Short Communication

Adenomatous Polyposis Coli Gene Mutation Alters Proliferation through its β -Catenin-Regulatory Function in Aggressive Fibromatosis (Desmoid Tumor)

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Aggressive fibromatosis is a monoclonal proliferation of spindle (fibroblast-like) cells. A subset of lesions contain somatic truncating adenomatous polyposis coli (APC) gene mutations, and all of the lesions contain an elevated β -catenin protein level. A major function of APC is to regulate β -catenin protein level. β -Catenin has a dual function in the cell: it is a member of the adherens junction, and it binds transcription factors in the tcf-lef family, transactivating transcription. Cell cultures from aggressive fibromatoses containing an APC mutation were studied. Transient transfection of the full-length APC gene caused decreased proliferation and β -catenin protein level in these cultures. To determine whether β -catenin protein level was responsible for the change in proliferation rate, stable transfections of Δ N89 β -catenin (a stabilized form that is not degraded by APC, but retains all other functions) were achieved in half of the cultures derived from each tumor, whereas the other half were transfected with an empty vector. Transfection of the full-length APC gene in cultures that were stably transfected with Δ N89 β -catenin did not result in a change in proliferation. The type I promoter of p56lck contains an HMG consensus region, to which members of the tcf-lef family can bind. p56lck was expressed in cultures not transfected with the full-length APC gene and in cultures transfected with the full-length APC gene and Δ N89 β -catenin, but not in cultures transfected with only the full-length APC

gene. These data show that APC truncating mutations give aggressive fibromatosis cells a proliferative advantage through β -catenin and suggest that β -catenin acts to transactivate transcription. (*Am J Pathol* 1998, 153:709–714)

Aggressive fibromatosis (desmoid tumor) is a locally invasive soft-tissue lesion composed of a monoclonal proliferation of spindle (fibroblast-like) cells.^{1–3} The tumor cells demonstrate an elevated level of β -catenin protein compared with surrounding normal marginal tissues, and a subset of tumors contain somatic mutations in the adenomatous polyposis coli (APC) gene.⁴ The somatic APC mutations are likely responsible for the proliferative advantage held by the tumor cells.

The APC gene was initially identified as mutated in the familial preneoplastic syndrome, familial adenomatous polyposis, which predisposes to colonic neoplasia. Many sporadic colon tumors, including polyps, were also found to contain somatic APC mutations. APC has several potential functions, one of which is to regulate the protein level of β -catenin. When β -catenin is present at high levels, it binds to APC protein. The serine-threonine kinase GSK3 β also binds APC, and this triprotein complex results in β -catenin protein degradation through phosphorylation of sites on APC.⁵ APC mutations usually result in an early stop codon, producing a predicted truncated protein product. The protein product of these truncating APC mutations typically loses the ability to degrade β -catenin, resulting in an elevated β -catenin protein level.⁶

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β -Catenin may function as a modulator of transcription. When present at high levels, it localizes to the nucleus and binds to members of the tcf-lef family of transcription factors.⁷⁻⁹ These factors are "architectural" transcription factors that bend DNA, allowing other factors to bind and activating transcription.¹⁰ They are members of the high mobility group (HMG) related group of transcription factors binding to the HMG consensus region. Members of the tcf-lef family are usually expressed only by lymphoid cells; however, they are expressed by some neoplasms, most notably colonic neoplasia.¹¹ Transcription factors in this family alter the way they transactivate transcription when bound to β -catenin. One factor in the family, tcf-4, is expressed in colon cancers and will transactivate transcription only if bound to β -catenin.⁸

p56lck is a member of the src family of tyrosine kinases, the function of which is not completely known, although it plays an important role in T-cell activation.¹¹⁻¹³ The type I promoter for p56lck contains the HMG consensus binding region to which tcf-lef transcription factors may bind. p56lck is expressed by colonic neoplasia but not normal colonic epithelial cells. Its expression in colon neoplasia may be due to activation by a member of the tcf-lef family,¹⁴ although an alternative mechanism of activation, coexpression of another HMG-related transcription factor, such as Sox-4, with Ets-1 may also be responsible.¹⁵ Although there is a potential alternative mechanism for promoter activation, the expression of p56lck is a marker for transcription activation by HMG-related transcription factors.

