Vasorin, a transforming growth factor β-binding protein expressed in vascular smooth muscle cells, modulates the arterial response to injury *in vivo*

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Growth factors, cell-surface receptors, adhesion molecules, and extracellular matrix proteins play critical roles in vascular pathophysiology by affecting growth, migration, differentiation, and survival of vascular cells. In a search for secreted and cell-surface molecules expressed in the cardiovascular system, by using a retrovirus-mediated signal sequence trap method, we isolated a cell-surface protein named vasorin. Vasorin is a typical type I membrane protein, containing tandem arrays of a characteristic leucine-rich repeat motif, an epidermal growth factor-like motif, and a fibronectin type III-like motif at the extracellular domain. Expression analyses demonstrated that vasorin is predominantly expressed in vascular smooth muscle cells, and that its expression is developmentally regulated. To clarify biological functions of vasorin, we searched for its binding partners and found that vasorin directly binds to transforming growth factor (TGF)-β and attenuates TGF-β signaling *in vitro*. Vasorin expression was down-regulated during vessel repair after arterial injury, and reversal of vasorin down-regulation, by using adenovirus-mediated *in vivo* gene transfer, significantly diminished injury-induced vascular lesion formation, at least in part, by inhibiting TGF-β signaling *in vivo*. These results suggest that down-regulation of vasorin expression contributes to neointimal formation after vascular injury and that vasorin modulates cellular responses to pathological stimuli in the vessel wall. Thus, vasorin is a potential therapeutic target for vascular fibroproliferative disorders.

Vascular smooth muscle cells (VSMCs), the major cell type in the vessel wall, show a spectrum of phenotypes, depending on environmental cues. Various injurious stimuli provoke the proliferation of differentiated medial VSMCs, which migrate to the intima and produce extracellular matrix proteins, resulting in the narrowing of the vascular lumen. These processes, called VSMC phenotypic modulation, play a key role in development of atherosclerotic diseases, such as postangioplasty restenosis, vein graft disease, and transplant vasculopathy. Whereas tremendous progress has been made in identifying growth factors and transcription factors that regulate the vascular response to injury, much information is lacking regarding cell-surface molecules that are involved in the pathogenesis of vascular fibroproliferative disorders. The signal sequence trap (SST) is a strategy to identify cDNAs containing signal sequence that encode secreted and type I membrane proteins (1, 2). We recently developed a refined SST system based on retrovirus-mediated expression screening (SST-REX) (3). In a search for secreted and cell-surface molecules expressed in the cardiovascular system, by using SST-REX, we identified a TGF-β binding protein, vasorin. Vasorin is predominantly expressed in VSMCs and modulates the vascular response to injury, at least in part, by attenuating TGF-β signaling *in vivo*. Here, we describe the molecular and functional characteristics of vasorin.

Methods

Cells and Reagents. A murine pro-B cell line Ba/F3 was maintained in RPMI medium 1640 containing 10% FCS and 2 ng/ml murine IL-3 (R & D Systems). Chinese hamster ovary (CHO) cells were grown in DMEM supplemented with 5% FCS and 1% nonessential amino acids (Invitrogen). Stable transfectants were established by the retrovirus expression system, by using a bicistronic retroviral vector pMX-IRES-EGFP as reported (4). Rat aortic VSMCs, prepared from 8-week-old Wistar rats by using the explant method (5), were grown in DMEM supplemented with 10% FCS. Primary antibodies used in this study were anti-Flag monoclonal antibody (M2, Sigma), anti-Smad2/3 monoclonal antibody (BD Transduction Laboratories), anti-phospho-Smad2 polyclonal antibody (Upstate, Charlottesville, VA), and anti-rat CD45 monoclonal antibody (BD Pharmingen).

Screening of a Human Heart cDNA Library by SST-REX and Cloning of the Full-Length cDNA Encoding *Vasorin*. A human heart cDNA library was screened by SST-REX as described (3). Briefly, cDNA was synthesized from poly(A)$^+$ RNA of human hearts (Clontech), by using the SuperScript Choice system (Invitrogen). The synthesized cDNA was separated based on size, and fractions >600 bp were inserted into *Bst*XI sites of the pMX-SST vector, by using *Bst*XI adapters (Invitrogen). Ba/F3 cells were infected with high-titer retroviruses expressing the human heart cDNA library, and the integrated cDNA fragments were isolated from factor-independent Ba/F3 clones by genomic PCR. All cDNA fragments were sequenced and analyzed. Subsequently, a human heart cDNA library in the pME18S vector was screened by using the ^{32}P-labeled cDNA fragment of a clone so as to isolate the entire coding region.

RNA, Protein, and Histological Analyses. Northern blot, *in situ* hybridization, semiquantitative RT-PCR, immunoprecipitation, Western blot, and histological analyses were done as described

Abbreviations: VSMC, vascular smooth muscle cell; TGF, transforming growth factor; EGF, epidermal growth factor; SST, signal sequence trap; SST-REX, retrovirus-mediated SST; CHO, Chinese hamster ovary; RA, retinoic acid; En, embryonic day *n*; LRR, leucine-rich repeat; Ad, adenovirus; Ad-vasorin, Ads expressing vasorin-Flag; PDGF, platelet-derived growth factor.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY166584).

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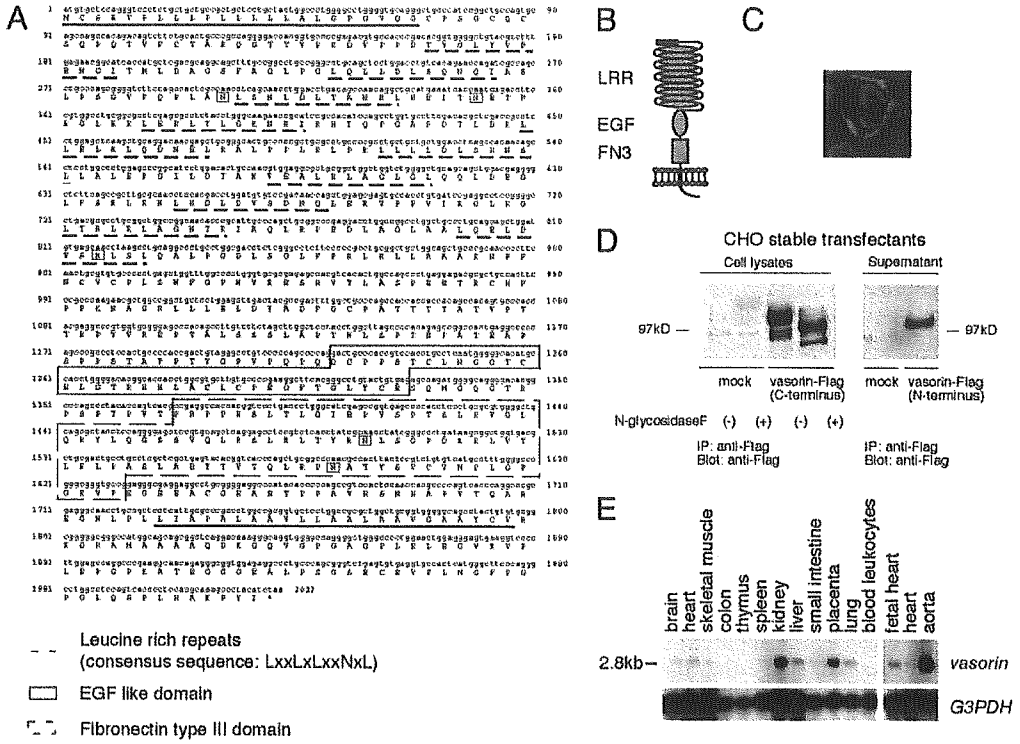

