonal antibody after preincubation with the blocking peptide. These data indicate that the anti-Wnt-2 monoclonal antibody can bind specifically to the native form of Wnt-2 protein. Using the Atlas human cancer profiling dot blot, Wnt-2 mRNA was found to be overexpressed in six of eight melanoma tumors when compared with their matched noncancerous skin tissues (Fig. 1D).

The Monoclonal Anti-Wnt-2 Antibody Induces Apoptosis in Melanoma Cells. We used the anti-Wnt-2 monoclonal antibody to treat the four human melanoma cell lines (LOX, FEM, FEMX, and SK-Mel-2). After 3–5 days of incubation, we found significant cell death in all these cell lines (>90% cell death at 10.0 µg/ml antibody; P < 0.005; Fig. 2A). We observed no noticeable cytopathic effect in these cell lines when the monoclonal antibody was preincubated with blocking peptide or after control monoclonal antibody treatment. Moreover, no effect was noted in the normal human epithelial keratinocytes treated with the same antibody. Cell killing was due largely to induction of apoptosis (46.0–68.2% of apoptotic cells after 72 h of incubation; Fig. 2B). As a control, we examined induction of apoptosis by using monoclonal antibody blocked by overnight preincubation with blocking peptide (30-fold over the antibody) in LOX and FEMX cells (Fig. 2C). After 72 h of incubation, we found that the apoptosis induced by the anti-Wnt-2 antibody could be inhibited significantly by blocking peptide (P < 0.003). The same dose of the blocking peptide (300.0 µg/ml for 72 h) alone had no effect on cell viability. After 72 h of treatment with the control monoclonal antibody (10.0 µg/ml), no significant apoptosis induction was detected.

The Monoclonal Anti-Wnt-2 Antibody Inhibits Wnt Signaling. We have shown that Dvl-3 is overexpressed in lung cancer and mesothelioma (23, 24). We found that both cytosolic β-catenin and Dvl-3 were down-regulated after anti-Wnt-2 monoclonal antibody treatment in the melanoma cell lines FEMX and LOX (Fig. 2D). In FEMX and LOX cells in which the anti-Wnt-2 monoclonal antibody

Fig. 2. The anti-Wnt-2 monoclonal antibody induces apoptosis in cancer cell lines. A, 0.5% crystal violet staining of normal human epithelial keratinocytes (NHEK) and four human melanoma cell lines (LOX, FEM, FEMX, and SK-Mel-2) after anti-Wnt-2 antibody treatment (10.0 µg/ml for 72 h). Primary tumor cultures freshly made from patients with malignant melanoma were treated for 7 days with the anti-Wnt-2 monoclonal antibody. B, anti-Wnt-2 monoclonal antibody induces apoptosis in different human cancer cell lines. This panel shows examples of apoptosis induction using flow cytometry. FEMX and LOX cancer cells were treated with 10.0 µg/ml control antibody and 10.0 µg/ml anti-Wnt-2 antibody, respectively, for about 72 h. FL1-H represents annexin V-FITC staining. C, specific cell killing by anti-Wnt-2 monoclonal antibody in cancer cell lines. This panel shows the percentage of dead FEMX and LOX cells after about 72 h of treatment with monoclonal antibody alone (10.0 µg/ml) and with monoclonal antibody blocked by preincubation with blocking peptide (30-fold over the antibody). As another control, FEMX and LOX cells were also treated with the same dose of blocking peptide. After 72 h of incubation, cells were collected for flow cytometry analysis. Results are the means ± SD (error bars). D, Western analysis before and after anti-Wnt-2 antibody treatments (10.0 µg/ml for 48 h). Two melanoma cell lines, LOX and FEMX, were treated with anti-Wnt-2 antibody. Expression of Dvl-3, β-catenin, survivin, cyclin D1, cytochrome c, pro-caspase-3, and cleaved caspase-3 was assessed. Actin served as a loading control. Cytosolic proteins were prepared. Whole cell protein was used for cyclin D1 analysis. c-Myc and fibronectin genes were down-regulated after anti-WNT-2 monoclonal antibody treatments (10.0 µg/ml for 48 h) in the LOX melanoma cell line. Total RNA was used for hybridization.
induced apoptosis, we found that the cleaved (active) form of caspase-3 was up-regulated. Consistent with this caspase-3 activity, we detected increased levels of cytosolic cytochrome c in these cells after anti-Wnt-2 antibody treatment. In addition, we found that an inhibitor of apoptosis protein, survivin, was also down-regulated after anti-Wnt-2 antibody treatment. In addition, we found that an inhibitor of apoptosis protein, survivin, was also down-regulated in these cells after antibody treatment. The β-catenin–T-cell factor-targeted genes, cyclin D1, c-Myc, and fibronectin, were also down-regulated after Wnt-2 siRNA treatment in LOX cells (Fig. 3C).