This study aims to determine whether APC truncation increases proliferation through its β -catenin protein-regulatory function in aggressive fibromatosis. Elevated β -catenin may form a transcription-activating complex, activating an HMG-related transcription factor, ultimately resulting in loss of regulation of proliferation. Primary cell cultures from aggressive fibromatoses containing somatic APC mutations will be used in transfection studies to determine whether transfection of the full-length APC gene decreases proliferation and whether transfection of both the full-length APC gene and a stabilized form of β -catenin that cannot be degraded by APC continues to have a high proliferation rate. Expression of p56lck will be determined in the cultures. p56lck expression in cultures with a high proliferation rate and high β -catenin level suggests that β -catenin acts to transactivate transcription.

Materials and Methods

Cell Cultures

Primary cell cultures from four aggressive fibromatoses containing somatic APC mutations were obtained at the time of surgical resection. The APC mutations in two lesions were previously published.⁴ One has a frameshift mutation in codon 1324, and the other a frameshift in codon 1371. Both tumors demonstrated loss of heterozygosity for the other allele and a lack of immunohistochemical staining with an antibody to the carboxyl terminus

portion of APC protein. A protein truncation test for APC was utilized to screen for mutations in the other cases, using a previously reported technique.¹⁶ A truncation within segment three (of exon 15) was identified in both. Both had a homozygous APC mutation. The cultures were established using mechanical dissociation in RPMI (Life Technologies, Inc., Grand Island, NY) and 50% fetal calf serum. After the cells achieved near confluence, they were divided into multiple culture dishes and grown in RPMI with 10% fetal calf serum. Two culture dishes were tested to ensure that the cells were representative of the primary lesion, by performing immunohistochemistry using specific antibodies for the carboxyl- and amino-terminal portions of APC protein. All four cultures showed cytoplasmic staining for the amino-terminal but not the carboxyl-terminal portion of APC.

Transfection of APC and β -Catenin in Cell Culture

The full-length APC gene in a CMV-Neo-Bam vector was obtained from Bert Vogelstein and Kenneth Kinzler¹⁷ and a β -catenin gene with the first 89 codons deleted (Δ N89 β -catenin) in a CMV-neo-Bam vector was obtained from Paul Polakis.¹⁸ Empty vectors were produced by removing APC or Δ N89 β -catenin using restriction endonucleases. Δ N89 β -catenin was chosen as it lacks the ability to be degraded by APC, but its protein product retains the other functions of the full-length product.¹⁸ Transfection conditions were initially optimized using a human fibroblast cell line (MRC-5; American Type Culture Collection, Manassas, VA) and then using additional fibromatosis cell cultures. We and others were unable to achieve stable APC transfected cells, presumably because the cells transfected with the full-length gene proliferate at a slower rate, and the cells that are not successfully transfected have a proliferative advantage. We found we could achieve transfection rates of 85% 4 days after transfection, whereas only 40% were transfected 8 days after transfection. Stable Δ N89 β -catenin transfections were achieved using antibiotic selection, and stable expression was obtained 3 weeks after transfection. Thus, we transfected cells with Δ N89 β -catenin or an empty vector, grew the cells in antibiotic-containing media, and at 3 weeks transiently transfected the cells with APC. Determination of proliferation and apoptosis, protein extraction, and RNA isolation was carried out 4 days after the APC transfection.

