Role of the Adenomatous Polyposis Coli Gene Product in Human Cardiac Development and Disease*

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Expressed sequence tag (EST) and digital Northern analyses of human fetal, adult, and hypertrophic heart cDNA libraries revealed ESTs with high homology to adenomatosis polyposis coli (APC) and its associated protein, β-catenin, as well as their differential expression. Thus, we hypothesize that the APC/β-catenin pathway may play a role in cardiac development and disease. Reverse transcriptase-polymerase chain reaction analysis exhibited a higher APC expression in adult compared with fetal and hypertrophic heart but no significant difference in β-catenin mRNA level. However, β-catenin protein level was higher in fetal and hypertrophic heart compared with adult heart, suggesting the post-translational regulation of β-catenin by APC in the cardiovascular system. In vitro antisense inhibition of APC resulted in a higher β-catenin protein expression leading to an incomplete myotube formation, suggesting APC/β-catenin pathway involvement in myotube development. Western blot analysis further reveals three novel isoforms, APC-F, APC-A, and APC-D, ubiquitously expressed in fetal, adult, and hypertrophic heart, respectively. Isoform switching during development and disease pathogenesis suggests functionally distinct roles for each isoform. These data (i) demonstrate the usefulness of genome-based expression analysis for rapid discovery of differentially expressed genes, (ii) implicate the APC/β-catenin pathway in the cardiovascular development, and (iii) demonstrate APC isoform switching during cardiac development and disease.

In mammalian hearts, cardiomyocytes withdraw permanently from the cell cycle by adulthood, losing the ability to regenerate after injuries such as myocardial infarction (1–5). This phenomenon, leading to an increased risk in mortality (6), has been associated with numerous molecular factors. Although significant strides have been made toward elucidating these factors that regulate the permanent withdrawal of cardiomyocytes from the cell cycle, the underlying mechanisms are poorly understood. Thus, the identification and characterization of key regulatory genes of the cardiovascular system are necessary in understanding the underlying mechanisms of cardiac development and disease. Conventional approaches, which focus on identifying single genes, are lengthy and tedious processes. However, the combination of technological advances in bioinformatics and molecular biology has given investigators an opportunity for large scale gene discovery. An explosion of sequence-based genome research using expressed sequence tags (ESTs) has led not only to the discovery of novel transcripts with unknown functions but also to the identification of genes that are expressed in very low levels that were not previously reported in the cardiovascular system. The EST approach has been established as a highly efficient technique for large scale gene identification. In our laboratory alone, we have generated over 50,000 ESTs from 11 cDNA libraries representing different cardiac developmental and diseased states. Sequence analysis of these ESTs from the libraries found several cDNA clones with significant sequence similarities to adenomatosis polyposis coli (APC) and its interacting protein, β-catenin (7, 8). Further, computer-based digital Northern analysis of data from fetal, adult, and hypertrophic cDNA libraries suggested differential expressions of the aforementioned genes during cardiac development and disease.

APC is conventionally known as a 310-kDa protein encoded by 15 exons of the APC gene. The coding region of the human APC gene is highly conserved in mouse APC gene, mutations of which lead to colorectal cancer (9–11), commonly found in familial adenomatosis polyposis (FAP) (12, 13). In some cases of congenital colorectal cancer, the pathology also includes patches of hypertrophy of the retinal pigment epithelium (14). Although the role of APC is unknown, its association with a variety of developmental processes (17). This regulates the nuclear accumulation of β-catenin, which forms a complex with the LEF/TCF family of transcription factors (which have now been implicated in a variety of developmental processes) and in turn directly affects gene expression (15–18). Recently, several isoforms of APC have been reported in brain (non-dividing tissue), suggesting additional or novel functional roles in the control of growth and/or differentiation (9, 10). This evidence along with our identification of differential expression of APC during cardiac development and disease led us to speculate an involvement of APC in these processes.

