β-Catenin Overexpression Reduces Myocardial Infarct Size through Differential Effects on Cardiomyocytes and Cardiac Fibroblasts

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β-Catenin is a transcripational regulator of several genes involved in survival and proliferation. Although previous studies suggest that β-catenin may be involved in the process of preconditioning and healing after myocardial infarction (MI), little is known regarding the role of β-catenin in cardiomyocytes and cardiac fibroblasts. We investigated the role of β-catenin in cardiomyocytes and cardiac fibroblasts and whether β-catenin overexpression could reduce MI size. Adenovirus-mediated gene transfer of nonphosphorylatable constitutively active β-catenin (Ad-catenin) decreased apoptosis in cardiomyocytes and cardiac fibroblasts with increased expression of survivin and Bcl-2. Although Ad-catenin increased the percentage of cells in the S phase with enhanced expression of cyclin D1 and E2 in both cell types, the increase in cell number was only evident in cardiac fibroblasts, whereas hypertrophy and binuclear cells were more prominent in cardiomyocytes. All of these effects of β-catenin gene transfer were blocked by inhibition of its nuclear translocation. Furthermore, Ad-catenin enhanced the expression of vascular endothelial growth factor in both cells and induced differentiation of cardiac fibroblasts into myofibroblasts. In a rat MI model, injection of Ad-catenin into the infarct border zone resulted in a significant decrease in MI size with ant apoptotic effect and cell cycle activation in both cardiomyocytes and myofibroblasts. β-Catenin may play an important role in the healing process after MI by promoting survival and cell cycle not only in cardiomyocytes but also in cardiac fibroblasts with its differentiation into myofibroblasts.

β-Catenin is known to have dual functions. Membrane-bound β-catenin maintains tissue architecture and cell polarity at adherens junctions by linking the cadherin cytoplasmic tail to the actin cytoskeleton (1). Cytoplasmic β-catenin translocates into the nucleus where it forms a complex with transcription factors of Tcf/Lef family and activates the expression of specific genes involved in cell survival and proliferation (1, 2). Glycogen-synthase kinase 3β (GSK3β)3 constitutively phosphorylates cytoplasmic β-catenin resulting in proteosomal degradation (3), and Wnt signaling inhibits GSK3β, leading to cytoplasmic accumulation of β-catenin (4).

Although the critical roles of β-catenin during development (5) and in neoplastic disease have been well described previously (6), relatively little is known about the role of β-catenin in cardiomyocytes and cardiac fibroblasts, which are not only the principal cells comprising the myocardium but also key cells involved in remodeling after myocardial infarction (MI). Recent studies have suggested that β-catenin is capable of regulating survival/apoptosis and hypertrophy of cardiomyocytes (7, 8). The inactivation of GSK3β by statins has been shown to inhibit cardiomyocyte apoptosis (7), whereas activated GSK3β was shown to attenuate cardiac hypertrophy in vivo (8). In addition, Wnt/β-catenin pathways have also been implicated in fibroblast proliferation (9). Wnt-1 induced accumulation of cytosolic β-catenin, and the resultant Tcf/Lef transcriptional activation was correlated with enhanced proliferation, survival, and growth in Rat-1 fibroblasts (10). Taken together, previous data suggest that β-catenins enhance survival, growth, and proliferation in cardiomyocytes and cardiac fibroblasts and may play a role in the healing process after MI.

The modulation of upstream signals of β-catenin in the myocardial ischemia model has been investigated previously. These studies suggest that β-catenin may have a role in the process of preconditioning and healing after myocardial infarction. Inactivation of GSK3β reduced MI size (11) and resulted in the

3 The abbreviations used are: GSK3β, glycogen-synthase kinase 3β; MI, myocardial infarction; GFP, green fluorescence protein; Ad-catenin, adenoviruses expressing triple mutant β-catenin construct; Ad-GFP, adenovirus encoding GFP; NCadΔC, dominant negative N-cadherin; DMEl, Dulbecco’s modified Eagle’s medium; WST, water-soluble tetrazolium salt; BrdUrd, bromodeoxyuridine; FACS, fluorescence-activated cell sorter; VEGF, vascular endothelium growth factor; LVEDD, left ventricular end diastolic dimension; LVEF, left ventricular ejection fraction; ATW, anterior wall thickness; FS, fractional shortening; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PECAM, platelet-endothelial cell adhesion molecule; α-SMA, α-smooth muscle actin; PCNA, proliferating cell nuclear antigen; FBS, fetal bovine serum; LV, left ventricular.
accumulation of β-catenin in the cytosol and nucleus in a preconditioning model (12). Overexpression of FrzA, an antagonist of the Wnt pathway, induced the activation of GSK3β and reversed the benefit of ischemic preconditioning after MI (13). On the other hand, overexpression of FrzA reduced MI size and improved cardiac function in a non-reperfused model (14). In these studies, myocardial ischemia models were different, and none of these studies overexpressed β-catenin directly. The direct modulation of β-catenin may more clearly suggest that β-catenin plays a pivotal role in the healing process after MI.

