Proof-of-Principle: Oncogenic β-Catenin Is a Valid Molecular Target for the Development of Pharmacological Inhibitors

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Abstract

Activation of β-catenin is a critical step in the pathogenesis of many common human cancers and is the initiating event in adenocarcinoma of the colon. Because activation of β-catenin provides a gain-of-function, it is tempting to speculate that specific pharmacological inhibition of activated β-catenin might reverse the tumorigenic properties of human cancer cells and therefore form the basis of an effective anticancer strategy. In an effort to provide proof-of-principle for such a strategy, we used a novel clonal growth assay based on human somatic cell gene targeting to determine whether activated β-catenin remains a necessary oncogenic stimulus in advanced human cancer cells. Using this approach, we demonstrate that β-catenin is a necessary oncogene in human SW48 and DLD1 colon cancer cells but not in HCT116 cells. These data indicate that activated β-catenin can remain a critical oncogenic stimulus throughout the progression of human colon cancer and suggest that the small molecule inhibitors of activated β-catenin currently under development will be effective anticancer therapeutics in a subset of malignant colon cancers.

Introduction

Activation of the β-catenin oncogene is critical to the pathogenesis of many common human cancers. β-catenin can be activated either by intragenic mutations that abolish inhibitory phosphorylation sites or by extragenic mutations in its binding partners APC and Axin (1). Intragenic mutations in β-catenin are found in a variety of human tumor types, including colon cancer, hepatocellular cancer, thyroid cancer, and endometrial cancer (2–5). Extragenic mutations in either APC or Axin are found in colon cancer, breast cancer, and hepatocellular cancer (6–8).

β-catenin is a component of the Wnt signaling pathway (9). Normally, β-catenin is found in two intracellular pools: bound to E-cadherin at the cell membrane, and in a complex with other proteins, including APC and Axin, in the cytoplasm. Activation of β-catenin is thought to dramatically increase its stability and cause it to be translocated to the nucleus. Nuclear β-catenin then binds to members of the TCF family of transcription factors. These heterodimers between a TCF (providing the DNA binding domain) and β-catenin (providing the transcriptional activation domain) drive the transcription of Wnt target genes. Numerous candidate genes have been proposed as critical downstream effectors of Wnt signaling in cancer, including c-myc, cyclin D1, and BMP4 (10–13).

Oncogenes such as β-catenin represent, at least in theory, extremely attractive molecular targets for the development of anticancer pharmaceuticals. There are several reasons for this. First, and very importantly, activation of β-catenin is a gain-of-function. As such, it is feasible to use well-established techniques in drug development to develop inhibitors. Second, β-catenin is activated in many common human cancers. Therefore, effective inhibitors of β-catenin would be predicted to be broadly effective against a variety of common cancers. For these reasons, numerous academic laboratories and pharmaceutical companies are working to develop pharmacological inhibitors of activated β-catenin with the hope that they will represent effective anticancer pharmaceuticals.

However, it has not yet been unambiguously demonstrated that specific inhibition of oncogenic β-catenin can actually reverse the tumorigenic properties of human cancer cells. The technologies used to date have either simultaneously inhibited both the wild-type and activated forms of the protein (14, 15) or inactivated TCF4 instead, a protein thought to be functional surrogate for oncogenic β-catenin (16, 17). As such, it has remained a possibility that although activation of β-catenin is a critical initiating event in the pathogenesis of a human tumor, it eventually becomes superfluous in the face of additional mutations in other oncogenes and tumor suppressor genes. If this were true, pharmacological inhibition of oncogenic β-catenin would be ineffective as a treatment for advanced human cancers.

To test whether complete, specific inhibition of oncogenic β-catenin can reverse the transformed properties of human cancer cells, we used a novel clonal growth assay based on human somatic cell gene targeting. The assay is the converse of the well-accepted clonal growth assay that is commonly used to measure the growth-suppressing activity of transfected tumor suppressor genes, such as p53 (18). However, instead of measuring the reduction in colony number after the addition of an ectopically expressed tumor suppressor gene, our assay measures the clonal growth potential of cells after the subtraction of an endogenous activated oncogene. By applying this assay to three commonly studied
colon cancer cell lines harboring activated β-catenin, we demonstrate that oncogenic β-catenin is required for clonal growth in a subset of human cancer cell lines. As such, we provide proof-of-principle that specific inhibition of oncogenic β-catenin is a feasible strategy for the treatment of human cancer.

