Rescue of hereditary form of dilated cardiomyopathy by rAAV-mediated somatic gene therapy: Amelioration of morphological findings, sarcolemmal permeability, cardiac performances, and the prognosis of TO-2 hamsters

Tomie Kawada*, Mikio Nakazawa†, Sakura Nakauchi‡, Ken Yamazaki‡, Ryoichi Shimamoto‡, Masashi Urabe§, Jumi Nakata‡, Chieko Hemmi‡, Fujiko Masui‡, Toshiaki Nakajima‡, Jun-Ichi Suzuki‡, John Monahan¶, Hiroshi Sato*, Tomoh Masaki‖, Keiya Ozawa§, and Teruhiko Toyo-oka‡**

‡Department of Organ Pathophysiology and Internal Medicine, Cardiovascular Medicine, University of Tokyo, Tokyo 113-0033, Japan; *Pharmacy Division, Niigata University Medical Hospital, and †Department of Medical Technology, School of Health Sciences, Niigata University, Niigata 951-8520, Japan; §Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi 329-0498, Japan; ‖Research Institute, National Cardiovascular Institute, Suita 565-8565, Japan; and ¶Avigen, Inc., Alameda, CA 94502

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The hereditary form comprises ≈1/5 of patients with dilated cardiomyopathy (DCM) and is a major cause of advanced heart failure. Medical and socioeconomic settings require novel treatments other than cardiac transplantation. TO-2 strain hamsters with congenital DCM show similar clinical and genetic backgrounds to human cases that have defects in the δ-sarcoglycan (δ-SG) gene. To examine the long-term *in vivo* supplement of normal δ-SG gene driven by cytomegalovirus promoter, we analyzed the pathophysiologic effects of the transgene expression in TO-2 hearts by using recombinant adeno-associated virus vector. The transgene preserved sarcolemmal permeability detected *in situ* by mutual exclusivity between cardiomyocytes taking up intravenously administered Evans blue dye and expressing the δ-SG transgene throughout life. The persistent amelioration of sarcolemmal integrity improved wall thickness and the calcification score postmortem. Furthermore, *in vivo* myocardial contractility and hemodynamics, measured by echocardiography and cardiac catheterization, respectively, were normalized, especially in the diastolic performance. Most importantly, the survival period of the TO-2 hamsters was prolonged after the δ-SG gene transduction, and the animals remained active, exceeding the life expectancy of animals without transduction of the responsible gene. These results provide the first evidence that somatic gene therapy is promising for human DCM treatment, if the rAAV vector can be justified for clinical use.

In spite of a steady progress in the pharmaceutical treatment of dilated cardiomyopathy (DCM), the patient's prognosis is still poor (1). Cardiac transplantation is the most life-saving therapy of DCM at the advanced stage, although it includes a wide variety of medical and socioeconomic problems. Another potential strategy including gene therapy is urgently required (2), particularly in the infantile or juvenile cases when it is difficult to repeat cardiac transplantation along their growth. An animal model is useful for developing a new treatment. Cardiomyopathy (CM) hamster is a representative model of human hereditary CM (3) and is divided into hypertrophic CM (BIO 14.6 strain) and DCM-inbred sublines (TO-2 strain), both of which descended from the same ancestor (4). In 1997, two groups independently identified the responsible gene as δ-sarcoglycan (δ-SG) in the strain BIO 14.6 (5, 6). We also have determined the breakpoint of δ-SG gene at the intron 1 in both BIO 14.6 and TO-2 strains (6). In human cases with DCM, the similar δ-SG gene defect has been reported in four families, and one member required heart transplantation (7).

Gene therapy might be promising for the DCM treatment of hereditary origin. Both the limited area and transient duration after the *in vivo* gene transfer has disturbed a functional evaluation of the transfected hearts (8, 9). The *in vivo* transduction of normal δ-SG gene by recombinant adeno-associated virus (rAAV) has made it possible to induce both the transcript and transgene in appreciable amounts and ameliorate cardiac dysfunction up to 10 and 20 weeks (Ref. 10; Fig. 1). This vector has been proven nonpathogenic (11, 12) and has been tried for the therapy of human patients with cystic fibrosis (13) or hemophilia B (14). We hypothesized that supplementation of normal δ-SG before the onset of disease in the DCM animals by a mean of *in vivo* gene transfer may rescue the animals from the development and progression of the disease. Here, we report that an efficient rAAV-mediated δ-SG gene transfer into hearts of TO-2 hamsters resulted in a dramatic rescue of animals from developing the disease, with long-term improvements of morphological lesions, physiological indices at both the cellular and organ levels, and the prognosis.