When cells reached 30% confluence, half were transfected with Δ N89 β -catenin, whereas the other half were transfected with the empty vector. Lipofection (Lipofectamine; Life Technologies, Inc.) with 2 μ g DNA at a 1:10 DNA to liposome ratio with a 5-hour incubation was utilized. Cells were grown under antibiotic suppression (G418; 400 μ g/ml; Life Technologies, Inc.), and one culture dish from the set transfected with Δ N89 β -catenin and one from the set transfected with the empty vector underwent protein extraction and Western analysis using an antibody to the C-terminal portion of β -catenin (Transduction Laboratories, Lexington, KY) to verify production

of the shorter, transfected protein product 3 weeks after the transfections.

Three weeks after Δ N89 β -catenin transfection, half of the cells transfected with Δ N89 β -catenin and half of the cells transfected with an empty vector were transfected with the full-length APC gene. The remainder of the cells were transfected with the empty vector. APC was transfected using 10 μ g of DNA with a 1:10 DNA to liposome ratio and a 6-hour incubation. Four days after this second transfection, separate dishes of cell cultures were tested for APC transfection (by immunohistochemistry to the carboxyl terminus portion of APC), proliferation (by bromodeoxyuridine incorporation), apoptosis (by *in situ* end labeling to detect fragmented DNA using the TdT *in situ* apoptosis detection kit (TACS), (Genzyme, Cambridge, MA), protein level of β -catenin (by Western analysis), and expression of p56lck (by reverse transcription-polymerase chain reaction (RT-PCR)).

APC Immunohistochemistry

Cell cultures were fixed for 5 minutes in 0.3% hydrogen peroxide in absolute methanol, washed in phosphate-buffered saline, and blocked with 1% horse serum for 30 minutes. They were incubated with monoclonal antibody to the amino terminus of APC (APC-3; Oncogene Science, Cambridge, MA) or the carboxyl terminus (APC-4; Oncogene Science) overnight at 4°C at 1.0 μ g/ml as previously described.⁴ The tissues were washed with phosphate-buffered saline and incubated with a secondary anti-mouse immunoglobulin, and detected using immunoperoxidase (Vector Laboratories, Burlingame, CA).

Western Blot for β -Catenin

Cells were scraped off the dishes and homogenized in lysis buffer (1% sodium dodecyl sulfate, 10 mmol/L Tris/HCl, pH 7.4) followed by 5 minutes of centrifugation at 12,000 $\times$ g. Equal amounts of total protein were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and stained to verify equal amounts of transferred protein. Western blot was performed using a monoclonal antibody to amino acid residues 571 to 781 of β -catenin (Transduction Laboratories) as previously described.⁴ Hybridization was carried out overnight at 4°C and detected using anti-mouse immunoglobulin G-horseradish peroxidase secondary antibody and chemoluminescence.

Proliferation Using Bromodeoxyuridine Incorporation

Bromodeoxyuridine (25 μ g/ml; Sigma) was added to the cell culture medium for a 12-hour period, after which cells were washed in phosphate-buffered saline and fixed in alcohol. Immunohistochemistry was performed using an anti-bromodeoxyuridine antibody as previously described.¹⁹ Light microscopy was utilized to count cells

with and cells without nuclear staining. Cells were counted over 10 high-powered fields to determine the percentage of cells with positive nuclear staining.

Apoptosis Using *in Situ* End Labeling to Detect DNA Fragmentation

A commercially available kit to detect DNA fragmentation (TACS, Genzyme) was utilized.²⁰ Cells were trypsinized and cytopins produced. These were processed according to manufacturer's instructions along with positive and negative control slides using manganese detection. Slides were observed under light microscopy and observed for staining compared with the control slides.

RNA Extraction and RT-PCR for p56lck Expression

RNA was extracted using Trizol reagents (Life Technologies, Inc.) and converted to cDNA using reverse transcriptase with a poly-T primer. PCR primers pairs that amplify a product that crosses introns for reduced glyceraldehyde-phosphate dehydrogenase and p56lck were chosen from previous publications.^{19,21} PCR was performed using previously published conditions for the primers.^{19,21} Resultant products were electrophoresed, stained with ethidium bromide, and photographed under ultraviolet light.