Fig. 1. Vasorin, an identified cell-surface protein. (*A*) Deduced amino acid sequence of human vasorin. The putative signal peptide (underlined), the LRRs (dotted underlines), the EGF motif (boxed), the fibronectin type III motif (dotted boxes), the transmembrane sequence (underlined), and five putative N-glycosylation sites (boxed) are indicated. (*B*) Structural model of vasorin. (*C*) Immunofluorescence analysis of subcellular localization of vasorin. Vasorin was expressed on the cell surface. (*D*) Cell lysates and supernatants of CHO cells stably expressing vasorin-Flag were subjected to immunoprecipitation and Western blot analysis by using an anti-Flag antibody. An ≈110-kDa protein for membrane-bound vasorin with C-terminal tag and a ≈100-kDa protein for soluble vasorin with N-terminal tag were detected under reducing conditions. N-glycosidase F treatment revealed that vasorin is N-glycosylated. (*E*) Northern blot analysis of adult human tissues. A single intense 2.8-kb band was detected and the strongest expression was observed in the aorta.

in *Supporting Text*, which is published as supporting information on the PNAS web site.

Production of the Recombinant Vasorin-Fc Chimera Protein. The bicistronic retroviral vector containing the *vasorin*-Fc chimera, pMX-*vasorin*-Fc chimera-IRES-EGFP, was constructed by inserting the cDNA for the whole-extracellular domain of human *vasorin* and the cDNA for the human Ig Fc region. CHO cells stably expressing the *vasorin*-Fc chimera were expanded in the medium supplemented with 2.5% Ultralow IgG FCS (Invitrogen) and 1% nonessential amino acids (Invitrogen). Secreted recombinant vasorin-Fc chimera protein was purified from the media of infected CHO cells by using Hitrap protein A columns (Amersham Biosciences).

Surface Plasmon Resonance Analysis. The BIAcoreTM2000 system (BIAcore, Uppsala) was used to characterize the interaction and to determine binding characteristics between the recombinant vasorin-Fc and TGF-β1, according to the manufacturer's instructions. To immobilize TGF-β1 on CM5 sensor chips, recombinant human TGF-β1 (R & D Systems) solution in 10 mM acetic acid (pH 4.0) was injected until the desired amount of coupled TGF-β1 was achieved, by using the standard amine-coupling procedure. All experiments were performed at room temperature by using the KINJECT command at a flow rate of 20 μl/min. Responses obtained on control chips were subtracted from those obtained on chips coupled with TGF-β1. Sensorgrams were analyzed by using BIAEVALUATION software (version 3.0).

Transient Transfection and Reporter Assay. CHO stable transfectants were transiently transfected with TGF-β1-responsive luciferase reporter plasmid (p3TP-lux) together with a β-galactosidase reporter plasmid driven by Rous sarcoma virus-LTR as an internal control, by using FuGENE6 (Roche Diagnostics, Roskilde, Denmark). After 24 h, the cells were stimulated for 24 h by adding TGF-β1 (R & D Systems) and were then assayed for luciferase and β-galactosidase activities. Experiments were performed several times and representative data are shown.

Rat Arterial Balloon-Injury Model. Adult rats (weighing 400–450 g) were anesthetized with chloral hydrate (370 mg/kg i.p.). Balloon denudation of the left common carotid artery was done by using a 2F Fogarty catheter (Baxter Edwards Healthcare, Irvine, CA), as described (6). The right common carotid artery served as a control. Rats were killed 3 or 14 days after injury, and the carotid arteries were perfused with 4% paraformaldehyde/PBS. Each injured left carotid artery was excised from the proximal edge of the omohyoid muscle to the carotid bifurcation. The middle third of the segment was isolated for subsequent analyses.

Generation of Recombinant Adenovirus (Ad) Expressing Vasorin. Replication-incompetent Ads expressing vasorin-Flag (Ad-vasorin) were prepared by using the Adeno-X system (Clontech) according to the manufacturer's instructions. Viral titer was measured by end-point dilution assay by using 293 cells.

Statistical Analysis. Quantitative values are expressed as the mean ± SD. Comparisons of means were made by using Student's *t* test for unpaired values; when >2 means were compared, an ANOVA with repeated measurements was used. If a significant *F* value was found, the Scheffé post hoc test for multiple comparisons was used to identify any differences among groups.

Results

Cloning of *Vasorin*. We screened a human heart cDNA library, by using SST-REX (ref. 3 and Table 1, which is published as supporting information on the PNAS web site), and identified a type I membrane protein of 673 aa (Fig. 1*A*). The extracellular region was composed of a putative hydrophobic signal sequence, 10 tandem arrays of a leucine-rich repeat (LRR), an epidermal growth factor (EGF)-like domain, and a fibronectin type III-like domain, and the short intracellular region contained no obvious signaling domain (Fig. 1 *A* and *B*). We termed this protein vasorin, based on the expression pattern described below. By using human vasorin as a probe, we identified homologous mouse and rat protein sequences in the EMBL/GenBank/DDBJ database (accession nos. AK012169 and XM 220168, respectively).

To observe the subcellular localization of vasorin, we stained CHO cells expressing human vasorin-Flag with an anti-Flag antibody (Fig. 1*C*), and confirmed this molecule to be expressed on the cell-surface membrane. Western blotting of cell lysates from CHO stable transfectants revealed a ≈110-kDa protein (Fig. 1*D*), which was larger than the predicted molecular mass. To determine whether this increase in molecular mass was due in part to N-linked glycosylation, immunoprecipitates of vasorin were treated with N-glycosydase F. An apparent shift in molecular mass of vasorin was observed, suggesting that vasorin is a cell-surface glycoprotein (Fig. 1*D*). Next, we examined the supernatant from CHO cells expressing human vasorin-Flag, and soluble vasorin was also detected (Fig. 1*D*).

***Vasorin* Is Predominantly Expressed in VSMCs.** Tissue distribution of *vasorin* was examined by using Northern blot analysis of adult human tissues. The highest expression was detected in the aorta, and moderate expression was detected in the kidney and placenta (Fig. 1*E*). We also performed Northern analysis of various human cell lines. Interestingly, *vasorin* was not expressed abundantly in any cell lines we examined (Fig. 6, which is published as supporting information on the PNAS web site).