Materials and Methods

Experimental Animals and Specific Antibodies. Normal ($n = 12$) and TO-2 strain hamsters ($n = 50$) with the early onset of DCM (4, 6, 9) were purchased from Bio Breeders (Fitchburg, MA). All of the animals were male and 5 weeks old at the gene transduction, housed under diurnal lighting, and allowed food and tap water *ad libitum*. TO-2 strain hamsters were divided into the following three subgroups: (*i*) totally untreated animals ($n = 6$); (*ii*) transfected by the reporter gene, Lac Z, alone ($n = 24$); and (*iii*) cotransfected by Lac Z and δ-SG gene with normal sequence ($n = 20$). Polyclonal and site-directed antibody to δ-SG was prepared in high titer with synthetic peptide (GPKAVEAY-GKKFEVKT) as a specific epitope of which amino acid sequence was deduced from the cloned cDNA (6). Monoclonal antibody to β-Gal was obtained from NovoCastra, Newcastle, U.K.

Abbreviations: DCM, dilated cardiomyopathy; CM, cardiomyopathy; δ-SG, δ-sarcoglycan; rAAV, recombinant adeno-associated virus; LVDs, left ventricular systolic dimension; LVDd, left ventricular diastolic dimension; LVP, left ventricular pressure.

**To whom reprint requests should be addressed at: Second Department of Internal Medicine, Tokyo University Hospital, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan. E-mail: toyooka-2im@h.u-tokyo.ac.jp.

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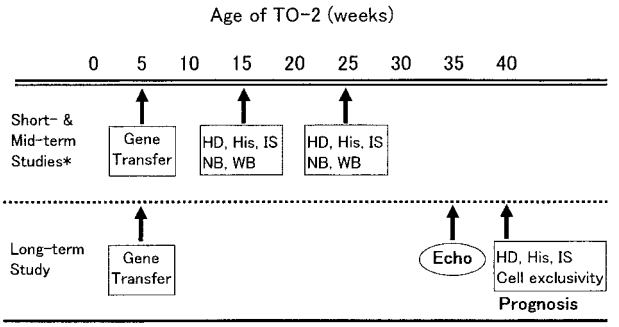

Fig. 1. Protocol for the assessment of gene therapy using rAAV vector. Unlike a previous report (10), the present study was focused mainly on the long-term efficacy and improvement of the animal's prognosis that might be the most important to verify a rationale to develop a novel therapy. HD, hemodynamics; His, histological examinations; IS, immunostaining; NB, Northern blot; and WB, Western blot.,

rAAV Vector Construction and Protocol for Gene Delivery *in Vivo*. pW1, an rAAV plasmid containing a reporter gene, Lac Z, flanked by the inverted terminal repeats of AAV genome, pHLP19, a helper plasmid with rep and cap genes, and pladeno-1, harboring adenovirus E2A, and E4 and VA genes were used for the rAAV-reporter gene production (15). pWSG harboring the δ-SG expression cassette driven by the same cytomegalovirus promoter was prepared for rAAV-δ-SG biosynthesis. These rAAVs were produced in 293 cells in culture and purified. The titer of each vector was determined, as described (10).

Under open chest surgery with constant volume ventilation (Model 683, Harvard Bioscience, South Natick, MA; ref. 10), rAAV-reporter gene (Lac Z) alone or the mixture of rAAV-Lac Z and rAAV-δ-SG gene was administered intramurally to the cardiac apex and two sites in the left ventricular free wall (10 μl each: 8.4×10^{10} and 6×10^{10} copies for Lac Z and δ-SG gene in total, respectively). Then, animals were cared for 35 weeks after the transduction in the Infection Research Laboratory under Guidance for Animal Facility: Maintenance and Housing Conditions.

The long-term protocol to follow both physiological and pathological effects *in vivo* and postmortem after the gene transfer is summarized in Fig. 1. Because the present study was addressed mainly to the examination of sarcolemmal integrity and myocardial contractility (both *in vivo*), we analyzed leaky cardiomyocytes secondary to the degradation of transmembrane dystrophin-related proteins (DRP, ref. 9) and wall motion by high-resolution echocardiography, in addition to the hemodynamic studies and prognosis analysis.