Materials and Methods

Creation of Human β-Catenin-targeting Vectors. β-cateninKO-neo is a previously described high-efficiency human gene-targeting vector designed to delete β-catenin exons II-IV and replace them with an in-frame promotortless neo<sup>+</sup> gene (13). β-cateninKO-hyg is identical to β-cateninKO-neo except that it contains a promotortless hyg<sup>+</sup> gene instead of a promotortless neo<sup>+</sup> gene. It was created using an identical cloning strategy using homologous recombination in Saccharomyces cerevisiae.

Tissue Culture and Transfection. SW48, HCT116, and DLD1 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in McCoy’s 5A media (Life Technologies, Inc., Carlsbad, CA) at 37°C in 5% CO<sub>2</sub>, in media containing 10% fetal bovine serum (Mediatech, Herndon, VA) and 1% penicillin-streptomycin (Life Technologies, Inc.).

To create heterozygous KOs in SW48 cells, ~20 25 cm<sup>2</sup> flasks were transfected with Not I linearized β-cateninKO-neo (New England BioLabs, Beverly, MA) using LipofectAMINE (Invitrogen, Carlsbad, CA), following the manufacturer’s protocol. Clones were selected in 0.3 mg/ml G418 for ~3 weeks, changing the media every 3 days. Each flask of pooled clones was then individually trypsinized, counted, and plated out at limiting dilution in 96-well plates to create individual clones. Clones were allowed to grow for ~3 weeks. Individual colonies were transferred first to wells in a 24-well plate, then to a 25 cm<sup>2</sup> flask. Cells in confluent flasks were trypsinized, and approximately one-third of the cells was used for preparation of genomic DNA, whereas two-thirds were used for cryopreservation.

To create DLD1 heterozygous KO clones, cells were transfected with Not I-linearized β-cateninKO-neo using LipofectAMINE (Invitrogen), following the manufacturer’s protocol. To obtain stably transfected clones by limiting dilution, cells were trypsinized ~18 h after transfection, mixed with media containing 1 mg/ml G418 (Invitrogen), and distributed to 96-well plates. After ~2 weeks of growth, individual colonies were transferred first to wells in a 24-well plate, then to a 25 cm<sup>2</sup> flask. Cells in confluent flasks were trypsinized, and approximately one-third of the cells was used for preparation of genomic DNA, whereas two-thirds were used for cryopreservation.

In an effort to create DLD1 homozygous KO clones, several independently derived DLD1 heterozygous KO clones were transfected with β-cateninKO-hyg, and individual hyg<sup>+</sup> colonies were selected by limiting dilution in 0.2 mg/ml hygromycin B (Invitrogen) as described above. Cells in confluent flasks were trypsinized, and approximately one-third of the cells was used for preparation of genomic DNA, whereas two-thirds were used for cryopreservation.

Genomic PCR, Southern Blots, and DNA Sequencing. Preparation of genomic DNA, PCR, Southern blots, and automated sequencing were all performed using standard techniques. Taq Platinum (Invitrogen) was used for PCR, according to the manufacturer’s instructions. For Southern blots, 5 μg of genomic DNA were digested with Sph I, separated on a 1% agarose gel, and transferred to a Zeta-Probe nylon membrane (Bio-Rad, Hercules, CA). The membrane was prehybridized overnight at 60°C, then hybridized for 24 h at 60°C using a radiolabeled, PCR-generated probe for β-catenin intron VI. The blot was then washed and imaged using a Molecular Dynamics phosphorimager.

Xenograft Growth Assay. Tumors were established by s.c. injection of 2 × 10<sup>6</sup> cells suspended in HBSS in the flanks of an immunodeficient mouse. Tumor growth rate was determined by measuring three orthogonal diameters of each tumor twice a week, and the tumor volume was estimated at π/6 (D<sub>1</sub>D<sub>2</sub>D<sub>3</sub>).

Results

Targeted Deletion of β-Catenin in HCT116 Cells. HCT116 cells were initially isolated from an invasive, metastatic colonic adenocarcinoma and harbor a common heterozygous activating mutation in β-catenin, an in-frame deletion of serine 45 (2, 19). We described recently derivatives of HCT116 cells in which either the wild-type or mutant allele of β-catenin had been deleted by gene targeting (13). The wild-type and mutant alleles were targeted with virtually equal frequency.