Therefore, the aim of this study was to elucidate the role of β-catenin in myocardial healing after infarction. We investigated and compared the role of β-catenin in cardiomyocytes and cardiac fibroblasts along with the downstream signaling pathway of β-catenin. To confirm that the effects observed were mediated by the transcriptional activity of β-catenin, nuclear translocation of β-catenin was inhibited. Furthermore, we studied the effect of direct β-catenin overexpression in a rat MI model.

EXPERIMENTAL PROCEDURES

Construction of Adenoviral Vectors Expressing Triple Mutant β-Catenin—Adenoviruses expressing the triple mutant β-catenin construct were produced using AdEasy™ kits (Qbiogene, Inc.). Human wild type β-catenin plasmid was generously provided by Dr. Bert Vogelstein of The Johns Hopkins University. To overexpress β-catenin, we produced a β-catenin triple mutant plasmid using PCR-based targeted mutagenesis, which contains three mutations in phosphorylation sites (S33Y/S37F/T41A) resulting in resistance to degradation by GSK3β. β-Catenin triple mutant plasmid was subcloned into a shuttle vector (pAdTrack-CMV). The recombinant shuttle vector was cotransfected with the adenoviral genome (pAdEasy-1) containing green fluorescence protein (GFP) gene into Escherichia coli (B)5183 where homologous recombination occurred. The recombinant adenovirus β-catenin triple mutant (Ad-catenin) was transfected to 293a cells (Microbix Biosystem Inc.) to amplify viral particles, which were purified by CsCl (Sigma) ultracentrifugation and dialysis. Successful construction of Ad-catenin was confirmed by immunoblot analysis of β-catenin, and transfected cells were determined by the coexpression of GFP. Hemagglutinin was tagged to the adenoviral vector, indicating exogenous β-catenin. Adenovirus encoding GFP (Ad-GFP) was used as control.

Inhibition of β-Catenin-mediated Transcription Activation by Cadherin Derivatives—Dominant negative N-cadherin (NCadΔC), which lacks most of the extracellular domain, was used to suppress β-catenin-mediated transcriptional activity (15). The NCadΔC construct was obtained from Dr. C. S. Shin with the permission of Dr. Jeffrey Gordon (Washington University, St. Louis). NCadΔC was inserted into the BglII/EcoRI site of pMSCV-IRESC-3′GFP retroviral vector, giving pMSCV-NCadΔC-IRESC-3′GFP. 293T cells were transfected with the retroviral vectors pMSCV-NCadΔC-IRESC-3′GFP or control empty vector, pMSCV-IRESC-3′GFP, using Lipofectamine Plus reagents (Invitrogen). Viral supernatant was collected 48 h later, centrifuged at 1,000 X g for 5 min, and stored at −80 °C.

Cardiomyocytes and cardiac fibroblasts were transfected with the retroviral vectors before adenoviral transfection to block β-catenin-mediated transcriptional activity.

Primary Cell Cultures from Neonatal Rat Hearts—Primary cultures of neonatal rat cardiomyocytes and cardiac fibroblasts were prepared as described previously (16). Briefly, 2-day-old Fisher 344 rats were euthanized, and hearts were excised and washed several times with phosphate-buffered saline (Sigma). Ventricles were minced and trypsinated. After centrifugation, cell pellets were resuspended. Floating cardiomyocytes and attached fibroblasts were separated. To confirm adequate separation, cardiomyocytes were stained with anti-troponin T (Santa Cruz Biotechnology) and fibroblasts with an anti-fibroblast-specific marker (AF5110-1; Acris) (data not shown).

Cell Viability and Proliferation Assays—Cell viability and proliferation were measured using WST-1 (WST; high water-soluble tetrazolium salt; Roche Applied Science) assay and incorporation of bromodeoxyuridine (BrdUrd; Roche Applied Science) according to the manufacturer’s instructions. Cardiomyocytes and cardiac fibroblasts were seeded in 96-well plates at 2 X 104 cells per well in DMEM (Invitrogen) and transfected with retroviral vectors and recombinant adenoviral vectors (100 multiplicities of infection). Cells were then placed in 0.5% FBS for 40 h or in 1 µM staurosporine, a strong inducer of apoptosis, for 24 h to measure apoptosis or in 10% FBS for 40 h to measure proliferation. At the end of the incubation period, 10 µl/well of WST-1 or BrdUrd was added and incubated for another 4 h in the same incubator.