To determine the expression pattern of *vasorin* within the aorta, we performed *in situ* hybridization analyses. By using the antisense probe, strong expression of *vasorin* was detected in the tunica media of the proximal ascending aorta (Fig. 2*A*), the descending thoracic aorta (Fig. 2*B*), the abdominal aorta (Fig. 2*C*), and the coronary arteries (Fig. 2 *E* and *F*), suggesting that *vasorin* is expressed in VSMCs of different origins. We also performed *in situ* hybridization analyses in the kidney. *Vasorin* expression was detected in interstitial cells (Fig. 2 *G* and *H*).

Developmental Regulation of *Vasorin* Expression. The developmental regulation of *vasorin* was investigated by using Northern blot and *in situ* hybridization analyses of staged mouse embryos. As shown in Fig. 3*A*, *vasorin* mRNA expression was detected in embryonic day (E)10.5 embryos, with increasing levels of expression observed at subsequent stages (E13.5 and E17.5). With *in situ* hybridization analyses, we examined the expression pattern of *vasorin* during aortic development (Fig. 3*B*). The expression of *vasorin* increased gradually in parallel with the differentiation of VSMCs in aortas at different stages of development (E11.5, E13.5, and E17.5).

When VSMCs are established in culture, a rapid transition from a contractile differentiated phenotype to a synthetic dedifferentiated phenotype occurs (5). To investigate the influence of this phenotypic modulation on the expression of *vasorin*, semiquantitative RT-PCR was performed to compare the expression of *vasorin* in the adult rat aorta with that in cultured rat aortic VSMCs. The expression of *vasorin* was significantly downregulated in the cultured VSMCs (Fig. 3*C*).

P19 embryonal carcinoma cells differentiate into SMCs when given retinoic acid (RA) treatment. Recently, this *in vitro* differentiation system was improved by generating stable cell lines of P19 carrying a smooth muscle α-actin promoter/puromycin resistance gene cassette to enrich SMC lineage cells, by using RA plus puromycin selection. One such stable line, designated as A404, shows a high propensity for SMC differentiation even before puromycin selection (7). As expected, *vasorin* gene expression was induced by RA-treatment in A404 cells (Fig. 3*D*).

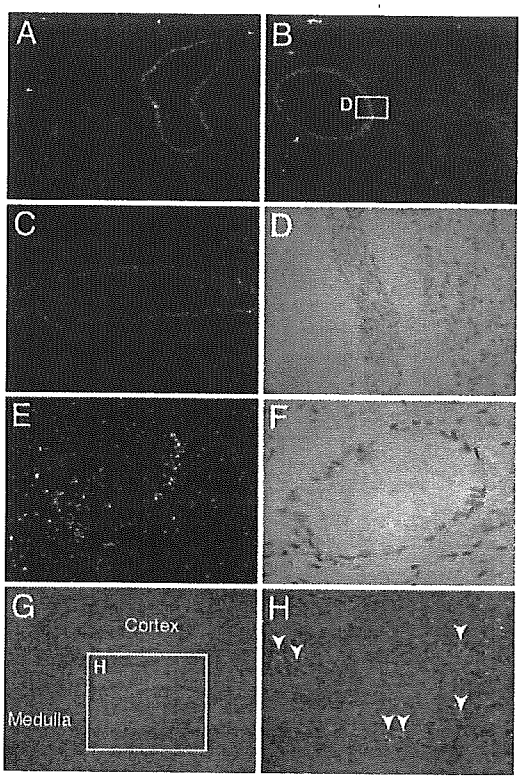

Fig. 2. *In situ* hybridization analysis of *vasorin*. Sections of adult mouse aorta at different levels (*A–D*), the coronary artery (*E* and *F*), and the kidney (*G* and *H*) are shown. *Vasorin* is expressed in VSMCs of different origins. White spots represent hybridization signals. (*A*) The proximal ascending aorta. (*B*) The descending thoracic aorta. (*C*) The abdominal aorta. (*D*) Partial magnification of bright-field image of *B*. Black spots within the elastic fibers represent hybridization signals. (*E*) The coronary artery. (*F*) A bright-field image of the coronary artery. Black spots represent hybridization signals. (*G*) The kidney. (*H*) Partial magnification of *G*. *Vasorin* is expressed in interstitial cells.

Vasorin Directly Binds to TGF-β and Modulates TGF-β Signaling *in Vitro*. An LRR, an EGF-like domain, and a fibronectin type III-like domain are characteristic motifs involved in protein–protein interactions (8). To clarify biological functions of vasorin, we generated recombinant vasorin-Fc fusion protein (Fig. 4*A*), and searched for binding partners of vasorin by using vasorin-Fc as a probe. When comparing the extracellular domain of vasorin with the EMBL/GenBank/DDBJ database, several other LRR protein family members, including decorin, were found to share a significant homology with vasorin. Decorin is a small leucine-rich proteoglycan that interacts directly with TGF-β (9, 10). Considering that vasorin has the same number of LRRs as decorin, and that TGF-β plays an important role in vascular pathophysiology, we tested whether TGF-β binds directly to vasorin. By using a surface plasmon resonance biosensor, we found that the extracellular domain of vasorin directly binds to TGF-β1 in a specific and significant manner (Fig. 4 *B* and *C*). The equilibrium dissociation constant (K_d) was calcu-

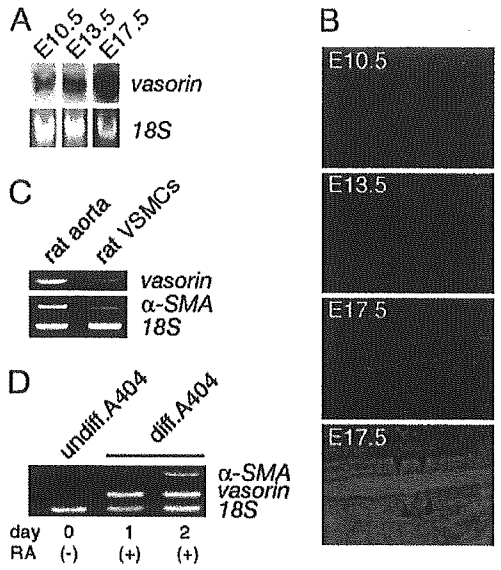

Fig. 3. Developmental regulation of *vasorin*. (*A*) Northern blot analysis of staged mouse embryos (E10.5, E13.5, and E17.5). (*B*) Expression pattern of *vasorin* during aortic development examined by *in situ* hybridization analyses. The fourth image is the corresponding bright-field image of the third representation. Arrowheads indicate the aorta in the mouse embryo (E17.5). (*C*) Semiquantitative RT-PCR comparing the expression of *vasorin* in the adult rat aorta with that in cultured rat aortic VSMCs. Rat α-smooth muscle actin (α-SMA) and 18S rRNA were used as a positive and an internal control, respectively. (*D*) Semiquantitative RT-PCR showing the induction of the *vasorin* gene in RA-treated A404 cells. Rat α-SMA and 18S rRNA were used as a positive and an internal control, respectively.