Results

Stable Δ N89 β -catenin transfections were achieved in all four cultures, as determined using Western blot with a C-terminal antibody to β -catenin. Transient APC transfections were successfully achieved, with 75 to 85% of cells expressing the C-terminal portion of APC 4 days after transfection (Figure 1).

Proliferation was decreased with transfection of the full-length APC gene in cultures not transfected with Δ N89 β -catenin, compared with cells not transfected with wild-type APC. Cultures only transfected with the empty vector had between 48 and 65% of nuclei stained for bromodeoxyuridine, whereas cultures transiently transfected with the wild-type APC had between 14 and 30% positively stained nuclei. When the cultures were stably transfected with Δ N89 β -catenin, there was no difference in proliferation when wild-type APC was transiently expressed (41 to 61% positive staining *versus* 42% to 60% positive staining). Thus, when cells expressed a stabilized form of β -catenin, expression of the wild-type APC was unable to decrease proliferation (Figure 2).

β -Catenin protein level decreased in cells transiently transfected with wild-type APC, compared with transfection with an empty vector. The β -catenin decrease was greater for cultures with a higher transfection rate (Figure 3). There was no change in mRNA level for β -catenin between any of the cultures (data not shown). RNA for p56lck was detected using RT-PCR in cultures that were not transfected (except with empty vectors), or that were

Figure 1. Demonstration of successful transfection of the full-length *APC* gene and Δ N89 β -catenin. Shown is immunohistochemistry using a C-terminal antibody to APC from cultures transfected with an empty vector (A) and transfected with the full-length *APC* gene (B). Cytoplasmic staining for the C-terminal portion of APC is visualized in almost every cell in B, with 85% of cells overall showing cytoplasmic staining for APC 4 days after transfection. C: Western blot using a C-terminal antibody to β -catenin. The left lane (C) is loaded with protein from cells transfected with an empty vector, and the right lane (T) is loaded with cells transfected with Δ N89 β -catenin. The second, shorter-length, band (Δ N89) in the right lane is Δ N89 β -catenin.

transfected with the wild-type *APC* and the Δ N89 β -catenin. p56lck was not detected in the cultures transfected with wild-type *APC* alone (Figure 3).

Apoptosis (as determined by *in situ* end labeling for DNA fragmentation) was low in all of the cultures compared with controls and did not vary between any of the transfections (data not shown).

Discussion

There are a number of potential functions for APC in the cell, including complexing with the cytoskeleton, binding factors involved in cell adhesion, and binding growth-

regulatory genes. These transfection studies show that *APC* truncation results in an elevated β -catenin protein level and an elevated proliferation rate. The demonstration that Δ N89 β -catenin transfection "rescues" the decreased proliferation caused by transfection of the full-length *APC* gene into these tumors shows that *APC* truncation gives these cells a proliferative advantage by elevating β -catenin level.

β -Catenin has two potential functions in the cell: it can bind cadherins in adherens junction and play a role regulating cell adhesion, and it can transactivate transcription by binding a transcription factor. The demonstration of p56lck expression by cultures containing a high

Figure 2. Proliferation, measured using bromodeoxyuridine incorporation and immunohistochemistry. P, positive nuclear staining; N, negative nuclear staining. (A) is from cells transfected with only empty vectors, (B) is from cells transfected with the full-length *APC* gene, and (C) is from cells transfected with the full-length *APC* gene and Δ N89 β -catenin. Over 10 high-powered fields, 65% of cells from cultures shown in (A), 20% from (B), and 55% from (C) showed bromodeoxyuridine incorporation, thus illustrating that Δ N89 β -catenin transfection "rescues" the decrease in proliferation from APC transfection.