Flow Cytometry—Apoptosis and cell cycle status were evaluated by fluorescence-activated cell sorter (FACS), as described previously (17). Cells were transfected with retroviral vectors and 100 multiplicities of infection of recombinant adenoviral vectors and then placed in 0.5 or 10% FBS. Cells were harvested and fixed at 40 h for analysis of apoptosis and cell cycle progression. Flow cytometric analysis was performed on a FACStar Plus (BD Biosciences). Histograms of DNA contents were analyzed using Modfit LT software (Verity Software) to characterize population fractions in each phase of the cell cycle.

Assessment of Ploidity, Protein Synthesis, and Hypertrophy—Cell cycle distribution of DNA content was determined by flow cytometry as described previously (18). Flow cytometry was carried out using a Coulter Elite ESP flow cytometer and analyzed using CellQuest software (BD Biosciences). For each analysis, 104 cells were analyzed. [3H]Leucine incorporation was carried out as described previously (19). Briefly, cells were made quiescent by incubation in 0.5% FBS for 24 h. The cells were then placed in 10% FBS for 40 h. Four hours before harvesting, [3H]Leucine (1 µCi/ml) (PerkinElmer Life Sciences) was added to the media. [3H]Leucine incorporation was determined using an LS 3801 scintillation counter (Beckman Instruments). Cell hypertrophy was analyzed as described previously (20). Cell area was quantified from images of unstained cells as follows: 10 fields were chosen randomly, and 10 cells were measured per field.

Western Blotting—Immunoblot assays were performed as described previously (21). The primary antibodies used were anti-total β-catenin antibody (1:500 dilution; Cell Signaling Technology, Inc.), anti-survivin antibody (1:500 dilution;
Novus), anti-Bcl-2 antibody (1:1000; Pharmingen) anti-cyclin
D1 antibody (1:500 dilution; Santa Cruz Biotechnology), anti-
cyclin E2 antibody (1:500 dilution; Santa Cruz Biotechnology),
anti-VEGF antibody (1:500 dilution; Santa Cruz Biotechnol-
ygy), and anti-\nH9251\nH9251-tubulin antibody (1:500 dilution; Oncogene).
The secondary antibody was anti-mouse IgG/horseradish per-
oxidase conjugate (1:2500 dilution; Amersham Biosciences).

**MI Model and Adenovirus-mediated Gene Transfer**—Female
Sprague-Dawley rats (12 weeks old), weighing 220–250 g (Daehan
Biolink Co.), were fed and given water ad libitum. All ani-
mal experiments were performed after receiving approval from
the Institutional Animal Care and Use Committee of the Clin-
ical Research Institute in Seoul National University Hospital,
and all protocols complied with the National Research Council.
Myocardial infarction was induced in rats as described previ-
ously (22). Either Ad-catenin or Ad-GFP was injected to the
infracted area and the MI border zone.

**Echocardiographic Studies**—The rats were anesthetized,
and echocardiographic examination was performed. Left ventric-
ular end diastolic dimension (LVEDD), left ventricular end sys-

tolic dimension (LVESD), and anterior wall thickness (ATW)
were measured from at least three consecutive cardiac cycles on
the M-mode tracings, based upon the American Society for
Echocardiography leading edge method. Fractional shortening
(FS) was calculated as \( \frac{(\text{LVEDD}-\text{LVESD})}{\text{LVEDD}} \). 

**Histologic Analysis and Immunohistochemistry**—At 7 days
after MI, rats were euthanized by pentobarbital overdose.
Infarct size was determined by image analysis system (Scion
Image, Scion Corp.) on Masson’s trichrome-stained slides. The
infarct area was measured in five different sections from each
animal. Immunohistochemistry was performed as described
previously (23). The heart was excised, embedded in OCT com-
pound, frozen in liquid nitrogen, and stored at \(-70^\circ\text{C}\). To eval-
uate apoptosis, the terminal deoxynucleotidyltransferase-me-
diated dUTP nick-end labeling (TUNEL) assay was performed.
TUNEL-positive cells were counted in 10 different microscopic
fields of at least three different sections from each animal.
Immunofluorescent staining was performed as described pre-
viously with minor modifications (24). Sections were washed,
fixed, and incubated with monoclonal antibodies against

