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ORIGINAL ARTICLE

Dominant-negative c-Jun inhibits rat cardiac hypertrophy induced by angiotensin II and hypertension

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Cardiac activator protein-1 (AP-1), composed of c-Jun, is significantly activated by hypertension or angiotensin II (AngII). This study was undertaken to elucidate whether c-Jun could be the potential target for treatment of cardiac hypertrophy. We constructed recombinant adenovirus carrying dominant-negative mutant of c-Jun (Ad.DN-c-Jun). Using catheter-based technique of adenoviral gene transfer, we achieved global myocardial transduction of DN-c-Jun in rats, to specifically inhibit cardiac AP-1. (1) AngII (200 ng/kg/min) infusion in rats caused cardiac hypertrophy, increased cardiac p70S6 kinase activity by 1.3-fold ($P<0.05$) and enhanced the gene expression of cardiac hypertrophic markers. Ad.DN-c-Jun, which was transferred to the heart 2 days before AngII infusion, prevented cardiac hypertrophy ($P<0.01$), decreased p70S6 kinase phosphorylation ($P<0.05$), and suppressed cardiac gene expression of brain natriuretic peptide, collagen I, III, and IV, monocyte chemoattractant protein-1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1) ($P<0.01$). (2) In genetically hypertensive rats with cardiac hypertrophy, cardiac gene transfer of Ad.DN-c-Jun, without affecting hypertension, regressed cardiac hypertrophy ($P<0.05$), and suppressed p70S6 kinase phosphorylation by 20% ($P<0.05$) and suppressed the enhanced expression of collagen I, III, and IV, MCP-1 and PAI-1. These results provided the first evidence that in vivo blockade of cardiac c-Jun inhibits pathologic cardiac hypertrophy.
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Introduction

c-Jun is an important component of transcription factor activator protein-1 (AP-1), and is activated by a variety of stress stimuli and plays the pivotal role in cellular responses including cell growth, apoptosis, differentiation, and gene expression.[1] Previously, we constructed dominant-negative c-Jun (DN-c-Jun) as the tool to specifically inhibit c-Jun and examined the effect of DN-c-Jun gene transfer on balloon-injured rat carotid artery *in vitro*[2] or cultured vascular smooth muscle cells *in vitro*.[3] We found that blockade of c-Jun by gene transfer of DN-c-Jun inhibited vascular smooth muscle cell proliferation and migration *in vivo* or *in vitro*, thereby proposing that suppression of c-Jun is useful for treatment of vascular hyperplasia.[2,3] Furthermore, we have determined the activities of cardiac AP-1 in angiotensin II (AngII)-infused rats[4] and stroke-prone spontaneously hypertensive rats (SHRSP).[5] We have found that cardiac AP-1 activity, related to c-Jun, is activated in AngII-infused rats and SHRSP. However, the significance of c-Jun for cardiac diseases remains to be defined, mainly because of the lack of the specific and potent pharmacological inhibitor of c-Jun. In the present study, to define the contribution of c-Jun to cardiac hypertrophy and gene expression induced by AngII or hypertension, we carried out cardiac transfer of adenoviral vector containing DN-c-Jun (Ad.DN-c-Jun) *in vivo*. We obtained the evidence that c-Jun is a potent therapeutic target for cardiac disease.

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Methods

Animals and experimental design

All procedures were in accordance with institutional guidelines for the care and use of laboratory animals. All rats were fed a standard laboratory chow (CE-2, Clea Japan) and given tap water *ad libitum*. In the first experiment, 9-week-old male Sprague–Dawley rats (Clea Japan) were used. At 2 days after rats received the transfer of Ad.DN-c-Jun or adenovirus containing bacterial β-galactosidase gene (AD.LacZ) as described later, AngII (200 ng/kg/min) was subcutaneously

infused to rats via osmotic minipump, as described by us,[6] to examine the effect of DN-c-Jun gene transfer on AngII-induced hypertension, cardiac hypertrophy, and gene expressions. In the second experiments, 8-week-old male SHRSP (SLC Japan) were used as the model of hypertensive cardiac hypertrophy. SHRSP were subjected to cardiac transfer of Ad.DN-c-Jun or Ad.LacZ as described later, to examine the effect of DN-c-Jun on hypertensive cardiac hypertrophy.