In this report, the differential expression of APC and its interacting protein (β-catenin) in fetal, adult, and hypertrophic hearts was investigated using reverse transcriptase-polymerase chain reaction (RT-PCR). These proteins were further char-

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1 The abbreviations used are: EST, expressed sequence tag; APC, adenomatosis polyposis coli; FAP, familial adenomatosis polyposis; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
acetylated by Western blot to determine their status during post-translational regulation. In an effort to ascertain the function of APC, a cell culture model of cardiomyocytes was set up using the C57Bl6 cell line. C57Bl6 cells are often utilized to simulate cardiomyocytes because of their ability to differentiate into contracting myotubes and to express cardiac specific genes (19). Antisense oligonucleotide specifically designed for APC was then used to examine the effect of translational blockage of APC on the proliferation and differentiation in vitro. This study provides significant data supporting the involvement of the APC/β-catenin pathway in cardiac development and disease.

EXPERIMENTAL PROCEDURES

**Total RNA Extraction from Tissue and Cell Culture**—Total RNA was extracted from hearts of BALB/C mice (Jackson Laboratory, Bar Harbor, ME) at midgestation, late gestation, neonate, 1, 5, and 10 days old, and adult. Animals were sacrificed by instant decapitation as an approved method in accordance with the Animal Welfare Act. Hearts were quickly removed, freeze-clamped, and stored in liquid nitrogen. Human fetal, adult, and hypertrophied hearts were obtained from pooled pathological specimens and were also stored in liquid nitrogen. The tissues were subsequently powdered using a stainless steel mortar precooled to the temperature of liquid nitrogen prior to RNA isolation. Tissue cultures were washed twice with 1× PBS before RNA extraction. Total RNA from samples was extracted using Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. The quantity and the purity of each sample were determined by spectrophotometry at 260 and 280 nm. Integrity of RNA was examined on a 1% formaldehyde-agarose RNA gel.

cDNA Library Construction and Large Scale Sequencing of cDNA Libraries—Poly(A)^+ enriched RNA was isolated using oligo(dT)-cellulose chromatography according to the manufacturer’s protocol (Amer sham Pharmacia Biotech). The concentration and purity of the mRNA were examined in a similar manner as with total RNA. mRNA was then used to construct the cDNA libraries, and large scale sequencing was performed in our laboratory according to protocols described previously (7, 20–23).

**Sequence and Digital Northern Analysis**—ESTs sequences were compared against the nonredundant public data bases, GenBank/EMBL/DDBJ and dbEST, for sequence similarity using BLAST. ESTs were considered to be a match to a known gene if E value was < 10^-10. Computer-based digital Northern analysis was performed in 24 well tissue culture plates (Corning) to achieve a differential expression of a gene “tagged” in a specific library. Frequencies were estimated as a ratio of the number of times a gene is tagged per total number of cDNA clones sequenced from each library.

**RT-PCR**—RT-PCR was carried out to compare the mRNA level of APC and its interacting proteins at different developmental and diseased stages of cardiac tissue. RT-PCR was performed using the Ti tan^® one-tube RT-PCR system (Roche Molecular Biochemicals) according to manufacturer’s protocol in a total volume of 50 μl containing 0.1 μg of RNA in the presence of primer pairs for the following: human APC, forward 5'-TCATGATAAGATGATAGTTGCC-3' and reverse 5'-AACTCTGAATGCTCTGTAC-3'; human β-catenin, forward 5'-ACCTCTAGAAGTTGCTTGGC-3' and reverse 5'-AGTTGTCATGGCGTCTTGAG-3'; human GAPDH, forward 5'-GGGCTGCTTGAACATTAAGA-3' and reverse 5'-TGCTGGTTGACATGGCGTCTTGAG-3'; human β-catenin, forward 5'-AGGCTGCTTGAACATTAAGA-3' and reverse 5'-AGTTGTCATGGCGTCTTGAG-3'; mouse APC, forward 5'-GAAGCTGCTTGAACATTAAGA-3' and reverse 5'-TCTGGCTGGCTGTGC-3'; mouse β-catenin, forward 5'-ACAGGAGATGCAGTCTGAG-3' and reverse 5'-GGACTCATGCTATCTGCTGGT-3'.

RT-PCR was carried out under the following conditions: one cycle of 60 °C for 30 min; one cycle of 94 °C for 2 min; 40 cycles of 94, 60, and 68 °C for 2 min each; one cycle of 68 °C for 5 min.