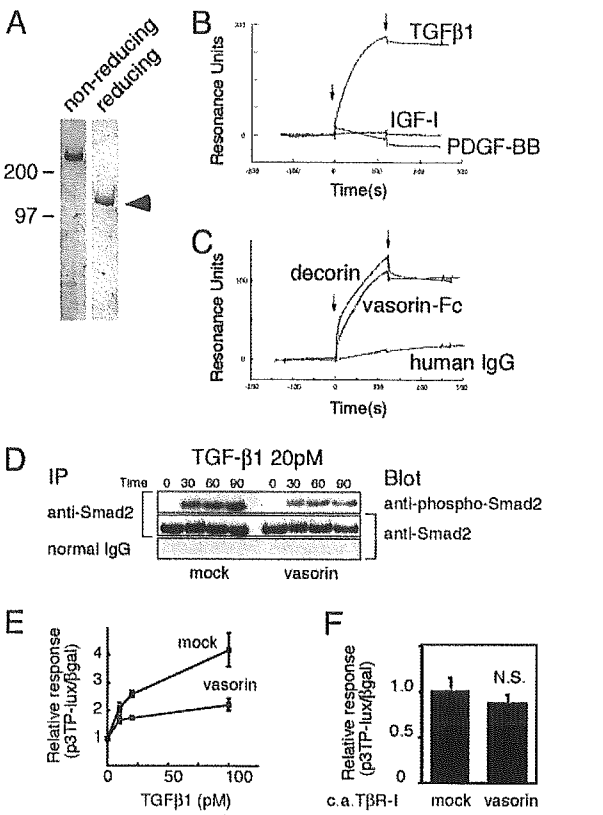

Fig. 4. Vasorin directly binds to TGF-β and modulates TGF-β signaling *in vitro*. (*A*) Purified recombinant vasorin-Fc fusion protein was free of protein contamination, as estimated by SDS/PAGE, followed by Coomassie blue staining. (*B*) Sensorgrams obtained from injection of vasorin-Fc on immobilized TGF-β1, PDGF-BB, and insulin-like growth factor I (IGF-I) are shown. (*C*) Sensorgrams obtained from injection of vasorin-Fc, decorin, and human IgG on immobilized TGF-β1 are shown. Arrowheads indicate initiation and termination of injections. (*D*) TGF-β-induced Smad2 phosphorylation was significantly inhibited in vasorin-expressing cells. Stable transfectants were treated with TGF-β1 (20 pM), and then immunoprecipitated with an anti-Smad2/3 antibody, followed by blotting with an anti-phospho-Smad2 antibody. (*E*) A reporter assay was performed by using the TGF-β-responsive reporter p3TP-lux. Stable transfectants were stimulated with TGF-β1 at various concentrations, and vasorin inhibited TGF-β-induced reporter gene activation. (*F*) Vasorin inhibited TGF-β signaling at the extracellular and/or cell-surface level. The p3TP-lux reporter and the constitutively active TβR-I were cotransfected into stable transfectants. Transfection of the constitutively active TβR-I activated the p3TP-lux reporter, but vasorin did not significantly inhibit this activation. N.S., not significant.

lated to be 0.86 nM. We also tested the binding of TGF-β2 and TGF-β3 to vasorin. TGF-β2 and TGF-β3 showed a specific binding to vasorin with a similar binding affinity to that of TGF-β1 (data not shown).

Next, we examined the functional role of vasorin in TGF-β signaling. First, stable transfectants expressing vasorin were stimulated by TGF-β1 (20 pM). Cells expressing vasorin showed a significant reduction in Smad2 phosphorylation (Fig. 4*D*). Second, we did a reporter assay, by using the TGF-β-responsive reporter p3TP-lux. TGF-β1 activated this reporter in a dose-dependent manner, and vasorin significantly inhibited this effect (Fig. 4*E*). This inhibitory effect of vasorin was specific to TGF-β signaling because vasorin did not affect the cellular responses to irrelevant cytokine stimulation (data not shown). Stable transfectants were also stimulated by using the constitutively active TGF-β type I receptor (constitutively active TβR-I) instead of TGF-β1 stimulation. Transfection of constitutively active TβR-I activated the p3TP-lux reporter, but vasorin did not significantly inhibit this activation (Fig. 4*F*). These findings indicate that vasorin inhibits TGF-β signaling at the extracellular and/or cell-surface level.

Vasorin Expression Was Down-Regulated During Vessel Repair After Arterial Injury, and Reversal of Vasorin Down-Regulation, by Using Ad-Mediated *in Vivo* Gene Transfer, Significantly Reduced Neointimal Formation at Least in Part by Modulating TGF-β Signaling in the Vessel Wall. To investigate *in vivo* functions of vasorin, we used a rat arterial balloon-injury model, because it is a well characterized atherosclerosis model of VSMC-derived lesions, and it is well established that TGF-β contributes to neointimal formation by promoting fibrosis.

Adult rats were subjected to balloon injury with a catheter inserted through the external carotid artery. Vascular injury provokes fibroproliferative activity in quiescent VSMCs, and the phenotypic modulation of VSMCs is induced. Because the fibroproliferative activity of VSMCs peaks at 3 days after injury (6), balloon-injured carotid arteries were harvested at 3 days after insertion to examine the expression levels of *vasorin* by semiquantitative RT-PCR. Consistent with our findings described above (Fig. 3 *A–D*), down-regulation of *vasorin* expression was induced by mechanical vascular injury (Fig. 5*A*). In contrast, the expression of several cytokines, including TGF-β, was up-regulated by vascular injury, and the ratio of TGF-β to vasorin was increased (Fig. 5*A*).

Next, we investigated the functional role of vasorin down-regulation in neointimal formation. To restore *vasorin* expression during vessel repair after injury, we did Ad-mediated *vasorin* gene transfer to balloon-injured rat carotid arteries. Replication-defective Ad-vasorin were constructed, and after denudation with a balloon catheter, the vessel wall was exposed to the adenoviral solution (1×10^9 pfu) for 20 min to deliver *vasorin* gene locally. First, arteries were harvested 3 days after-