Construction of recombinant adenovirus containing DN-c-Jun

The dominant-negative mutant of c-Jun, called TAM67, was generated by removal of the transactivational domain of amino acids 3–122 of wild-type c-Jun by polymerase chain reaction, as described.[2,7] TAM67 has the DNA-binding domain of wild-type c-Jun. Recombinant replication-defective E1 and E3 adenoviral vectors expressing the TAM67 gene (Ad.DN-c-Jun) were constructed by using an adenovirus expression vector kit (Takara Biomedicals), according to the method of Miyake *et al*.[8] cDNA encoding TAM67 was placed into a cassette cosmid vector, PaxCAwt, which possesses the CAG promoter, composed of a cytomegalovirus enhancer, chicken β-actin promoter, and rabbit α-globin poly(A) signal. A recombinant adenovirus was constructed by *in vitro* homologous recombination in 293 cells by using the above cosmid vector, PaxCAwt, containing TAM67 cDNA and the adenovirus DNA-terminal protein complex. Ad.LacZ was also constructed as the negative control of Ad.DN-c-Jun, in the same way as Ad.DN-c-Jun. All the adenoviral vectors were purified with CsCl gradient centrifugation, dialyzed with a buffer containing 10 mM Tris (pH 7.5), 1 mM $MgCl_2$ and 10% glycerol, and stored in aliquots at −70°C.[9] The titer of the virus was determined by plaque assay and expressed as PFU/ml.

Adenoviral delivery protocol

Cardiac adenoviral delivery was performed according to the method of Hajjar *et al*.[10] Rats were anesthetized with sodium pentobarbital i.p. and placed on a ventilator. The chest was entered from the left side through the third intercostal space. The pericardium was opened and the aorta was identified. A 24G catheter containing 200 μl of adenovirus containing approximately 10^{10} plaque-forming units of either Ad.LacZ or Ad.DN-c-Jun was advanced from the apex of the left ventricle to the aortic root. The aorta was clamped distal to the site of the catheter and the solution injected. The clamp was maintained for 10 s. This procedure allows the solution that contains the adenovirus to circulate down the coronary arteries and perfuse the heart without direct manipulation of the coronaries. After 10 s, the clamp on the aorta was released. After removal of air and blood, the chest was closed, and animals were extubated and transferred back to their cages.

In the preliminary experiments, by Western blot analysis, we compared the expression of β-galactosidase in cardiac tissue at 1, 2, 5, and 7 days after cardiac transfer of Ad.LacZ to determine when the maximal protein expression is achieved in cardiac tissue. We found that the maximal β-galactosidase expression occurred at 2 days after the cardiac gene transfer. Therefore, we carried out cardiac gene transfer of Ad.LacZ or Ad.DN-c-Jun, 2 days before AngII infusion.

Western blot analysis

Protein extracts from LV were separated on a 12% SDS-polyacrylamide gel and immobilized on polyvinylidene difluoride membrane, as described in detail by us.[2] The membrane was immunoblotted with anti-c-Jun antibody (sc-44; Santa cruz), anti-β-galactosidase, anti-phospho-p70S6 kinase (Promega), anti-p70S6 kinase (Promega), and anti-α-tubulin, using the enzyme-linked chemiluminescence (ECL) method as described previously.[2]

RNA preparation and Northern blot analysis. All procedures were performed, as described in detail in our previous reports.[6,11] In brief, 20 μg of total RNA samples from individual left ventricle were subjected to 1% agarose gel electrophoresis, transferred onto nylon membrane, and hybridization was carried out with (^{32}P)dCTP-labeled cDNA probe for collagen type I, collagen type III, collagen type IV, monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and (^{32}p)ATP-labeled oligonucleotides complimentary to α-myosin heavy chain (α-MHC), and β-myosin heavy chain (β-MHC).[6,11] The densities of an individual mRNA band were measured by using a bioimaging analyzer (BAS-2000, Fuji Photo film Co., Tokyo, Japan).