**Quantification of RT-PCR Results**—Six microliters of RT-PCR products was electrophoresed on a 1% agarose gel. The bands were then quantified using a gel documentation system (Bio-Rad) as counts × mm². All results were normalized as a ratio to the level of GAPDH and β-actin.

**Immunoblotting Analysis**—Immunoblot analysis was carried out to determine the protein expression levels and to examine the differential expression levels at the protein level. Total protein was extracted from tissues or cells using sample buffer containing 62.5 mM Tris, 2.3% SDS, 5% β-mercaptoethanol, and 10% glycerol at pH 6.8. Fifty micrograms of protein was electrophoresed on a 3–12% gradient and 8% polyacrylamide gel. Proteins were then electroblotted to polyvinylidene difluoro membrane (Millipore) using a semidry transfer (Buchler instruments). The efficiency of transfer was verified by Ponceau Red staining of the membrane. Proteins were subsequently incubated with polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:200 for APC (N-terminus) and 1:200 for β-catenin (N-terminus) and overnight at 4 °C. Membranes were then washed with horseradish peroxidase-conjugated secondary antibody, dilated 1:5000 for 30 min at room temperature. Each membrane was stripped prior to reprobing with polyclonal actin antibody serving as a housekeeping internal control. Proteins were detected using ECL and ECL PLUS Western blotting detection system (Amersham Pharmacia Biotech). To eliminate the likelihood of nonspecific binding or degradation, only those bands that appear in both N terminus and C terminus blots are analyzed as possible isoforms. Imaging densitometry (Bio-Rad) was used to quantify the intensity of each band in counts × mm². The values were then normalized as a ratio to corresponding β-actin. The up-regulation or down-regulation was expressed as a percentage.

**Cell Culture**—Murine C2C12 myoblasts (ATCC) were cultured in high glucose Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Life Technologies, Inc.) and penicillin/streptomycin (1:100) antibiotic (Life Technologies). Differentiation was induced by switching the medium to high glucose Dulbecco’s modified Eagle’s medium containing 2% horse serum (Life Technologies, Inc.) and antibiotic. Cultures were maintained in an appropriate medium in an incubator containing 95% O₂ and 5% CO₂ at 37 °C.

**Antisense and Uptake Study**—Antisense and random misense oligonucleotides were custom designed by Chemicon International, spanning between nucleotide positions of 3000 and 3050 of constitutively expressed exon 15 to inhibit APC protein translation. Fluorescence-labeled phosphorothiate oligonucleotides were used for an uptake study to establish the time required for the uptake of the antisense oligonucleotides according to manufacturer protocol. Time points are the span between the addition of the oligonucleotide and termination of the incubation at 48, 24, 8, 4, 2, and 1 h. Two thousand cells in 150 μl of medium were seeded 1 h prior to the addition of 3 μl (100 μM stock) of fluorescein isothiocyanate-labeled oligonucleotides in cell culture dishes to allow slides to occur. Slides were washed in 4% paraformaldehyde/minimal medium to fix the cells. Subsequently, the slides were photographed using a Nikon fluorescence microscope.

**Cellular Proliferation Assay**—The effect of antisense oligonucleotides on cellular proliferation was determined by the rate of proliferation using direct cell counts in a period of 10 days. Cells were grown in 3.5% fetal bovine serum to ensure submaximal growth. Ten thousand cells/well were seeded in 24 well tissue culture plates (Corning) to achieve near confluence in 48 h. Each well was labeled as P, C, or A for no treatment, random oligonucleotides, or antisense oligonucleotides treatment, respectively. Cultures were passed every 2 days. In 10,000 cells/well with refreshment of oligonucleotides. Oligonucleotides were added at 2 μM concentration 2 h after seeding of the cells to allow proper attachment. Detached cells were also counted to monitor apoptotic response to antisense oligonucleotides. Cells were processed by the addition of trypsin blue 1:1 (v/v) using a hemacytometer.