To determine whether deletion of the oncogenic allele of β-catenin affected the malignant properties of HCT116 cells, we measured the ability of the parental cells and β-catenin gene-targeted derivatives to grow in vitro and in vivo as xenografts in immunodeficient mice. HCT116 parental cells and several independently derived heterozygous KO clones lacking either wild-type β-catenin or oncogenic β-catenin displayed similar in vitro growth rates (data not shown). Strikingly, both the parental cells and two independently derived gene-targeted derivatives lacking oncogenic β-catenin formed tumors with virtually complete efficiency (Figs. 1A and C), and the tumors grew at similar rates (in fact, the gene-targeted cells grew somewhat faster: Fig. 1B). These surprising data, which are similar to those reported recently by Chan et al. (20) and Sekine et al. (21), indicate that complete inhibition of oncogenic β-catenin is insufficient to revert the transformed properties of HCT116 cells.

Targeted Deletion of β-Catenin in DLD1 Cells. We next attempted to confirm these surprising data acquired in HCT116 cells using a second cell system with a different mechanism of activation of β-catenin. DLD1 cells were chosen for this, because they are a colon cancer cell line with activated β-catenin by virtue of homozygous inactivating mutations of the APC tumor suppressor gene and are well established as being suitable for human somatic cell gene targeting (22, 23). Because in DLD1 cells, β-catenin is activated by extragenic mutations in APC, it was necessary to delete both copies of β-catenin to completely inhibit the activated form of β-catenin.
Initially, DLD1 cells were transfected with β-cateninKO-neo to create heterozygous KO cell lines, as described in “Materials and Methods.” Individual genotin-resistant colonies were obtained, expanded into clonal cell lines, tested by PCR, and confirmed by Southern blots to determine whether the targeting vector had integrated into the β-catenin locus by homologous recombination (Fig. 2A). In 8 of 40 (20%) of the cell lines tested, the targeting vector had integrated via homologous recombination. In each of the eight gene-targeted cell lines tested, the targeting vector had integrated into the already targeted allele (Fig. 2B; examples in Fig. 2A, Lanes 5–7), indicating that homozygous deletion of activated β-catenin is incompatible with clonal growth of the cells ($P = 0.005$).

These data demonstrated that complete inhibition of activated β-catenin led to a reversal of the most fundamental transformed property of DLD1 cells: their ability to grow in vitro. However, definitive interpretation of these data were complicated by the fact that homozygous deletion of β-catenin simultaneously removed both the wild-type and activated functions of the protein. It remained a distinct possibility that the oncogenic functions of β-catenin were dispensable in DLD1 cells but that wild-type β-catenin was simply an essential gene.

**Targeted Deletion of β-Catenin in SW48 Cells.** In an effort to reconcile these data, we decided to target oncogenic β-catenin in a third cell line. SW48 cells were chosen because they are a near diploid human colon cancer cell line harboring a well-characterized heterozygous activating mutation of the β-catenin oncogene (S33Y; Ref. 2). Therefore, as in the case of HCT116 cells, it is theoretically possible to specifically delete the activated form of β-catenin while leaving the wild-type allele of β-catenin intact.

SW48 cells were transfected with the β-cateninKO-neo human promotorless targeting vector as described in “Materials and Methods.” A group of 290 individual genotin-resistant colonies was obtained, expanded into clonal cell lines, and tested by PCR to determine whether the targeting vector had integrated into the β-catenin locus by homologous recombination (Fig. 3A). In 33 of 290 (11%) of the cell
In addition to providing an important proof-of-principle, the results and reagents described herein provide several possible avenues for future β-catenin-targeted anticancer drug discovery. First, the isogenic set of β-catenin gene-targeted HCT116 cells studied here are potentially valuable as the basis of a cell-based screen for identifying small molecules that specifically kill cells harboring activated β-catenin. Such compounds presumably would not inhibit activated β-catenin directly but might instead modulate other important molecules that govern Wnt signaling. The advantage of such a cell-based screen is that it makes no assumptions regarding the biochemical function of active lead compounds; instead, it would identify in an unbiased way molecules that selectively kill cancer cells harboring activated β-catenin. Second, it may be possible to validate inhibitors of β-catenin identified in more conventional in vitro screens by testing their activities against a panel of SW48 and HCT116 cells. On the basis of the data described herein, compounds that inhibit the growth of SW48 cells, but not HCT116 cells, are good candidates as specific, effective inhibitors of activated β-catenin.

In summary, we describe a novel clonal growth assay based on human somatic cell gene targeting and demonstrate that the β-catenin oncogene is a necessary growth stimulus in a subset of human colon cancer cell lines. These results have significant relevance to understanding the function of activated β-catenin in the pathogenesis of human cancer, and provide an important proof-of-principle guiding the development of anticancer pharmaceuticals.

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References


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