**Wnt-2 siRNA Leads to Programmed Cell Death in Melanoma Cells by Inhibiting Wnt Signaling.** Wnt-2 targeted siRNA was used to study the effect of Wnt-2 mRNA silencing following the protocol described by Elbashir et al. (22). Similar to the anti-Wnt-2 monoclonal antibody, treatment with Wnt-2 siRNA for 3–5 days induced apoptosis in all melanoma cell lines expressing Wnt-2 (Fig. 3). Significant apoptosis was induced by 100 nM Wnt-2 siRNA, and no apoptosis was induced by nonsilencing siRNA control (100 nM; P < 0.01; Fig. 3A). We confirmed the silencing of Wnt-2 expression after Wnt-2 siRNA treatments (100 nM for 72 h) by Western blot analysis (Fig. 3B). In these studies, nonsilencing siRNA served as control (100 nM for 72 h). To determine whether the apoptotic effects correlated with the inhibition of Wnt-2 signaling, expression levels of Dvl-3, cytosolic β-catenin, and survivin were examined and found to be down-regulated after Wnt-2 siRNA treatment (Fig. 3B). The β-catenin–T-cell factor-targeted genes, c-Myc and fibronectin, were also down-regulated after Wnt-2 siRNA treatment in LOX cells (Fig. 3C).

The **Monoclonal Anti-Wnt-2 Antibody Suppresses Tumor Growth in Vivo.** Malignant melanoma LOX cells were injected s.c. into nude mice. The animals were then treated with 250 μg of the anti-Wnt-2 monoclonal antibody, control antibody, or PBS control via i.p. injections twice a week. The monoclonal anti-Wnt-2 antibody significantly inhibited tumor growth versus control (Fig. 4). Suppression of tumor growth was seen when the anti-Wnt-2 monoclonal antibody injection was started after the tumors were already established (3 days after tumor cell inoculation; P < 0.005; Fig. 4, A and B). The tumor tissues were harvested and analyzed via terminal deoxynucleotidyl transferase-mediated nick end labeling staining, and apoptotic cells were detected in tumors treated with the anti-Wnt-2
monoclonal antibody (Fig. 4, C and D). Furthermore, several organs including the lung, liver, heart, and intestine of mice treated with the antibody were examined microscopically. Blinded analysis by an experienced pathologist failed to demonstrate noticeable toxicities or abnormalities in Wnt-2 monoclonal antibody-treated mice. Finally, the mice treated with the Wnt-2 antibody appeared completely normal after 4 weeks of treatment.

DISCUSSION

The Wnt gene family contains at least 19 secreted, cysteine-rich glycoproteins that play a critical role in development and oncogenesis (25). Several Wnt proteins, including Wnt-2, have been shown to be overexpressed in a number of cancers. Our Wnt-2 expression data are consistent with these recent findings. For instance, Wnt-2 mRNA expression was only detected in the placenta and fetal lung (14). Importantly, we have shown that several human melanoma cell lines overexpress the Wnt-2 protein. Although the connection has been made between APC/β-catenin mutations and human cancer, little is known regarding the role that the Wnt-2 ligand plays in human carcinogenesis.

Our data also indicate that an anti-Wnt-2 monoclonal antibody can induce apoptosis in these human cancer cells, suggesting that the antitumor effect is due to the blockade of Wnt signaling and inhibition of downstream signaling intermediates. The apoptotic cell death induced by anti-Wnt-2 antibody not only correlates with decreased Wnt-2 expression but is also accompanied by inhibition of canonical Wnt signaling, as evidenced by a decrease in Dvl and cytosolic β-catenin levels in these cells. Conversely, both Dvl and cytosolic β-catenin protein levels remain unchanged in normal cell lines after anti-Wnt-2 antibody treatment. To further elucidate the possible mechanism(s) through which the anti-Wnt-2 monoclonal antibody induces apoptosis in human melanoma cells, we examined other possible components in the apoptotic pathway. For instance, cytochrome c indirectly activates caspase-3, and inhibitors of apoptosis mediate inhibition of several caspasess, including caspase-3 (26, 27).

We observed increased levels of cytochrome c in the cytosol of melanoma cells after anti-Wnt-2 antibody treatment. Our results indicate that both caspase-3 and cytochrome c are most likely involved in this anti-Wnt-2 antibody-induced apoptosis through suppression of survivin and/or other inhibitors of apoptosis, as well as direct caspase activation.

Our findings also suggest that the Wnt-2 antibody may not only directly induce apoptosis in melanoma cells that overexpress Wnt-2 but may also partially reverse chemotherapy-induced drug resistance. Thus, Wnt-2 up-regulation may function as a survival mechanism by inhibiting normal apoptotic machinery in these melanoma cells. Overexpression of survivin is a common feature of many human cancers, including melanoma. Survivin is expressed in the G2-M phase, and its interaction with the mitotic spindle is essential for its antiapoptotic function (28). Targeting survivin increases the sensitivity of tumor cells to cytotoxic drugs (29). Previously, we have shown that antisense oligonucleotide to survivin is sufficient to cause apoptosis in human mesothelioma cells (30). Others have shown that antisense to survivin induces apoptosis and synergizes with chemotherapy in the melanoma cell line LOX (31, 32). Anti-Wnt-2 antibody should, theoretically, synergize with the effect of cytotoxic chemotherapy in melanoma cells.

In summary, this study indicates that Wnt-2 blockade induces apoptosis in human melanoma cells and that Wnt-2 may be an attractive and active target for the treatment of malignant melanoma. The in vitro and in vivo data suggest the anti-Wnt-2 antibody can induce selective apoptosis in melanoma cells through inhibition of Wnt pathway(s). These results support future development of the anti-Wnt-2 monoclonal antibody as a therapeutic strategy for the treatment of cancer.

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