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**FIGURE 1. The effects of \(\beta\)-catenin on survival in cardiomyocytes and cardiac fibroblasts.**

A–D, adenovirus-mediated constitutively stable \(\beta\)-catenin
(Ad-catenin) transfection significantly decreased apoptosis from serum deprivation and staurosporine, which was reversed by NCadΔC, suggesting that the anti-apoptotic effect was mediated by the transcriptional activity of \(\beta\)-catenin. After transfection, cardiomyocytes and fibroblasts were cultured in DMEM with 0.5% FBS for 40 h or in 1 \(\mu\text{M}\) staurosporine for 24 h. Ad-catenin transfection reduced apoptosis under serum deprivation by 41% in cardiomyocytes (A) and 17% in cardiac fibroblasts (C) as measured by FACS analysis. The effects of \(\beta\)-catenin on cell survival were confirmed by WST-1 assay \((n = 5)\) in cardiomyocytes (B) and cardiac fibroblasts (D). \(*, p < 0.05\), Ad-catenin versus Ad-GFP; \#, \(p < 0.05\), Ad-catenin versus Ad-catenin NCadΔC.) E and F, immunoblot analysis showed that enhanced and prolonged expression of survivin and Bcl-2 in Ad-catenin-transfected cardiomyocytes (E) and cardiac fibroblasts (F), which was inhibited by NCadΔC. CMC indicates cardiomyocytes; CFB indicates cardiac fibroblasts. Control means retrovirus encoding GFP.
hemagglutinin, platelet-endothelial cell adhesion molecule (PECAM)-1 (Pharmingen) for capillary density, proliferating cell-nuclear antigen (PCNA), troponin T, cyclin D1 (Cell Signaling Technology), and cyclin E (Santa Cruz Biotechnology) for 30 min at room temperature. Afterward, cells were stained with goat anti-rabbit IgG antibodies (Molecular Probes), conjugated with fluorescein isothiocyanate. The fluorescent image was obtained with a fluorescence microscope (Axiovert 200 microscope; Carl Zeiss). PECAM-1 positive cells were counted in 10 different microscopic fields of at least three different sections from each animal.

**Statistical Analysis**—All data were expressed as means ± S.E. Comparisons of means were performed using the Student t test or analysis of variance test with Bonferroni post hoc test. All calculations were performed using the SPSS software version 11.0, and \( p < 0.05 \) was considered statistically significant.

**RESULTS**

**Confirmation of Adenoviral Construct and Successful Gene Transfer and Confirmation of Retroviral Vector**—After transfection of cardiomyocytes and cardiac fibroblasts with adenoviral vectors, we confirmed GFP expression in more than 95% of the cells. Ad-catenin transfection resulted in overexpression of total β-catenin on Western blotting analysis in contrast to Ad-GFP, which showed no changes in β-catenin expression, confirming successful formation of Ad-catenin construct and gene transfer. After retroviral transfection, we confirmed GFP expression in about 70% of the cells, both in cardiomyocytes and cardiac fibroblasts.

**β-Catenin Reduces Apoptosis in Cardiomyocytes and Cardiac Fibroblasts**—Under serum deprivation, the subdiploid apoptotic fraction of DNA as measured by flow cytometry analysis significantly decreased in both cardiomyocytes and cardiac fibroblasts transfected with Ad-catenin compared with Ad-GFP-transfected cells, which was reversed by NCadΔC (Fig. 1, A and C). Accordingly, WST-1 assay showed a significantly enhanced cell viability in Ad-catenin-transfected cells compared with Ad-GFP-transfected cells (absorption: 135 ± 12% versus 100 ± 12% in cardiomyocytes and 148 ± 2% versus 100 ± 10% cardiac fibroblasts for Ad-catenin versus Ad-GFP, \( p < 0.05 \); Fig. 1, B and D), which was inhibited by NCadΔC. When challenged by staurosporine, Ad-catenin also enhanced cell viability (absorption: 129 ± 11% versus 100 ± 1% in cardiomyo-
cytes and 123 ± 1% versus 100 ± 2% cardiac fibroblasts for Ad-catenin versus Ad-GFP, p < 0.05; Fig. 1, B and D). To investigate the possible downstream mechanism of the anti-apoptotic effect of β-catenin overexpression, we examined survivin and Bcl-2, anti-apoptotic proteins. The expression of survivin and Bcl-2 increased after Ad-catenin transfection, which was also inhibited by NCadΔC (Fig. 1, E and F). Immunofluorescent staining confirmed that Bcl-2 protein was increased in the Ad-catenin group compared with the Ad-GFP group (supplemental Fig.). Collectively, β-catenin overexpression enhanced cell survival in cardiomyocytes and cardiac fibroblasts, and survivin and Bcl-2 are the downstream molecules to mediate the anti-apoptotic effect of β-catenin in these cells.