Evaluation of Pathological Alterations. The transgene expression was not restricted to the injected sites; a distant region also was transfected (10). Accordingly, wall thickness was measured at the four portions; interventricular septum, left ventricular free wall opposite to the septum, anterior and posterior free walls of the left ventricular cavity in the cross section after staining by hematoxylin and eosin and then summed. For the semiquantification of tissue calcification, we scored as follows, depending on the degree: 0 points, without calcified region; 1 point, with one calcified spot; 2 points, with two regions; and 3 points, with more than three regions and/or huge, elongated, or fused region. The score was summed in four cross sections between the apex and mitral annulus without notifying the observer as to which site and which vector was transfected.

Morphological Analyses and Evaluation of Sarcolemmal Permeability. For the immunostaining of the reporter transgene (β-Gal) and δ-SG, we used adjacent serial sections with a specific antibody to each protein because of the increased sensitivity and specificity (16). To evaluate the rAAV-δ-SG treatment, sarcolemmal integrity was analyzed by i.v. injection of 1% (vol/vol) Evans blue dye, which was kept circulating for 3 h before killing the animals at 35 weeks after the gene transduction. The dye was excluded cardiomyocytes that preserves normal sarcolemmal permeability but is taken up by the cardiomyopathic cells with leaky cell membrane (17, 18). The immunostaining of δ-SG by FITC-labeled second antibody to rabbit IgG (NovoCastra, Newcastle, U.K.) and Evans blue were visualized under double fluorescence microscopy with a Nikon F800 equipped with a green activation filter (546 nm with 12 nm band-pass) for the excitation and barrier filter (590 nm) for the emission. The site where β-Gal, a transgene of reporter, was identified by immunostaining (Fig. 2), recorded by digital camera at 200× magnification, and used for the analysis of other transgene expression or Evans blue uptake. The areas of positive cytoplasm for Evans blue or δ-SG were measured by planimetry (10) for the assay of membrane permeability and efficacy of the transduction, respectively.

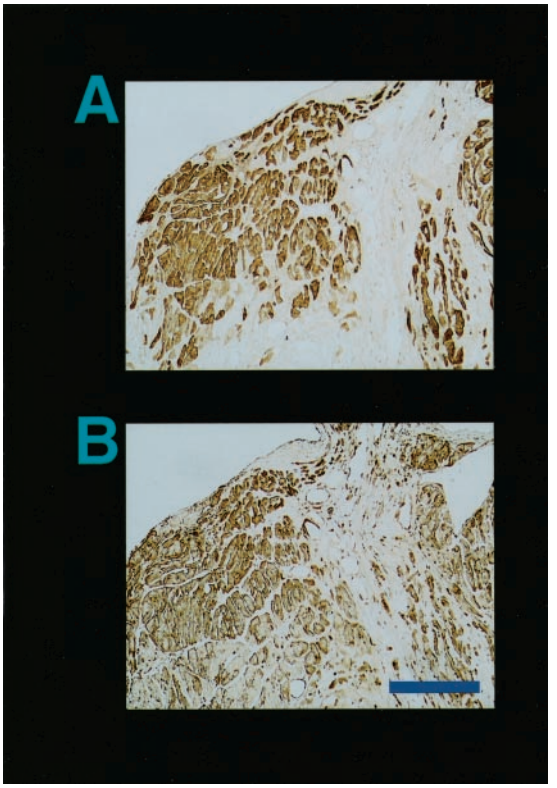

Fig. 2. Efficient expression of reporter β-Gal (*A*) or δ-SG (*B*) in serial sections after the rAAV-mediated gene transduction to the TO-2 hamster hearts that had lacked δ-SG gene (6, 9). Original magnification, ×4. (Bar = 100 μm.)

***In Vivo* Assessment of Cardiac Contractility and Hemodynamics.** Mechanical performances were determined by several observers who were not aware of the administered vectors and the injection site. Before both echocardiographic and hemodynamic measurements, the concentration of the gas anesthetic isoflurane was reduced to 1% and maintained for 20 min to stabilize the hemodynamics (19). Both the left ventricular systolic dimension (LVDs) and left ventricular diastolic dimension (LVDd) were determined by high-frequency (13 MHz) echocardiography (EUB 6000, Hitachi, Tokyo) under visualizing short axis of the left ventricle at 30 weeks after the gene transfer. Because these dimensions were still too inaccurate to determine the actual diameter of the left ventricular cavity even using the two-

dimensional view, we measured the percent fractional shortening (FS) and calculated the ejection fraction (EF) by Teicholz's formula.