**Cellular Differentiation Assay**—The effect of antisense oligonucleotides on cellular differentiation was determined by observing changes in the morphology of myotube formation and by direct measurement of myotube density under the microscope as “counts per field.” Counts were taken from nine fields to obtain the average myotube counts for each individual experiments. Antisense oligonucleotides were added 48 h prior to induction of differentiation with switching to medium containing 2% horse serum. Antisense oligonucleotides were added every 48 h with a fresh change of medium at 2 μM concentration. Cultures were maintained for 10 days after induction of differentiation. In addition, the blockage of the APC gene by antisense was determined by Western blotting with APC’s C-terminal and N-terminal antibodies. Furthermore, the membranes were stripped and followed by β-catenin antibody treatment to determine the effect of APC expression blockage on the level of β-catenin.

**Statistics**—Differences between groups were tested using unpaired Student’s t test. All values are reported as mean ± S.E.

**RESULTS**

**Sequence and Computer-based Digital Northern Analysis**—Sequence analysis of over 50,000 ESTs generated from 11 cDNA heart libraries revealed several cDNA clones significantly matching (p < 10^-10) APC and its interacting protein, β-catenin. Digital Northern analysis indicated a differential...
expression of these genes during cardiac development and disease as they were tagged at different frequencies in fetal, adult, and hypertrophic heart libraries as shown in Table I.

In Vitro Gene Expression Analysis (RT-PCR)—The expression of APC mRNA is significantly up-regulated in adult heart compared with fetal heart in both human \((p < 0.05, \text{Fig. 1A})\) and mouse \((p < 0.001, \text{Fig. 2A})\). In contrast, its expression in hypertrophic heart is down-regulated \((p < 0.05, \text{Fig. 1A})\). There was no significant difference in the mRNA expression level of \(\beta\)-catenin between adult and fetal heart of both species \((\text{Figs. 1B and 2B})\); however, it is significantly \((p < 0.05)\) down-regulated in the hypertrophic heart compared with normal adult heart \((\text{Fig. 1B})\). To determine the stage of development at which up-regulation occurs, mouse tissues from several developmental stages were used. The APC expression was shown to be the highest in neonate and especially in the 1-day-old heart. Following this, there is a progressive increase in the APC mRNA level from 5-, 10-, and 20-day-old heart with a higher expression level in adult heart compared with fetal stages \((p < 0.05, \text{Fig. 3})\).

Protein Expression Level (Western Blot)—Western blot analysis indicated a 46\% \((\text{human; Fig. 4A})\) and 30\% \((\text{mouse; Fig. 4B})\) down-regulation of \(\beta\)-catenin in adult heart compared with fetal heart. Hypertrophic heart, however, exhibited a 46\% up-regulation compared with normal adult heart \((\text{Fig. 4A})\). Further analysis of \(\beta\)-catenin protein level in APC antisense-treated cells demonstrated a 30\% higher expression of \(\beta\)-catenin in these treated cells \((\text{Fig. 6})\).

Western blot analysis of APC using antibodies against the last 19 amino acids of the C terminus and the first 20 amino acids of the N terminus revealed three novel isoforms of APC and their switching during human cardiovascular development and disease \((\text{Fig. 5A})\). Further analysis exhibited similar isoform expression patterns in the heart of both human and mouse during cardiac development. An 85-kDa APC isoform was identified to be ubiquitously expressed in fetal heart of both mouse and human and therefore was designated APC-F, for fetal isoform. Another isoform was ubiquitously expressed in normal adult heart at 60 kDa. This isoform was further up-regulated in hypertrophic hearts and therefore was called APC-D, for diseased form. The 45-kDa isoform is exclusively expressed in normal adult heart \((\text{not expressed in diseased adult heart})\). This isoform was designated as APC-A for adult isoform \((\text{Fig. 5B})\).