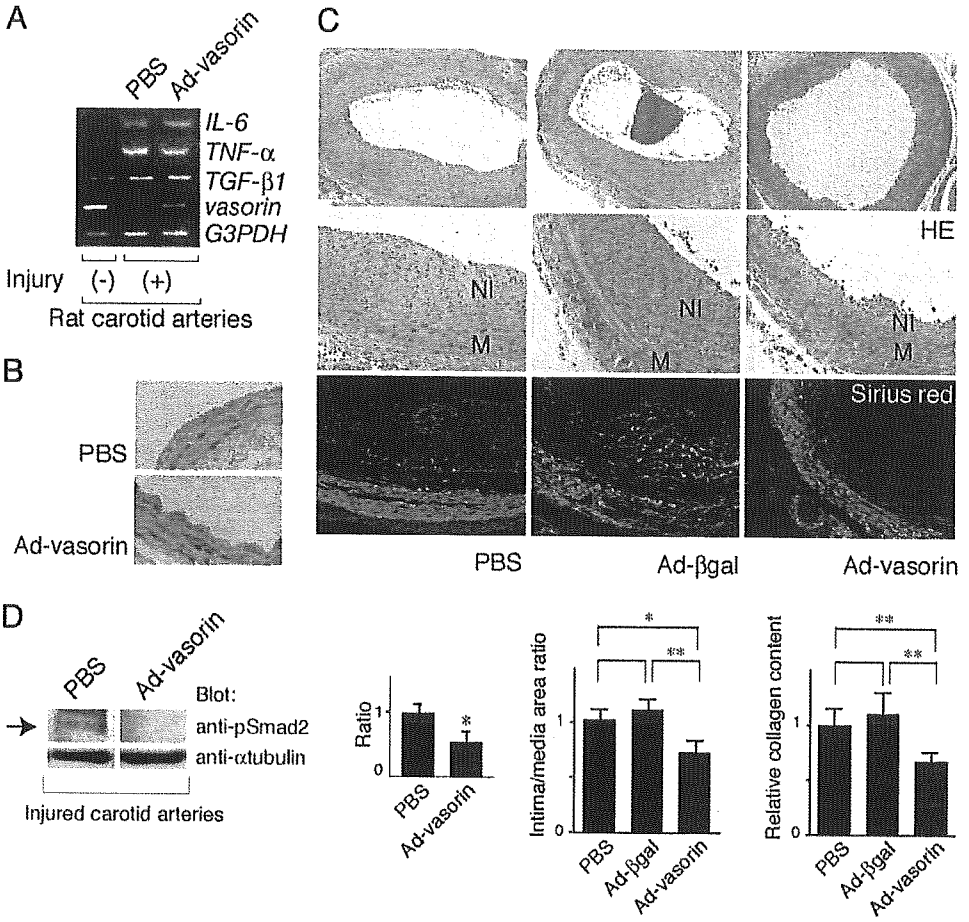

Fig. 5. Vasorin expression was down-regulated during vessel repair after arterial injury, and reversal of vasorin down-regulation significantly reduced neointimal formation, at least in part, by attenuating TGF-β signaling *in vivo*. (*A*) Rat carotid arteries were harvested at 3 days after injury to examine the expression levels of *vasorin* by semiquantitative RT-PCR analysis. Down-regulation of *vasorin* expression was induced by mechanical vascular injury, and Ad-vasorin treatment partially reversed this down-regulation. In contrast to vasorin, the expression of TGF-β1, TNF-α, and IL-6 was up-regulated by vascular injury and was not altered by vasorin administration. GAPDH was used as an internal control. (*B*) Vessels treated with Ad-vasorin were harvested 3 days afterward and were subjected to immunostaining to confirm protein expression by using the anti-Flag antibody. (*C*) Effects of Ad-vasorin on neointimal formation in rat carotid arteries at 14 days after injury ($n = 5$ arteries for each group). Representative hematoxylin/eosin-stained cross sections (*Top* and *Middle*) and Sirius red-stained cross sections (*Bottom*) of balloon-injured arteries treated with PBS (*Left*), Ad-β-galactosidase (*Center*), and Ad-vasorin (*Right*) are shown. (*Middle*) Partial magnifications of the respective *Top* images. Ad-vasorin administration significantly reduced the intima/media area ratio of injured arteries and collagen content in the lesions ($P < 0.01$), as compared with Ad-β-galactosidase administration. NI, neointima; M, media; HE, hematoxylin/eosin. *, $P < 0.05$; **, $P < 0.01$. (*D*) The inhibitory effects of vasorin administration on TGF-β signaling *in vivo*. Arteries were harvested 3 days after injury and were subjected to Western blot analysis by using the anti-phospho-Smad2 antibody. Representative data are shown. Smad2 phosphorylation was significantly reduced in all Ad-vasorin-treated arteries ($P < 0.05$). The blots were stripped and reprobed with the anti-α-tubulin antibody to ensure equal loading of proteins. The relative intensities of phospho-Smad2 bands were measured by densitometric scanning from three independent experiments. *, $P < 0.05$.

ward to examine the expression of vasorin by RT-PCR analysis (Fig. 5*A*), immunostaining (Fig. 5*B*), and Western blot analysis (data not shown). Ad-mediated *vasorin* gene transfer was successful (Fig. 5 *A* and *B*), and the expression of TGF-β, TNF-α, and IL-6 was not altered by vasorin administration (Fig. 5*A*). Second, arteries were harvested to assess the effect of *vasorin* gene transfer on neointimal formation ($n = 5$ arteries for each group) at 14 days after injury. The administration of Ad-vasorin significantly reduced the intima/media area ratio of injured arteries by 35% ($P < 0.01$), as compared with the administration of Ad-β-galactosidase (Fig. 5*C*), suggesting that restoration of vasorin expression had a significant inhibitory effect on neointimal formation. Because TGF-β stimulates extracellular matrix protein synthesis, and also functions as an antiinflammatory cytokine, we examined collagen content and leukocyte infiltration in the lesions quantitatively, by using Sirius red staining and CD45 staining, respectively. As shown in Fig. 5*C Lower*, vasorin administration significantly reduced collagen content in the lesions ($P < 0.01$), whereas leukocyte infiltration was not altered (data not shown).

Finally, we investigated whether *in vivo* vasorin administration inhibits TGF-β signaling in the vessel wall. Balloon-injured rat carotid arteries treated with Ad-vasorin were subjected to Western blot analysis, by using the anti-phospho-Smad2 antibody, when the fibroproliferative activity of VSMCs peaked (3 days after injury). Smad2 phosphorylation was significantly reduced in Ad-vasorin-treated arteries ($P < 0.05$; Fig. 5*D*). These results suggest that enhanced TGF-β signaling after vascular injury (Fig. 5*A*) is significantly inhibited by *in vivo* vasorin administration, and that vasorin inhibits neointimal formation at least in part by modulating TGF-β signaling in the vessel wall.

Discussion

VSMCs respond to various growth factors, and the best investigated situation in which VSMCs proliferate and produce extracellular matrix proteins *in vivo* is after vascular injury.

Whereas the rat carotid arterial balloon-injury model has been studied extensively, the underlying mechanisms that regulate vessel repair and neointimal formation appear to be the same in other arteries and in other species, including humans. The phenotype of VSMCs following arterial injury is similar to that observed during embryonic angiogenesis, and molecular mechanisms that regulate VSMC differentiation during embryonic development are thought to be recapitulated during vessel repair. *Vasorin* gene expression was developmentally regulated (Fig. 3 *A–D*), and, consistent with this finding, *vasorin* was down-regulated during vessel repair after arterial injury (Fig. 5*A*). This finding suggests its involvement in injury-induced vascular lesion formation. Therefore, we explored the *in vivo* functions of vasorin, by using a rat arterial balloon-injury model combined with Ad-mediated *in vivo* gene transfer. As shown in Fig. 5*C*, reversal of vasorin down-regulation had a significant inhibitory effect on neointimal formation. These results indicate that down-regulation of vasorin expression contributes to the fibroproliferative response to vascular injury.