Thirty-five weeks after the transduction, hamsters were anesthetized again, as described above (19). A catheter-tip transducer (SPR-671, Millar Instruments, Houston, TX) was inserted into the left ventricle through the right carotid artery to measure the left ventricular pressure (LVP), the left ventricular end-diastolic pressure (LVEDP) and the derivative of LVP (dP/dt). For the determination of the central venous pressure (CVP), a heparin-saline-filled polyethylene catheter connected to a pressure transducer was introduced into the superior vena cava through the right jugular vein. The hemodynamic parameters were recorded after A/D transduction on a Power Lab system (A. D. Instruments, Castle Hill, NSW, Australia) at a 1-kHz sampling rate (10, 19).

Evaluation of the Prognosis After Gene Therapy. The final effect of the gene therapy was evaluated on the life-saving action in the TO-2 animals ($n = 20$) with cotransduction of δ-SG plus reporter genes, comparing them with another animal group transfected by reporter gene alone ($n = 24$). All animals were operated on at age 5 weeks, randomly allocated for each treatment, and housed for 40 weeks, which exceeded the mean lifespan of TO-2 strain hamsters (4). The survival rate was evaluated by Kaplan-Meier analysis.

Statistical Analysis. Preliminary study has revealed that the morphological and physiologic effects of each gene were independent and did not show any additive or synergistic action (10). All values were expressed by the means ± SE and analyzed by paired Student's *t* test and ANOVA. A *P* value of less than 0.05 was considered significant.

Results

Wall Thickness, Calcified Lesion, and Transgene Expression. Wall thickness measured at four points of the left ventricular wall of TO-2 heart and summed revealed that the *in vivo* cotransduction of reporter and δ-SG genes ($n = 16$) increased the thickness from 4.4 ± 0.2 to 5.1 ± 0.3 mm ($P < 0.05$), compared with the heart transfected by the reporter gene alone ($n = 10$). These results confirmed previous data that cell width in the myocardium transfected by δ-SG gene was normalized, in part, by the present therapy (10).

The calcified lesion was homogeneously distributed throughout the ventricular wall at random. We semiquantitatively scored the lesion in each animal and summed. TO-2 hearts transfected by the Lac Z gene alone ($n = 10$) showed a 1.46-fold larger score than those treated by both Lac Z and δ-SG genes ($n = 16$, 9.9 ± 0.9 vs. 6.8 ± 1.0, $P < 0.05$). These results denote the physiological effect of δ-SG on the progression of calcification.

To identify the transgene of the reporter, immunostaining of β-Gal protein by specific antibody was more sensitive than the classic, histochemical reaction and did not disturb the immunodetection of SG protein secondary to blue color presentation of the reaction product after histochemistry (16). The β-Gal expression was observed at 35 weeks after the transduction (i.e., at age 40 weeks) in cardiac muscle of TO-2 hamsters. Combined with a previous report that the transgene also was documented at 10 and 20 weeks after the gene transfer (10), the present results indicate that the transgene expression with rAAV continued throughout the animal's life (4).

The transgenes of both reporter and δ-SG were clearly detected in the same part of serial sections (Fig. 2). The β-Gal was shown exclusively in the cytoplasm of cardiomyocytes (Fig. 2*A*), indicating that β-Gal did not require translocation after the biosynthesis. It should be noted that most myocardial cells presenting β-Gal matched those cells exhibiting δ-SG (Fig. 2*B*). In contrast, the expression of δ-SG was not restricted to sarcolemma, and cytoplasm in some cardiomyocytes also was stained, similar to skeletal muscle (17, 18). Furthermore, it might be quite meaningful to detect the transgene expression in ventricular working muscle cells, His-Purkinje bundle, and coronary smooth-muscle cells (Fig. 2*B*); ventricular myocytes look to be more preferentially transfected than the conduction system.