Cellular Growth and Differentiation Assay—To ensure the success of the antisense oligonucleotides to block the translation of APC, a 24-h uptake time was allowed prior to the initiation of any experiments although cellular uptake of antisense oligonucleotides reaches an experimentally competent level after 8 h of incubation \((\text{results not shown})\). To reveal the effect of antisense on cellular differentiation, a comparison of myotube counts and morphological features was made among cells treated with APC antisense oligonucleotide and cells receiving no or random oligonucleotide treatment. Myotube counts were significantly \((p < 0.05)\) reduced in antisense-treated cells for the entire duration of the experiment \((\text{Fig. 6})\). Morphologically, antisense-treated cells form shorter and rounder myotubes in larger proportion compared with \(\Phi\) and \(C\) in the first few days after the onset of differentiation. Nuclei in these cells tend to aggregate in the center of the cell rather than being evenly dispersed along the long axis as observed in normal myotubes. In addition, the onset of contractile activity was approximately 1 day later than normal in antisense-treated cells. The mode of contraction also differed in antisense-treated cells, since they showed weaker contraction without noticeable rhythm. Detectable contraction in cells with no treatment persisted from the onset on day 4 to the end of the experimental period, while in antisense-treated cells they ceased on day 8. The myotubes in the antisense-treated population were unable to achieve a length consistent with mature myotubes, leaving a significantly shorter myotube population compared with \(\Phi\) and \(C\). In addition, these cells were not able to fuse in the later stage of differentiation \((\text{Fig. 7})\). Western blot analysis was also carried out on the protein extract of \(\Phi\), \(C\), and \(A\) from 10-day differentiated cells, indicating a complete inhibition of the conventional 310-kDa APC in \(\Phi\) compared with \(\Phi\) and \(C\). The follow up Western blot analysis of \(\beta\)-catenin demonstrated a higher expression level of \(\beta\)-catenin in \(\Phi\) compared with \(\Phi\) or \(C\). Actin antibody treatment demonstrated equal protein loading of samples \((\text{Fig. 8})\). Furthermore, to assay cellular proliferation, cell counts over a 10-day period were obtained. Cells with no treatment \((\Phi)\) or control oligonucleotide treatment \((C)\) consistently showed a doubling time of 36 h, whereas cells treated with APC antisense oligonucleotide demonstrated a reduced rate of proliferation. After 4 days of expo-
sure to antisense oligonucleotide, there were significantly (p < 0.05) lower cell counts with virtually no proliferation by the 10th day (Fig. 9A). In addition, significantly more dead cells were observed with antisense-treated cells after 4 days of exposure (Fig. 9B). No difference in cell counts was observed between F and C (Fig. 9A and B).

**DISCUSSION**

During the course of random sequencing of cDNA clones from a human fetal heart cDNA library, we discovered several ESTs with significant sequence similarity to the APC gene (7, 8). Our digital Northern analysis suggested a possible differential expression of APC during cardiovascular development and disease. In this report, we have shown that transcripts encoding

APC are expressed at a higher level in adult heart compared with fetal heart in human and mouse. Furthermore, RT-PCR for APC expression in different developmental stages of mouse heart from midgestation to adult indicated a very low expression level in fetal stages compared with all postnatal stages. A gradual increase in APC expression was seen from 5- to 20-day old hearts, leveling off in adults, correlating with the observation that by day 15 of postnatal rodent development, all cardiomyocytes have exited the cell cycle (4, 24). Up-regulation of APC thus appears to play an important role in the cardiomyocytes' withdrawal from the cell cycle. This is further supported by the decrease of APC expression level in hypertrophic heart compared with adult heart. The unexpected high expression in neonatal and 1-day-old heart is probably a result of transition from fetal (minimal pulmonary flow) to neonatal (high pulmonary flow) circulation (25) causing reactivation of gene expres-
sion, which gradually adjusts itself during the first days of extrauterine life (26).

Although a similar mRNA expression level of β-catenin was observed in fetal and adult heart, its expression at the protein level was lower in adult heart. This can be explained by the higher APC expression level in the adult and hence β-catenin post-translation regulation by APC through the APC/β-catenin pathway. In addition, our RT-PCR indicates a lower expression level of β-catenin in the hypertrophic heart compared with adult. The follow-up Western blot analysis reveals a higher level of protein expression, which is consistent with the regulatory role APC plays on β-catenin, further supporting the post-translational regulation event, very possibly by the reduced levels of APC.