Numerous factors that regulate VSMC activity have been studied in the arterial-injury model, and the results of these investigations suggest the importance of pathological extracellular stimuli, such as the renin-angiotensin system, catecholamines, EGF, platelet-derived growth factor (PDGF), insulin-like growth factor I, endothelin 1, TGF-β, and oxidative stress. TGF-β is a context-dependent pleiotropic cytokine, which plays a key role in the vascular response to injury. Several studies using gene transfer techniques have shown that locally enhanced TGF-β signaling enables matrix-rich neointima to develop in uninjured normal arteries of rats (11, 12). In contrast, localized blockade of TGF-β signaling results in the inhibition of neointimal formation, accompanied by reduced extracellular matrix synthesis in a rat balloon-injury model (13, 14). Of clinical relevance is the observation that the expression levels of TGF-β mRNA in restenotic lesions are higher than those in primary atherosclerotic lesions (15). These investigations indicate that TGF-β functions as a fibrogenic cytokine in a balloon-injury model, and apparently aggravates neointimal formation by promoting fibrosis. In this paper, we found that vasorin directly binds to TGF-β and negatively modulates TGF-β signaling in the vessel wall (Figs. 4 and 5*D*). Considering the functional role of TGF-β in this model, it is reasonably assumed that the *in vivo* phenotype induced by Ad-vasorin administration is mediated at least in part by the inhibitory effects of vasorin on TGF-β signaling in the vessel wall.

The extracellular region of vasorin is composed of ten tandem arrays of an LRR, an EGF-like domain, and a fibronectin type III-like domain, that are known to be involved in protein–protein interactions (Fig. 1*B*). Secreted and cell-surface molecules containing those domains, such as extracellular matrix proteins and adhesion molecules, sometimes have multiple binding partners. PDGF and TGF-β are prominent growth factors that have been suggested to play an important role in neointimal formation after arterial injury, and we demonstrated here that vasorin directly binds to TGF-β, but not to PDGF (Fig. 4*B*). However, it is possible that vasorin has other binding partners and that vasorin affects not only TGF-β signaling but also other signaling pathways, through another yet-to-be-identified mechanism. Further investigations will be needed to clarify these issues.

In the present study, we found that down-regulation of vasorin expression was induced by acute vascular injury, and that reversal of vasorin down-regulation during vessel repair inhibits neointimal formation, at least in part, by modulating cellular responses to TGF-β. These data raise a possibility that the gene expression profile of cell-surface molecules is changed by mechanical vascular injury, and that altered cellular responses to growth factors in dedifferentiated VSMCs are in part due to this change. Thus, identification and modification of the pivotal gene expression of cell-surface molecules in VSMCs may be a potential therapeutic approach to vascular fibroproliferative disorders.

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Residual fibrosis affects a long-term result of left ventricular volume reduction surgery for dilated cardiomyopathy in a rat experimental study☆

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Abstract

Objectives: The aim of this study is to evaluate the relationship between left ventricular (LV) wall property and the results of LV volume reduction surgery (LVR) to treat dilated cardiomyopathy (DCM) in an experimental model. **Methods**: DCM was introduced in 18 Lewis rats by autoimmunization with cardiac myosin. Among them, 12 rats underwent LVR and the rest were served as controls. They were subjected to echocardiography and cardiac catheterization for dimensional and functional measurements. The animals were sacrificed 4 weeks after surgery, and the fraction of myocardial fibrosis was calculated in 4 divided parts of the LV wall. **Results**: Percent fibrosis varied widely from 4.7 to 45.2%. LV volume reduction surgery improved cardiac function immediately after surgery in all rats (E_{max}, 0.28±0.14 to 0.48±0.18 mmHg/μl; LV end-diastolic pressure, 21.0±6.1 to 13.3±5.1 mmHg, $P<0.05$, respectively). Four weeks later, 6 hearts remained in good shape with smaller LV end-diastolic dimension (Dd) than baseline values (LV Dd, 9.7±0.6 mm; fractional area change (FAC), 40.3±8.4%) and the other 6 had more redilation in diameter and more deterioration in function than baseline values (LV Dd, 10.9±0.6 mm; FAC, 25.8±6.9%; $P<0.05$, respectively). Percent fibrosis in the septum differed 11.1±3.4 vs. 27.8±2.8% between the two groups ($P<0.01$). There was a significant correlation between the ratio of LV redilatation after surgery and percent fibrosis in the septum ($r=0.951$, $P<0.01$). **Conclusions**: Although the initial benefit of LVR was confirmed, the long-term result was affected by the amount of residual fibrosis. This information suggests that surgical site selection is important to achieve a good result of LV restoration surgery for DCM.

Keywords: Dilated cardiomyopathy; Heart failure; Left ventricular restoration

1. Introduction

Initial enthusiasm of partial left ventriculectomy (PLV), so called Batista operation, to treat dilated cardiomyopathy (DCM) has quickly diminished partially due to an unpredictable mid-term result [1,2]

PLV works very well in some patients, but not so well or even worse in others. The reason why the effect of PLV is inconsistent remains unclear so far.

In our clinical practice, we noticed that the left ventricle of DCM was not always homogeneously affected as pointed out previously [3,4]. Some patients have inhomogeneous left ventricular (LV) wall property, and others have diffusely and homogeneously damaged LV wall [5,6].

The hypothesis is that the surgical benefit does not last long if fibrosis was prominent in the retained myocardium after PLV. The main purpose of PLV is to reduce the diameter and the volume of the left ventricle, and therefore we perform LV volume reduction surgery (LVR) as an alternative to PLV in this study. We have focused attention to the LV wall property as assessed by the amount of myocardial fibrosis and evaluated the relationship between the residual fibrosis of the retained myocardium and the surgical result of LV volume reduction surgery in a rat DCM model.

2. Materials and methods

2.1. Animal information

Young male Lewis rats were used in this study.

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All animals received humane care in compliance with Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute Council and published by the National Academy Press.

2.2. DCM model

Six weeks old Lewis rats were autoimmunized with purified cardiac myosin and later on developed DCM as reported elsewhere [7,8]. Animals have suffered from sort of acute myocarditis for 4–5 weeks and such inflammatory reaction almost disappeared within 8 weeks after autoimmunization [9].

Ten weeks after autoimmunization all animals were evaluated by means of echocardiography. Rats with dilated LV and severely depressed LV function confirmed by echocardiography were provided for this study. After all, 18 rats completed the study, among which 12 rats underwent surgical intervention and the rest were served as controls.