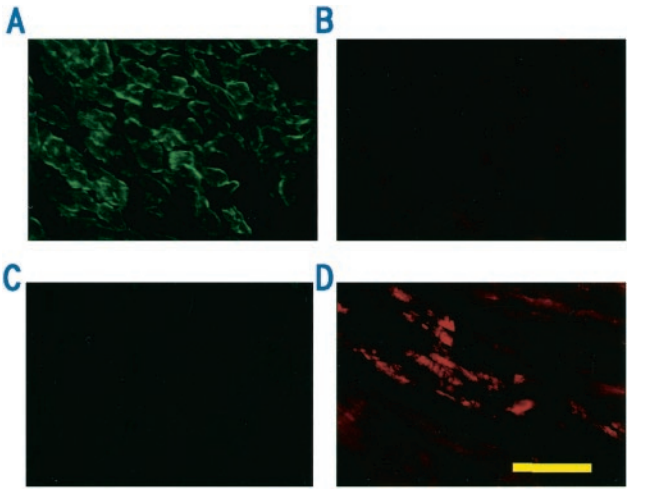

Fig. 3. Mutual cell-exclusivity of δ-SG expression and Evans blue uptake. After the cotransduction of reporter gene plus normal δ-SG gene (*A* and *B*) or transduction of the reporter gene alone (*C* and *D*) for 35 weeks to TO-2 hamster hearts, the transgene of δ-SG and the cells with leaky sarcolemma were detected by double fluorescence with FITC-labeled antibody (*A* and *C*) and Evans blue (*B* and *D*), respectively. Original magnification, ×200. (Bar = 100 μm.)

Amelioration of Sarcolemmal Permeability After the Gene Therapy. To examine the nonspecific effect of gene transfer on myocardium, the age-matched TO-2 hamsters transfected by reporter gene alone were used as controls of TO-2 hamsters cotransfected by reporter gene plus δ-SG gene. At 35 weeks after the transduction, cardiac tissue was collected and examined for δ-SG expression and Evans blue dye uptake by double fluorescence visualization. As expected, cardiac muscle from TO-2 hamsters treated by the reporter plus δ-SG genes revealed the site-specific expression of δ-SG transgene, where the reporter was detected in a serial section. The δ-SG (green fluorescence, Fig. 3*A*) was stained across cardiomyocytes that did not take up Evans blue (red fluorescence, Fig. 3*B*), distinctly showing the mutual cell-exclusivity of δ-SG expression and the dye uptake. In contrast, the TO-2 heart transfected by the reporter gene alone revealed the absence of δ-SG (Fig. 3*C*) and the extensive dye uptake (Fig. 3*D*). The control F1B heart demonstrated no uptake of Evans blue but clear immunostaining of δ-SG (data not shown).

It should be intensified that the rAAV-δ-SG treatment of TO-2 muscle achieved the protection of cardiomyocytes from sarcolemmal leakage as late as 40 weeks old, when some TO-2 hamsters died of heart failure (4). In the distant myocardium where β-Gal or δ-SG was not detected, Evans blue was strongly stained (data not shown). These results unequivocally demonstrate the physiological significance of δ-SG to protect cardiomyocytes from the sarcolemmal deterioration, similar to gene therapy for 10- and 20-week-old hamsters (10).

Quantitative assessment of the relationship between δ-SG staining and Evans blue uptake required counting every positive cell in composite photographs. These data demonstrate that Evans blue-positivity conferred a 5.88-fold protective effect of the gene therapy (20 specimens of 5 animals in each group), because the ratio decreased from 4.48 ± 0.27 to 0.83 ± 0.13 ($P < 0.01$).

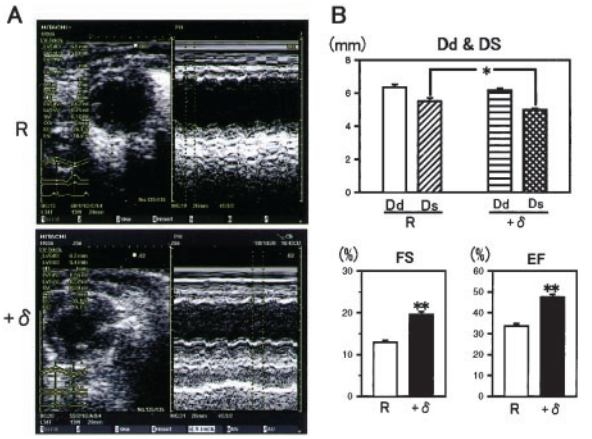

Fig. 4. (*A*) Short-axis view of the left ventricular cavity by high-frequency (13 MHz) echocardiography and M mode recording in TO-2 hamsters. The LVDs and LVDd were determined at 30 weeks after the transduction of reporter gene (R) alone or the cotransduction of R plus normal δ-SG gene (+δ) to TO-2 heart *in vivo*. (*B*) Summary of the LVDd (Dd), LVDs (Ds), fractional shortening (FS), and ejection fraction (EF) in a group transfected by the reporter gene alone (white bar, $n = 10$) or another group cotransfected by the reporter gene plus normal δ-SG genes (black bar, $n = 10$).