Figure 3. Western analysis for β -catenin and RT-PCR for p56lck. **A:** Western blot for β -catenin on equal quantities of extracted protein from cells transfected with an empty vector (lane C), and transfected with full-length APC at lower (50%, lane T1) and higher (85%, lane T2) efficiencies. A decrease in protein level with higher efficiency is demonstrated. **B:** RT-PCR data for p56lck and reduced glyceraldehyde-phosphate dehydrogenase from cultures transfected with the empty vector only (lane A), with full-length APC (lane B), or with full-length APC and Δ N89 β -catenin (lane C). Expression of reduced glyceraldehyde-phosphate dehydrogenase is present in all of the cultures, but p56lck is only expressed in lanes A, and C, which are the cultures with a high β -catenin protein level and high proliferation rate. Lane N is loaded with products from a normal fibroblast explant cell culture.

β -catenin protein level and a high proliferation rate, but not in cultures with a low β -catenin level and a low proliferation rate, suggests that β -catenin acts to regulate transcription in aggressive fibromatosis. A previous study,⁴ showing a lack of expression of E-cadherin and thus a lack of epithelial cell adherens junctions, and the demonstration that β -catenin localizes throughout the cell rather than to the cell periphery in aggressive fibromatosis, gives further support to β -catenin acting to modulate transcription.

Other neoplasms contain APC truncations, most notably colon tumors. There are differences between the clinical behavior of aggressive fibromatosis and colonic neoplasia, which suggests different cellular functions for the mutations in the different tumors. For instance, colon polyps can progress to colon carcinoma, whereas aggressive fibromatoses only very rarely become malignant. These differences may be due to the cellular environment containing the APC truncation. Perhaps the lack of epithelial cell adherens junctions in aggressive fibromatosis or the fact that β -catenin binds to a different transcription factor is responsible for the difference. Alternatively, a function of APC other than the β -catenin protein-regulatory function may play a more important role in the colonic epithelial cells than in the fibroblast-derived aggressive fibromatosis cells.

Primary cell cultures are not always representative of the lesion from which they are derived. Because aggressive fibromatosis is a monoclonal proliferation, cell cultures derived from this tumor should be representative of the initial lesion. The demonstration of a lack of staining using a specific C-terminal antibody to APC further sug-

gests that the cultures are representative of the primary tumor, which also lack staining using this antibody.

The expression of p56lck by lesions with a high proliferation rate (and a high β -catenin level) supports β -catenin acting to complex with a transcription factor, probably in the tcf-lef family, to transactivate transcription. However, studies showing an alternative pathway for p56lck transcription in colonic neoplasia suggest the possibility that its expression is not directly activated by members of the tcf-lef family, but that whatever is activated by the elevated β -catenin level activates other transcription factors (such as Ets and Sox-4), which leads to p56lck expression.

The lack of a difference in apoptosis with transfection of the full-length APC gene in our cultures suggests that APC truncation does not act to modulate apoptosis in aggressive fibromatosis. Thus, the proliferative advantage held by cells in this monoclonal lesion is probably not due to altered apoptosis. Previous study shows that β -catenin protein level is elevated in all aggressive fibromatoses compared with marginal tissues, regardless of whether lesions harbor an APC mutation or not.⁴ This information, along with the demonstration that the effect of APC truncation on cell proliferation is mediated by β -catenin protein level, suggests that β -catenin plays a central role in regulating proliferation in aggressive fibromatosis.

Replacement of the full-length APC gene into cases with a truncated APC results in decreased β -catenin protein level and decreased proliferation. This suggests that in the subset of aggressive fibromatoses containing somatic APC mutations, insertion of the full-length gene into the cells will reverse their proliferative advantage. Because this is a locally invasive lesion that does not metastasize, arresting the growth of the lesion may be an adequate therapy. Replacing the truncated APC gene with a full-length gene is a potential therapeutic strategy for this difficult-to-treat tumor.

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