**β-Catenin Propagates the Cell Cycle but Increases Cell Number Only in Cardiac Fibroblasts and Not in Cardiomyocytes—** Analysis of BrdUrd incorporation into DNA showed an increase in DNA synthesis in both cardiomyocytes and cardiac fibroblasts, which was completely inhibited by NCadΔC (absorption, 103 ± 5% versus 100 ± 3% in cardiomyocytes and 138 ± 6% versus 100 ± 3% cardiac fibroblasts for Ad-catenin versus Ad-GFP, p < 0.05; Fig. 2A). However, although the viable cell number increased in cardiac fibroblast after β-catenin transfection and subsequently decreased after NCadΔC transfection, there were no significant effects on the viable cell number of cardiomyocytes (absorption, 103 ± 5% versus 100 ± 3% in cardiomyocytes and 138 ± 6% versus 100 ± 3% cardiac fibroblasts for Ad-catenin versus Ad-GFP, p < 0.05; Fig. 2B), suggesting that β-catenin does not induce cellular division in cardiomyocytes even though it induced DNA replication. To investigate the differential effect of β-catenin on the DNA replication and cell proliferation of cardiomyocytes and cardiac fibroblasts, we examined the cell cycle and DNA content. Ad-catenin increased the percentage of cells in the S phase in both cell types (Fig. 2, C and D), concordant with the increased BrdUrd incorporation. To investigate the mechanism of cell cycle propagation after β-catenin overexpression, major regulators of G1-S tran-
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**FIGURE 4. The effects of β-catenin on the expression of VEGF in cardiomyocytes and cardiac fibroblasts and contractile protein in fibroblasts.** A, immunoblot analysis showed that enhanced expression of VEGF in Ad-catenin transfected cardiomyocytes and fibroblasts, which was inhibited by NCadΔC. B, proliferation of endothelial cells significantly increased after addition of conditioned media from cardiomyocytes and cardiac fibroblasts transfected with Ad-catenin as measured by WST-1 assay (n = 5). *, p < 0.05, Ad-catenin versus Ad-GFP. C, representative photographs of cardiac fibroblasts that express contractile protein, α-smooth muscle actin. DAPI, 4,6-diamidino-2-phenylindole. D, β-catenin overexpression led to the increased expression of α-smooth muscle actin, suggesting phenotypic alteration to myofibroblasts. CMC indicates cardiomyocytes; CFB indicates cardiac fibroblasts. Control means retrovirus encoding GFP.

sition, cyclin D1 and E2, were examined. In accordance with the increase in cells in the S phase, the expression of cyclin D1 and cyclin E2 increased in both cells transfected with Ad-catenin, which was reversed by NCadΔC (Fig. 2, E and F).

Because the number of cardiomyocytes was unchanged despite increased DNA synthesis, we examined the effects of β-catenin gene transfer on nuclear polyploidization and cellular hypertrophy. Ad-catenin resulted in an increase in the fraction of cells greater than 4 N DNA content compared with Ad-GFP, which was reversed by NCadΔC (Fig. 3, A and B). However, the increase in ploidity by β-catenin overexpression was more prominent in cardiomyocytes than in cardiac fibroblasts. Moreover, binuclear cells were more frequently observed in cardiomyocytes transfected with Ad-catenin compared with Ad-GFP but not in cardiac fibroblasts. In fibroblasts, cell size also increased, but to a lesser degree than cardiomyocytes, and binuclear cells were observed less frequently than in cardiomyocytes (Fig. 3, C and D). Analysis of [3H]leucine incorporation to protein demonstrated an increase in protein synthesis in both cardiomyocytes and cardiac fibroblasts, which was inhibited by NCadΔC (183 ± 6% versus 100 ± 1% in cardiomyocytes and 163 ± 1% versus 100 ± 5% cardiac fibroblasts for Ad-catenin versus Ad-GFP, p < 0.01; Fig. 3E). Accordingly, the effect of β-catenin on hypertrophy was more prominent in cardiomyocytes than in cardiac fibroblasts. Ad-catenin transfection induced a greater increase in cell size in cardiomyocytes compared with cardiac fibroblasts, which was reversed by NCadΔC (1.7 ± 0.1 versus 1.0 ± 0.1 in cardiomyocytes and 1.3 ± 0.1 versus 1.0 ± 0.1 cardiac fibroblasts for Ad-catenin versus Ad-GFP, p < 0.05; Fig. 3F).