Improvement of Myocardial Contractility and Hemodynamic Indices. High-frequency (13 MHz) echocardiography and its digital recording have made it possible to exactly evaluate the mechanical performances *in vivo* (Fig. 4). Operation procedure at 30 weeks before (Fig. 1) did not disturb visualization of the ventricular cavity (Fig. 4*A*). The *in vivo* cotransduction of reporter gene plus δ-SG gene ($n = 10$) to the TO-2 strain reduced the enlarged LVDs from 5.52 ± 0.18 to 4.98 ± 0.09 mm ($P < 0.05$), compared with the animals ($n = 10$) transfected by the reporter gene alone (Fig. 4*B*). In contrast, the LVDd did not change even after the gene therapy in both groups (6.33 ± 0.18, vs. 6.18 ± 0.11 mm). These results were reflected in the improvement of both percent fractional shortening (FS, 12.9 ± 0.5 vs. 19.5 ± 0.7, $P < 0.01$) and the left ventricular ejection fraction after the transfer of δ-SG gene (LVEF, 33.7 ± 1.2 vs. 47.4 ± 1.3; $P < 0.01$; Fig. 4*B*).

Open chest surgery for the gene transduction did not hamper the exact measurement of the hemodynamics at 35 weeks after the gene transduction (Fig. 5). Cotransduction of both the reporter and δ-SG genes ($n = 18$) distinctly improved the dP/dt_{min} (−3,269 ± 147 vs. −3,955 ± 183 mmHg/sec, $P < 0.05$), the LVEDP (20.8 ± 1.8 vs. 12.8 ± 1.9 mmHg, $P < 0.05$) and the CVP (3.72 ± 0.88 to 1.66 ± 0.43 mmHg, $P < 0.05$), compared with transduction of the reporter gene alone ($n = 12$). Gene therapy did not modify the LVP (86.8 ± 2.6 vs. 91.7 ± 2.4 mmHg), the dP/dt_{max} (4,629 ± 186 vs. 5,000 ± 162 mmHg/sec), or the HR (358 ± 8 vs. 350 ± 8 beats per min).

Prolongation of Life Expectancy After the Gene Therapy. At 5 weeks old, one group of TO-2 hamsters ($n = 20$) was administered reporter gene alone *in vivo* and another group ($n = 24$) was cotransfected by the reporter and δ-SG genes. All animals survived the open chest surgery, indicating that the operational procedure did not cause serious effect on their mortality or morbidity.

The group treated by reporter gene alone started to die at 34 days old and the number of deceased animals gradually increased from 171 to 228 days after the gene transfer (Fig. 6). The death timing supports the previous data in the same strain without gene manipulation (4). In contrast, all animals in another group cotransfected by the reporter plus δ-SG genes survived and remained active. We conclude that the present gene