Gel mobility shift assays

For DNA protein-binding reaction, gel mobility assays were performed as described in detail by us.[2–4] The sequence of double-strand consensus oligonucleotides of AP-1, NF-κB, Sp-1, and CREB were previously described. Supershift assay was performed with rabbit polyclonal anti-c-Fos IgG (sc-253-G, 2 μg), anti-c-Jun IgG (sc-822X, 2 μg) recognizing the amino acids from 56 to 69 (transactivation domain) of wild c-Jun, or anti-c-Jun IgG (sc-44X, 2 μg) recognizing the conserved DNA-binding domain of wild c-Jun (all from Santa Cruz).

Histochemistry

To evaluate the expression of β-galactosidase in the heart subjected to Ad.LacZ gene transfer, hearts were fixed with PBS containing 0.5% glutaraldehyde for 30 min and then in PBS with 30% sucrose for 30 min. The hearts were then permeabilized by incubation in solution containing sodium deoxycholate (0.01%) and Nonidet P-40 for 15 min. Then the hearts were incubated overnight in a solution containing 5-bromo-4-chloro3-indolyl-D-galactopyranoside (X-Gal), and 10-μm sections were then cut and examined under light microscopy.

To examine the expression of DN-c-Jun in the heart subjected to Ad.DN-c-Jun gene transfer, the heart was fixed with 4% paraformaldehyde and frozen in OCT-compound and the frozen sections were immunostained with the above-mentioned anti-c-Jun IgG (sc-822X) recognizing the transactivation domain of c-Jun, or anti-c-Jun IgG (sc-44X) recognizing the conserved DNA-binding domain of c-Jun, and then diaminobenzidine.

Statistics. Results were expressed as mean ± s.e.m. Statistical significance was determined by one-way ANOVA followed by Duncan's multiple range test. Differences were considered statistically significant at a value of $P < 0.05$.

Results

Cardiac gene transfer of Ad.LacZ and Ad.DN-c-Jun in vivo

Figure 1 shows the expression of LacZ protein 2 days following *in vivo* cardiac transfer of Ad.LacZ, using catheter based method of gene delivery. β-Galactosidase staining and Western blot analysis indicated that transferred LacZ protein was abundantly expressed in cardiac myocytes.

To quantify the transferred DN-c-Jun protein in cardiomyocyte, cardiac sections from Ad.DN-c-Jun-transferred group was immunostained with anti-c-Jun IgG (sc-44X) recognizing the conserved DNA-binding domain. As shown in Figure 2(c), immunoreactive c-Jun was negligible in the nucleus of cardiomyocyte from Ad.LacZ-transferred group. On the other hand, the nucleus of cardiomyocyte from Ad.DN-c-Jun-transferred group was significantly immunostained with anti-c-Jun IgG (sc-44X). The percent of cardiomyocyte nucleus immunoreactive to anti-c-Jun IgG (sc-44X) was 35.4 ± 7.4 in Ad.DN-c-Jun-transferred cardiac tissue while 1.9 ± 0.9% in Ad.LacZ-transferred tissue ($P < 0.01$, $n = 10$). Furthermore, cardiomyocyte nucleus from Ad.DN-c-Jun-transferred group was not immunostained with anti-c-Jun IgG (sc-822X) recognizing the transactivation domain, which can be explained by the fact that DN-c-Jun used in this work lacks the transactivation domain. Therefore, in the present work, we could specifically quantify DN-c-Jun by immunohistochemistry with anti-c-Jun IgG (sc-44X) recognizing the conserved DNA-binding domain and found that DN-c-Jun protein was overexpressed by our gene delivery method.