**β-Catenin Enhances Expression of VEGF in Cardiomyocytes and Cardiac Fibroblasts and Induces Contractile Protein in Cardiac Fibroblasts**—Because angiogenesis is an important feature in the healing process after MI, and because cardiomyocytes and cardiac fibroblasts are potential sources of VEGF, we also examined the effects of β-catenin on VEGF expression in both cell types. Transfection of Ad-catenin resulted in increased VEGF expression compared with Ad-GFP, which was reversed by NCadΔC (Fig. 4A). Proliferation of endothelial cells significantly increased after adding the culture supernatants from cardiomyocytes and cardiac fibroblasts (absorption, 155 ± 7% versus 100 ± 8% in cardiomyocytes and 124 ± 6% versus 100 ± 5% in cardiac fibroblasts for Ad-catenin versus Ad-GFP, p < 0.05; Fig. 4B). In addition, β-catenin gene transfer resulted in a significant increase in the fraction of α-SMA-expressing fibroblasts compared with Ad-GFP (52 ± 8% versus 11 ± 2% for Ad-catenin versus Ad-GFP, p < 0.05; Fig. 4C), and Western blotting showed that the expression of α-SMA increased in Ad-catenin-transfected cells than in Ad-GFP-transfected cells (Fig. 4D), suggesting that β-catenin overexpression may induce differentiation of fibroblasts into myofibroblasts.

**β-Catenin Reduces MI Size and Preserves LV Function**—From these data, we hypothesized that β-catenin overexpression could be beneficial in myocardial regeneration after injury.
Therefore, we performed gene transfer of either Ad-catenin or Ad-GFP in vivo, after inducing a MI in rats. At base line and day 3 after MI, there was no significant difference in the FS, LVESD, LVEDD, and end diastolic ATW between Ad-catenin-injected rats and Ad-GFP-injected rats as measured by echocardiography. At day 7, the LVEDD was significantly smaller (6.6 ± 0.5 mm versus 7.8 ± 0.5 mm, p < 0.05), and fractional shortening was significantly higher in Ad-catenin-injected rats compared with Ad-GFP-injected rats (31 ± 4% versus 25 ± 4%, p < 0.05; Fig. 5). Ad-catenin-injected rats also showed thicker end diastolic ATW (MI scar area) than control rats (1.03 ± 0.11 mm versus 0.82 ± 0.08 mm, p < 0.05) with no significant difference in posterior wall thickness (Fig. 5, A–D). Similar results were observed at day 14. On histological analysis, the infarcted area ratio was significantly lower in Ad-catenin-injected rats compared with control gene-injected rats at day 7 after MI (25 ± 7% versus 32 ± 7%, p < 0.05; Fig. 5, E and F). To confirm successful transfection, we performed immunofluorescent staining of hemagglutinin (indicating exogenous β-catenin), which showed that Ad-catenin was transfected successfully in vivo. Moreover, we observed strong intranuclear exogenous β-catenin expression in some cells, suggesting overexpressed β-catenin was translocated to the nucleus and resulted in transcriptional activation. Simultaneous staining of troponin T for cardiomyocytes revealed intranuclear exogenous β-catenin expression not only in cardiomyocytes (arrowheads) but also in myofibroblasts (arrows). HA indicates hemagglutinin; TnT indicates cardiac troponin T; DAPI, 4,6-diamidino-2-phenylindole.
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FIGURE 6. The effects of β-catenin gene transfer on apoptosis and capillary density in a rat myocardial infarction model. A, representative figure of tunnel staining at day 7. There were fewer TUNEL (+) cells (brown) in constitutively stable β-catenin (Ad-catenin)-injected rats compared with controls (Ad-GFP). B, quantitative analysis for apoptosis. TUNEL (+) cells were significantly fewer in Ad-catenin-transfected rats than Ad-GFP (n = 7). *, p < 0.05, Ad-catenin versus Ad-GFP. C, immunoblot analysis showed enhanced and prolonged expression of survivin and VEGF in vivo in Ad-catenin-injected rats after MI. N indicates cells from rats without MI; G indicates Ad-GFP-transfected rats; C indicates Ad-β-catenin-transfected rats. D and E, capillary density was significantly higher in Ad-catenin-injected rats than controls (n = 7). *, p < 0.01, Ad-catenin versus Ad-GFP. Immunofluorescence for PECAM-1 (red) in the border zone of myocardial infarction at day 7 showed much more capillaries in Ad-catenin-injected rats than Ad-GFP.