2.3. Echocardiographic study

Rats were anesthetized with ether and LV function was assessed by means of echocardiography with a 12 MHz phased-array transducer (SONOS 5500, Philips Medical Systems, Bothell, WA, USA) [10–12]. The following parameters were measured from B- and M-mode tracing: LV end-diastolic dimension (LV Dd, mm), LV end-systolic dimension (LV Ds, mm), fractional shortening (FS, %), and fractional area change (FAC, %). All parameters were measured by the American Society for Echocardiography leading-edge method from at least three consecutive cardiac cycles.

Echocardiography was performed before and immediately after surgery, following 2 and 4 weeks after surgery serially.

2.4. LV volume reduction surgery

Animals were orally intubated with ethyl ether gas and anesthesia was maintained with 1% isoflurane under control ventilation during operation. LV volume reduction surgery (LVR) was carried out by placation of the posterolateral wall. LV was gently pulled out from the pericardial cavity via left thoracotomy and the posterolateral wall of the left ventricle was plicated with several horizontal mattress sutures of 4-0 prolene without any kind of cardiac support. Surgical technique performed in this study was similar with the technique adapted to a large animal model, apex-preserving LVR [13]. Before and after surgery, echocardiography and cardiac catheterization were performed simultaneously and the efficacy of the surgical technique was evaluated.

2.5. Cardiac catheterization

A 2 French micromanometer-tipped catheter (Millar Instruments Inc, Houston, TX) was introduced from the right carotid artery to measure LV pressure and a 3 French occlusion balloon catheter was introduced from the right femoral vein to occlude the inferior vena cava. At the stable state, LV pressure and its first time derivative (dp/dt) were continuously monitored through the arterial catheter by using a multiple recording system during temporary caval occlusion. Simultaneously LV dimension was measured by echocardiography. The maximum time-varying elastance (LV E_{max}, mmHg/μl) and the time constant of isovolumic relaxation (Tau τ, ms) was calculated as an index of global systolic and diastolic function, respectively [10–12].

2.6. Histological study

At 4 weeks after surgery, final echocardiography was performed and all rats were sacrificed. Hearts were removed and transected at the base of the papillary muscles. The transverse sections were fixed with 10% formalin and stained with hematoxylin eosin and Masson's trichrome staining. In the same manner six DCM rats without LVR were served as control group.

The heart sections were divided into four parts: anterior, septal, posterior, and lateral part. Masson's trichrome stained sections were subjected to quantitative evaluation of the severity of interstitial fibrosis (percent fibrosis) using the point counting method.

2.7. Statistical analysis

Data were expressed as means ± SD. Statistical analysis was performed by using StatView (SAS Institute Inc, Cary, NC). Differences between two groups were assessed by the non-parametric test, Wilcoxon test and Mann–Whitney U-test. Correlation trends were assessed by Spearman rank correlation coefficient r. Values of P less than 0.05 were considered statistically significant.

3. Result

3.1. Echocardiography at baseline

Sixty-eight rats out of 100 rats which were autoimmunized with cardiac myosin survived until echocardiographic study at 8 weeks after initial manipulation. Fifty-two rats were diagnosed as DCM by echocardiography. The reasons of exclusion were as follows: LV cavity was not enough enlarged, LV wall motion was too much depressed (i.e. almost dying), or right ventricle was also severely enlarged or depressed.

Table 1 summarized echocardiographic data at baseline. LV Dd and LV Ds of DCM rats became bigger than those

Table 1
Echocardiographic data in control and DCM group

Variable	DCM ($n=12$)	Normal ($n=7$)	P-value
LV Dd (mm)	10.1±0.6	7.6±0.3	<0.001
LV Ds (mm)	7.8±0.7	3.3±0.2	<0.001
FS (%)	23.1±3.4	55.4±2.2	<0.001
FAC (%)	31.6±4.5	82.1±7.9	<0.001

Data are expressed as mean±SD. LV Dd, left ventricular end-diastolic dimension; LV Ds, left ventricular end-systolic dimension; FAC, fractional area change; FS, fractional shortening.

of health Lewis rats of the same age (normal control). FS and FAC of DCM rats were lower than those of normal control.

DCM rats had significantly larger hearts and poorer LV function than normal control group.

3.2. Cardiac catheterization before and after surgery

E_{max} and the maximal LV dp/dt increased after surgery. The LV end-diastolic pressure, minimum LV dp/dt, and τ decreased after surgery. The values measured by the catheterization were summarized in Table 2 and there was a significant difference between values before and after surgery.

Systolic function of LV improved after surgery without compromising diastolic function. In addition, the surgical technique used in the present study avoided excessive volume reduction of the left ventricle.

3.3. Serial echocardiographic change after surgery

Thirty-five rats underwent LV volume reduction surgery and 24 rats survived surgery. In 16 rats LVR was properly carried out and the efficacy of the surgery was confirmed by echocardiography and cardiac catheterization. Among them, 12 rats survived 4 weeks after surgery and were subjected to serial echocardiographic follow-up completely.

Comparing LV dimensions at 4 weeks after surgery with those at baseline, rats were divided into two groups as follows. One was Good result group, of which LV dimension at 4 weeks after surgery remained smaller than that at baseline. The other was Poor result group, of which LV dimension at 4 weeks after surgery became larger than that at baseline. LV Dd of Poor result group increased quicker and more severely than that of Good result group, as shown in Fig. 1.

Table 2
Cardiac catheterization data before and after surgery

	Pre	Post	P-value
LVEDP (mmHg)	21.0±6.1	13.3±5.1	<0.05
E_{max} (mmHg/μl)	0.28±0.14	0.48±0.18	<0.05
+LV dp/dt (mmHg/s)	3583±559	4430±724	<0.05
−LV dp/dt (mmHg)	−2385±196	−2797±507	<0.05
Tau τ (ms)	36.6±8.4	29.3±7.4	<0.05

Data are expressed as mean±SD. LVEDP, left ventricular end-diastolic pressure; LV E_{max}, maximal time-varying elastance; +LV dp/dt, maximal time-derivative; −LV dp/dt, minimal time-derivative; Tau τ, time constant of isovolumetric relaxation.