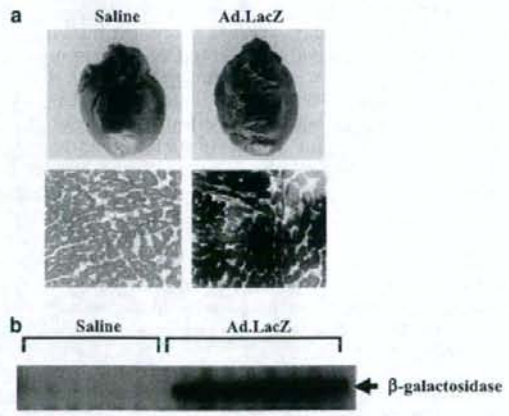

Figure 1 β-Galactosidase staining (**a**) and Western blot analysis of β-galactosidase protein (**b**) of rat heart subjected to adenoviral infection of LacZ gene (Ad.LacZ) or saline infusion. (**a**) At 2 days after adenoviral infection of LacZ gene to rat heart, β-galactosidase staining showed that LacZ protein was significantly expressed in the myocardium over the heart. (**b**) Western blot analysis with anti-β-galactosidase antibody indicated that LacZ protein was abundantly expressed in the heart received Ad.LacZ gene transfer.

Gel mobility shift analysis of left ventricular nuclear protein from rats subjected to gene transfer of LacZ or DN-c-Jun

Previously, we confirmed that Ad.DN-c-Jun employed in our work specifically inhibited transcriptional activity of AP-1, using luciferase assay.[2,3] As shown by gel mobility shift analysis in Figure 3, Ad.DN-c-Jun gene transfer increased AP-1 DNA-binding activity. However, the position of AP-1 band due to Ad.DN-c-Jun was upper to that of endogenous AP-1, being in good agreement with our previous papers.[2,3,12,13] As shown by supershift analysis, endogenous AP-1 band was supershifted with

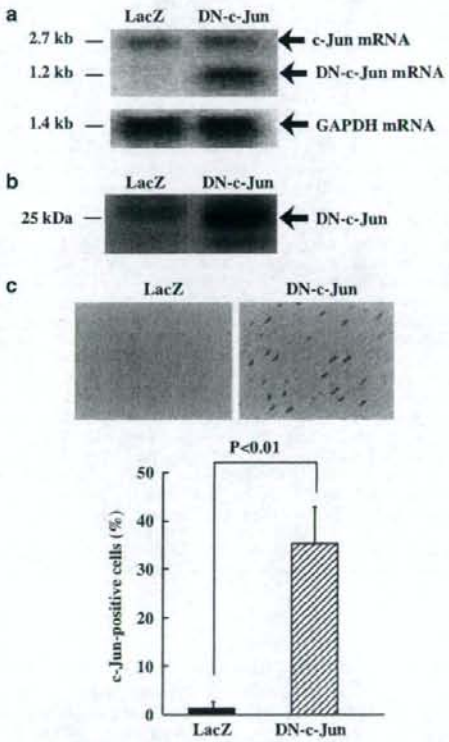

Figure 2 Northern blot analysis (**a**), Western blot analysis (**b**), and immunohistochemical staining (**c**) of dominant-negative c-Jun from heart received adenoviral infection of dominant-negative c-Jun (DN-c-Jun) or LacZ as the control. DN-c-Jun mRNA (**a**) and protein (**b**) were significantly detected in left ventricular extract from rat received adenoviral infection of DN-c-Jun. Immunohistochemistry of cardiac tissue from the rat subjected to cardiac gene transfer of LacZ or DN-c-Jun indicated that DN-c-Jun protein was overexpressed and localized in the nuclei of cardiomyocytes (**c**). DN-c-Jun was immunostained with anti-c-Jun IgG (sc-44X) recognizing the conserved DNA-binding domain. Values are means ± s.e.m. ($n = 10$).