gene transfer. The expression of the anti-apoptotic protein survivin increased significantly and for a longer period of time in Ad-catenin-injected rats compared with Ad-GFP-injected rats (Fig. 6C). To measure the effect of β-catenin gene transfer on angiogenesis after MI, we evaluated capillary density and VEGF expression. Capillary density was significantly higher (864 ± 187/mm² versus 523 ± 105/mm², p < 0.01; Fig. 6, D and E), and the expression of VEGF was significantly increased and prolonged in Ad-catenin-injected rats compared with Ad-GFP-injected rats (Fig. 6C). PCNA staining showed that the cell cycle was more active in Ad-catenin-injected rats than in Ad-GFP-injected rats (Fig. 7A). PCNA was observed in not only troponin T-positive cells but also cells in interstitium, suggesting that Ad-catenin propagated cell cycle in both cardiomyocytes and myofibroblasts. Cells positive for cyclin D1 and cyclin E2 were more frequently observed in rats injected with Ad-catenin compared with Ad-GFP (21.1 ± 2.2% versus 10.6 ± 1.9%, p < 0.01, and 5.9 ± 2.0% versus 3.0 ± 1.8%, p < 0.05, respectively) (Fig. 7B and C). Collectively, these results suggest that β-catenin overexpression may reduce infarct size through decreased apoptosis, increased capillary density, and enhanced cell cycle activity.

DISCUSSION

This study shows that β-catenin plays important roles in cardiomyocyte and cardiac fibroblast biology by promoting survival and the cell cycle progression, where inductions of survivin, Bcl-2, cyclin D1, and cyclin E2 are involved. The effects of β-catenin on survival or the cell cycle progression were blocked by NCadΔC, an inhibitor of nuclear translocation of β-catenin, suggesting that these effects were mediated by the transcriptional activity of β-catenin. Furthermore, β-catenin induced differentiation of cardiac fibroblasts into myofibroblasts and angiogenesis by VEGF induction in these cells. In vivo, β-catenin overexpression reduced infarcted area and preserved systolic function in a rat MI model through modulating survival, hypertrophy, or differentiation of cardiomyocytes or cardiac fibroblasts and angiogenesis by VEGF induction.

Differential Effects of β-Catenin on Cardiomyocytes and Cardiac Fibroblasts—We observed that cardiomyocytes and cardiac fibroblasts show different responses when cultured in a proliferative condition. Although cell number increased significantly in cardiac fibroblasts, increase in ploidity and cell size was more prominent in cardiomyocytes. Such differences in response to β-catenin overexpression may be due to the specific cell characteristics. Until recently, cardiomyocytes has been regarded as terminally differentiated cells, which have no mitotic activity. Cardiomyocytes lose their ability to undergo cytokinesis soon after birth, and an uncoupled karyokinesis and cytokinesis have been shown to result in binucleated cells (25). Another possible explanation may be the difference in proliferating capacity between cardiomyocytes and cardiac fibroblasts. Recent data have suggested that adult cardiomyocytes can undergo mitotic division. However, mitotic index was quite low, 0.08% in the regions adjacent to the infarcts (26). Interestingly, β-catenin overexpression increased expression of contractile protein in cardiac fibroblasts, suggesting phenotypic transformation to myofibroblasts. Transforming growth factor β1 induces α-SMA promoter activation and protein expression via β-catenin in tubular epithelium (27). Taken together, our data show that β-catenin is involved in the survival, cell cycle progression, growth, and phenotypic transformation of...
normal and differentiated cells such as cardiomyocytes and cardiac fibroblasts but may have some differential effects depending on the cell types.

Mechanism of Enhanced Survival of Cardiomyocytes and Cardiac Fibroblasts by β-Catenin—In this study, we demonstrated that survivin and Bcl-2 may be downstream of β-catenin. Survivin is a bi-functional member of the apoptosis inhibiting gene family (28). β-Catenin was reported to increase expression of the survivin gene in colorectal cancer cells (29). Although the role of survivin in cardiomyocytes and cardiac fibroblasts is relatively unknown, survivin was shown to be expressed by cardiomyocytes and was inversely associated with the presence of dilated cardiomyopathy and apoptosis of cardiomyocytes (30). In a recent study, the survivin increased after inhibition of GSK3β in cardiomyocytes (12). Bcl-2 is one of the representative molecules to inhibit apoptosis. β-Catenin also increased Bcl-2 expression in rat preconditioned myocardium (12). Collectively, these data suggest that β-catenin signaling may be involved in survivin and Bcl-2 expression in cardiomyocytes and cardiac fibroblasts. In this study, we found survivin and Bcl-2 to be significantly increased after β-catenin overexpression in both cell types, and this increase was inhibited by NCadΔC, suggesting that survivin and Bcl-2 may be under the control of β-catenin.