Analysis of the effect of antisense oligonucleotide inhibition of APC expression in differentiating cells demonstrated significant morphological and molecular changes. Morphologically, from day 4 onward, the antisense-treated cells show less myotube formation than nontreated and control antisense-treated cells. The Western blot analysis on the 10-day differentiating cells indicated complete inhibition of the 310-kDa APC. It has been suggested that proteins that associate with the cytoskeleton may have a regulatory function directly related to cell proliferation and carcinogenicity (27). In previous studies, it was suggested that cell-to-cell contact through various types of junctions might be the main method by which our body regulates cell growth. Signals received this way are often passed on to the nucleus by means of cytoskeleton-associated proteins such as APC (28). The inability of antisense-treated cells to develop into fully differentiated myotubes with respect to their length and width may be attributed to lowered APC concentration. At the molecular level, the β-catenin protein expression is affected by APC antisense treatment. The β-catenin is expressed at a higher level in antisense-treated cells, which can
be explained by the well-established fact that APC regulates the cytoplasmic level of β-catenin, a signaling molecule (17). Translocation of β-catenin from cytoplasm to nucleus results in the formation of a complex with LEF/TCF transcription factors in the nucleus, which directly changes the pattern of gene expression (15–18).

Inhibition of APC expression by antisense oligonucleotides affected the cellular proliferation rate, since it drastically reduced the number of cells from day 4 onward in the antisense-treated cells compared with control cells. In addition, there appears to be higher cell death in antisense treatment by virtue of a greater number of detached cells. This suggests that APC may also be involved in programmed cell death (i.e., apoptosis) as seen with many other tumor suppressors. For example, transcription factors such as c-Myc are intimately associated with cellular proliferation, and its constitutive expression increases the susceptibility of cells to apoptosis (29). Interestingly, a recent study has shown that APC is involved in the c-Myc pathway (30). Further insight into the direct involvement of APC in the c-Myc/apoptosis cascade is required.

The recent discovery of 16 APC transcripts and their differential expression in several mammalian tissues (10) suggests multiple functional and tissue-specific roles for APC isoforms, making APC a very interesting regulatory gene to study. Our Western blot analysis using APC antibody against the C terminus and N terminus, cross-reactive with both human and mouse APC, revealed similar expression patterns in the heart of both species during development. To eliminate the possibility of wrong isoform selection (due to nonspecific binding or degradation of larger forms), only the bands that appeared with both C and N terminus antibodies and in both species were analyzed. In the context of this assumption, an isoform switching event seems to take place during cardiovascular development and disease. Isoform switching is most apparent between a cardiovascular fetal enriched isoform, which is identified at 85 kDa (APC-F), an adult enriched isoform at 60 kDa (APC-D), which is up-regulated in diseased heart, and a 45-kDa isoform (APC-A), which is only expressed in normal adult heart. These results suggest that the APC-D may play a role in the maintenance of cell cycle exit and that APC-A may be involved in regulating and maintaining myocyte enlargement. Hypertrophy, an adaptive response to pressure overload, is characterized by the enlargement of existing myocytes rather than an increase in cell number (31, 32). The possibility of APC-D’s involvement in enlargement of myocytes can be ruled out, since it is also ubiquitously expressed in normal adult heart. These results further suggest isoform switching of these alternative APC transcripts during cardiac development and disease and, consequently, their functional significance in cardiovascular development and disease. The presence of one form in fetal and the others in adult heart as well as up-regulation of one in hypertrophic heart provides important clues for their specific roles. However, further studies are required to establish their role in the cardiovascular system.

In addition to the functional analysis of the conventional APC protein in cardiovascular system, this study has shown the important role publically available data bases play in finding key genes involved in regulation of cardiac development and disease. Furthermore, our study demonstrated the use of computer-based “digital Northern” analysis as a valuable tool for rapid identification of differentially expressed genes that can be further characterized by RT-PCR, Northern blot, and/or Western blot. This report also demonstrates the involvement of the APC/β-catenin pathway in cardiovascular development and process of disease. We have also utilized antisense technology to obtain conclusive confirmation that the APC-mediated down-regulation of β-catenin does in fact play important roles in cellular growth and differentiation. This report also illustrates isoform switching and substantiates the involvement of APC proteins in cardiac muscle as potential regulators of cardiac gene expression during cardiac development and hypertrophy. These findings contribute to a better understanding of molecular mechanisms that regulate cardiac development and differentiation in normal and diseased heart.

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