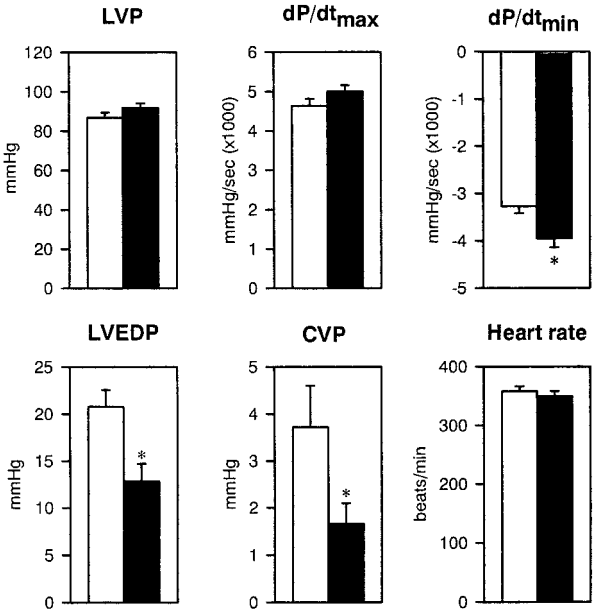

Fig. 5. Comparison of hemodynamic indices at 35 weeks after the gene transduction *in vivo*. LVP, CVP, and the heart rate were recorded under stable anesthesia (10). The LVP was digitized to calculate the maximum derivative (dP/dt_{max}) and the minimum derivative (dP/dt_{min}). White and black bars denote TO-2 hamsters transfected by the reporter gene alone (R) and cotransfected by the reporter plus δ-SG genes (+δ) for 30 weeks, respectively. *, statistical significance between the two groups ($P < 0.05$).

therapy prolonged the survival rate ($P < 0.01$), when the gene responsible for DCM was supplemented *in vivo*.

Discussion

The present study demonstrated that, upon rAAV-mediated efficient δ-SG gene transfer into the heart, TO-2 hamsters can be rescued from developing DCM and survive for at least 40 weeks, which exceeded the lifespan of TO-2 heart without responsible gene transduction, thus drastically improving the disease prognosis.

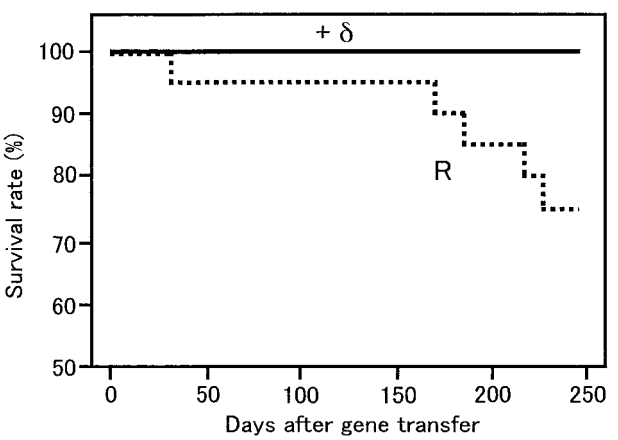

Fig. 6. Kaplan–Meier analysis of TO-2 strain hamsters' survival rate after the transduction of the reporter gene alone (R, $n = 20$) or cotransduction of the reporter plus normal δ-SG genes (+δ, $n = 24$) *in vivo*. Note that the animal group treated by R alone completely matched the prognosis of the animals without the gene manipulation (4), whereas another group treated by +δ survived the operational procedures for the *in vivo* gene transfer and remained active.

DRP links intracellular contractile machinery with extracellular matrix (20, 21). Gene defect and the corresponding protein disruption in the complex commonly induce muscle degeneration with or without cardiac symptoms. In fact, gene mutation of cardiac F-actin, dystrophin, each SG and laminin-α2 in addition to lamin A/C causes DCM in human cases as the chief symptom or a partial sign (20–23). Furthermore, acquired case in rat with myocarditis after enterovirus infection shows DCM-like symptoms secondary to the selective cleavage of dystrophin by protease 2A translated from the virus genome (24). We also have demonstrated that over-administration of isoproterenol to rats caused the selective cleavage of dystrophin, its translocation from sarcolemma to myoplasm, cardiomyocyte apoptosis, and finally acute or subacute heart failure (24). Accordingly, the interruption between intracellular F-actin and extracellular laminin-α2 would fail to preserve the integrity of sarcolemma, resulting in a DCM-like syndrome, irrespective of the hereditary or acquired origin.

Wall thickness was normalized after the gene therapy, and this fact might be a result of improved cell diameter (10) and/or the reduction of calcified lesions in the transfected site. Furthermore, the reduced calcification suggests that the pathogenesis is intrinsic to the deletion of the δ-SG gene *per se* and is not a result of additional deletions other than the δ-SG gene. Although the rAAV type 2 vector is potent for the widespread and long-lasting gene transduction *in vivo*, efficacy was still lower than the case after open chest surgery and confirms our previous results of the gene therapy for short or mid-term period (10). Other methods of gene transfer *in vivo* using intracoronary administration and/or electroporation did not exceed the present level of intramural administration (T.K., M.N., J.N., C.H., and T.T.-o., unpublished data). More efficient gene transfer through coronary circulation, as succeeded in heterotopically transplanted heart after the isolated perfusion (17), would completely restore these pathological alterations before the progression to irreversible degeneration. The apparent discrepancy between the reduced LVDs by echocardiography, and no effect on the LVP or dP/dt_{max} by hemodynamic study, might be explained by the insufficient gene delivery to cover whole heart. Escape of both atria from the gene transduction and their reduced contractility may decrease the preload of both ventricles and would not be reflected to the increment of LVP or dP/dt_{max}. We have reported that transduction of 30–40% cells and 20% protein amount were sufficient for improving hemodynamics, as was verified by immunohistology and Western blotting, respectively, for the level of δ-SG to rescue the animals (10). These results suggest the redundant expression of δ-SG in normal animals.