Mechanism of Cell Cycle Progression in Cardiomyocytes and Cardiac Fibroblasts by β-Catenin—The expression of both cyclin D1 and E2 was increased after β-catenin overexpression in cardiomyocytes and cardiac fibroblasts, which was slightly different from what was reported in cancer cells. Previously, it was reported in colon cancer cells that cyclin D1 is a direct target of the Tcf/LEF-1 pathway through a binding site in the cyclin D1 promoter region (31). In this study, NCadΔC reversed the expression of not only cyclin D1 but also E2, suggesting that in cardiomyocytes and cardiac fibroblasts both cyclin D1 and E2 may be downstream of β-catenin. β-Catenin increases cyclin E1 expression through direct and indirect mechanisms (32). First, increased cyclin D1 by β-catenin sequesters cdk inhibitors p21 and p27 from the cyclin E1-cdk 2 complex. Second, nuclear β-catenin coactivates liver receptor homolog-1-mediated transcription. Cyclin E2 is most closely related to cyclin E1 in a functional kinase complex (33). Cyclin E2 gene also has one mismatch liver receptor homolog-response element (TGACCTTGG). Therefore, cyclin E2 may be induced by β-catenin through similar mechanisms by which cyclin E1 is induced. The finding that β-catenin increases both cyclin D1 and cyclin E2 expression in cardiomyocytes and cardiac fibroblasts is a novel one, and may explain the increased percentage of cells in the S phase in our study.
Differential Effects of β-Catenin on Cardiomyocytes and Fibroblasts

Implication of β-Catenin in the Healing of Infarcted Myocardium—Previous studies have suggested that β-catenin may be involved in the process of preconditioning and healing after myocardial infarction; however, the results were not always concordant. A recent study reported that inhibition of GSK3β showed cytosolic accumulation of β-catenin and reduced infarct size (12). Barandon et al. (13) showed that overexpression of FrzA, an antagonist of the Wnt pathway, induced the activation of GSK3β and reversed the benefit of ischemic preconditioning after MI without modifying cytoplasmic accumulation of β-catenin. Meanwhile, overexpression of FrzA reduced infarct size and improved cardiac function with decrease of cytosolic β-catenin accumulation (14). The difference in myocardial ischemia model used and the modulation of upstream signals of β-catenin, instead of β-catenin itself, may explain these results. In this study, we directly overexpressed β-catenin using an adenoviral vector and reversed its action by sequestration with dominant negative cadherin. The direct modulation of β-catenin in our study more clearly suggests that β-catenin plays a pivotal role in the healing process after MI.

Possible Mechanisms of Infarct Size Reduction by β-Catenin—There are several possible mechanisms that could explain the reduction of MI size and preservation of LV systolic function by the transfer of the constitutively stable β-catenin gene. First is the anti-apoptotic effect by β-catenin on cardiomyocytes. Second, the enhanced VEGF expression and capillary density may also have contributed to the reduction of MI size. We and others have reported previously that β-catenin plays important roles in angiogenesis. GSK3β/β-catenin axis promotes angiogenesis through activation of VEGF signaling in endothelial cells (34), and β-catenin induced the expression of VEGF in skeletal myocytes, resulting in endothelial cell proliferation (35). In addition to the main mechanism mentioned above, β-catenin induced phenotypic transformation of cardiac fibroblasts to myofibroblasts, which could have contributed to the enhanced LV contractile function. Moreover, β-catenin decreased apoptosis and activated cell cycle in myofibroblasts. It has been shown previously that inhibition of myofibroblast apoptosis results in improvement of postinfarct remodeling and cardiac function (36). Finally, our study showed that β-catenin induced hypertrophy of cardiomyocytes. Youn et al. (22) reported that inhibition of hypertrophy seems to be detrimental for cardiac remodeling and myocardial function in the early phase after MI, and that cardiac hypertrophy seems to play a role in compensating for the loss of functioning myocardium, especially in the early stages after MI.

In conclusion, we show that constitutively stable β-catenin gene transfer decreases apoptosis and propagates the cell cycle in cardiomyocytes and cardiac fibroblasts. These direct effects along with induction of enhanced VEGF expression and contractile protein translate into reduced infarct size and preserved LV function after β-catenin gene transfer in a rat MI model. These data suggest that β-catenin may play an important role in cardiomyocyte and myofibroblast biology, and its modulation may improve the healing process after MI.

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