Based on the results of cardiomyocyte degradation in transgenic mice and their improvement by the pharmaceutical agent with coronary dilating action, K. P. Campbell and coworkers (25, 26) have presented the scheme that loss of δ- or β-SG but not α-SG would cause DCM secondary to the coronary spasm. These results are quite informative for the development of DCM, but may require further studies on the exact pathogenesis because of the following three reasons. (*i*) The same δ-SG gene deletion causes different phenotypes in hamsters; hypertrophic CM at the initial onset followed by DCM in BIO 14.6 strain (4, 6) and DCM as the first symptom in TO-2 strain (4, 6, 9, 10). It might be attractive to assume that an additional mutation in the TO-2 strain causes DCM, overcoming the compensatory hypertrophy that occurs in BIO 14.6 strain (*ii*). After the gene therapy, δ-SG was expressed in not only cardiomyocytes but also in smooth-muscle cells in the coronary artery (Fig. 2*B*). We found no significant difference in the coronary artery caliber between the δ-SG transfected and nontransfected arteries (data not shown). Measurements of local coronary flow with the vasospasm-inducing agents (e.g., acetylcholine or ergonovine) are mandatory to determine the contribution of coronary spasm (27). (*iii*) In addition, another kind of Ca^{2+} entry blocker, nifedipine, with more potent antispastic and more specific coronary dilating action (28) did not improve but rather aggravated the prognosis of this hamster (29).

Cardiac muscle is destined to repeat contraction and relaxation throughout the lifespan, and sarcolemma is supposed to be much more resistant to the expansion-shrinking cycle in the heart than in the skeletal muscle. The lack of a component in DRP is not lethal (20, 21), but its full set may be needed to keep both the membrane integrity and normal lifespan. Actually, present results demonstrate that the exogenously applied Evans blue dye permeated plasma membrane of cardiomyocytes that did not possess δ-SG (Fig. 3 *C* and *D*) when TO-2 hamsters started to die of heart failure (Fig. 6). In contrast, myocardial cells expressing δ-SG after the gene transduction did not take up the dye at the same age (Fig. 3 *A* and *B*). Continuous but gradual leakage of sarcolemma to Ca^{2+} in addition to the Ca^{2+} entry during slow inward current would elevate the intracellular Ca^{2+} level (30) because of the depletion of high-energy phosphates (31) and would activate the endogenous protease, calpain (Ca^{2+}-activated neutral protease, CANP; ref. 32). After that, α−, β− and γ-SG might be hydrolyzed at the posttranslation level (6, 9), because mRNAs for α−, β− and γ-SG were completely preserved (6), and because most of the cytoskeletal proteins, including SGs, are degraded (32) by isolated calpain (M. Koshimizu, H. Yoshida, and S. Takeo, personal communication). On the precise mechanism of DCM progression or coronary spasm, more detailed study would be required on sarcolemmal fluidity (31), humoral factors, or cytokines, including tumor necrosis factor α and/or endothelin (33, 34).

Recent data for some mice with lysosomal storage disease showed hepatocellular carcinoma or angiosarcoma were detected after intrauterine and i.v. administration of rAAV (35). In addition, we found a trace staining of β-Gal protein in liver, spleen, and kidney, suggesting extracardiac transduction of the reporter gene (S.N., T.K., and T.T.-o., unpublished data). Although species other than the transgenic animals described above did not demonstrate any tumor formation (35), and because the heart is one of the privileged organs in relation to tumorigenesis, further study is necessary to test the safety in primates. Present results distinctly indicate that somatic gene therapy with potent vector is promising for human DCM treatment, if the rAAV vector is available for clinical use.

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