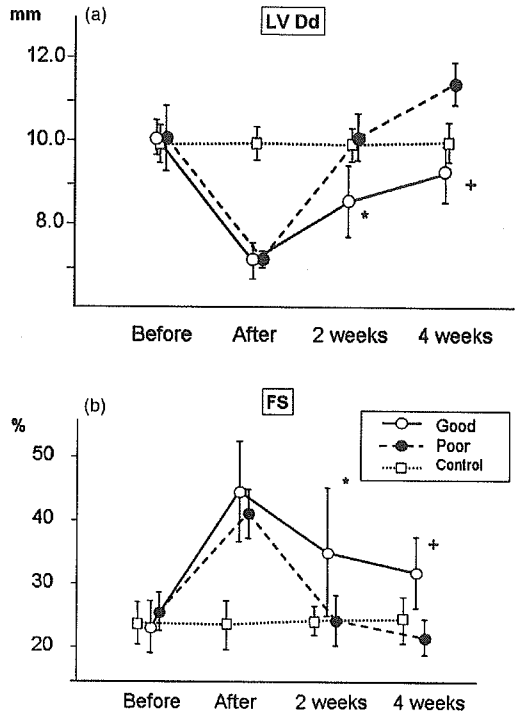

Fig. 1. (A) and (B) Serial echocardiographic change of cardiac function after surgery. (A) Change of left ventricular end-diastolic dimension (LV Dd): LV dimension once reduced by surgery increased time by time. LV redilatation of Bad result group was much more intense than that of Good result group. (B) Change of fractional shortening (FS): LV function once improved by surgery deteriorated time by time. LV function of Bad result group was more deteriorated than that of Good result group. *$P<0.05$, $^{+}P<0.001$ vs. Poor group.

The dimension of the left ventricle was uniformly reduced by surgery, following progressive redilatation time by time. LV Dd and LV Ds in Poor result group increased more quickly and severely than those in Good result group, as shown in Table 3 and Fig. 1. Similarly, FS and FAC of LV increased uniformly after surgery in both groups, following progressive deterioration time by time. FS and FAC of Poor result group deteriorated quicker and more severely than those of Good result group as shown in Table 3.

3.4. Histological study

3.4.1. Control DCM rats

Fractional fibrosis calculated as percent fibrosis varied widely from 4.4 to 45.2% part by part and localization of fibrosis varied. In the present model of DCM, fibrosis scattered around and localization of fibrosis differed rats by rats as shown in Table 4. The most fibrotic parts of each individual hearts differed rats by rats. The anterior part was the most fibrotic in one of 6 rats, the septum in 2, the posterior in one, and the lateral in 2. The anteroseptal wall

Table 3
Serial echocardiographic change after surgery

	Group	Pre	Post	2 Weeks	4 Weeks
LV Dd (mm)	Good	10.1±0.4	7.1±0.4	8.6±0.9*	9.7±0.6^{+}
	Poor	10.1±±0.8	7.2±0.2	10.2±0.6	10.9±0.6
LV Ds (mm)	Good	7.8±0.6	4.0±0.8	5.8±0.7^{+}	6.4±0.7^{+}
	Poor	7.8±0.8	4.4±0.4	8.0±0.8	9.1±0.7
FS (%)	Good	23.2±4.1	44.6±7.9	35.1±10.1*	33.9±6.1^{+}
	Poor	23.2±3.0	38.7±3.9	21.9±4.1	16.2±3.0
FAC (%)	Good	33.6±5.1	50.2±7.1	43.6±9.6	40.0±8.4^{+}
	Poor	31.6±4.5	47.0±5.8	32.1±9.2	25.8±6.9

Data are expressed as mean±SD. Pre, before surgery; Post, 4 weeks after surgery; Good, Good result group; Poor, Poor result group; LV Dd, left ventricular end-diastolic dimension; LV Ds, left ventricular end-systolic dimension; FS, fractional shortening; FAC, fractional area change. *$P<0.05$, $^{+}P<0.001$ vs. Poor group

was more fibrotic than posterolateral wall in 3 of 6 rats and the posterolateral wall was more fibrotic than the anteroseptal wall in 3. The LV wall of the DCM rats was not homogeneously damaged as shown in Fig. 2.

3.4.2. Four weeks after surgery

Percent fibrosis of the septum, which was far from surgical site and presumably least damaged by surgery itself, in Good result group was significantly smaller than that in Poor result group (11.3±3.4 vs. 27.8±2.8%, $P<0.0001$). Regarding percent fibrosis of the rest of the heart, which consisted of the anterior part and the posterior part, there was no big difference between both groups (14.9±4.9 vs. 22.7±8.1%, $P=0.07$).

Comparing the relationship between the ratio of LV redilatation (delta redilatation) and percent fibrosis of the septum, there was a strong correlation recognized between them ($r=0.951$, $P<0.001$) as shown in Fig. 3.

The ratio of LV redilatation was calculated as follows; (LV Dd after−LV Dd before)/LV Dd before×100 (%).

4. Discussion

Dr Batista introduced the surgical concept of resecting sound muscle and reducing LV diameter in order to improve LV function of enlarged DCM hearts according to the physics [14]. This concept seemed attractive to treat end-stage heart failure and brought broad attention not only to the surgical community but also to the entire cardiovascular medicine. A short-term result of PLV to treat a difficult patient group of end-stage heart failure was good enough to convince many surgeons to start performing PLV. However, unpredictable and inconsistent mid-term results have gradually been revealed and the initial hope to PLV has diminished quickly [1,2].

In 1996 we started performing PLV for DCM and achieved good initial results, following unpredictable mid-term result as occurred similarly around the world. We examined LV wall motion thoroughly by echocardiography with utilizing color kinetic method before surgery and intraoperatively and we noticed some patients had abnormal regional wall motion [5,6]. Abnormal LV wall motion and heterogeneity of LV wall property in DCM was pointed out previously [3,4]. According to the echocardiographic findings, we started SAVE operation, septal anterior ventricular exclusion, instead of PLV when LV wall motion of the anteroseptal part was worse than that of the posterolateral part [15,16]. Then left ventriculoplasty for DCM by careful selection of PLV and SAVE improved a mid-tem result. Four year survival rate of 61 elective operations was 69.3% [17].

Table 4
Histological data of control DCM rats

Part	% Fibrosis	%
Anterior	17.5±14.0	6.9–45.2
Septum	15.4±9.8	4.7–49.8
Posterior	12.3±5.3	4.4–18.1
Lateral	19.7±10.9	7.6–35.1
Total	16.2±10.2	4.4–45.2

Data are expressed as mean±SD. Part, each four divided part of the heart in the transverse section.

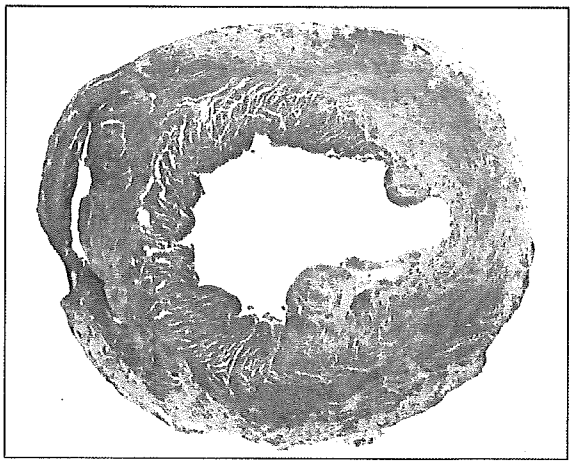

Fig. 2. Masson's trichrome staining of transected LV. In this specimen, fibrosis distributes prominently in the lateral wall. The lateral wall is more fibrotic than the anteroseptal wall. Fibrosis is not diffusely scattered and localized in the lateral wall.

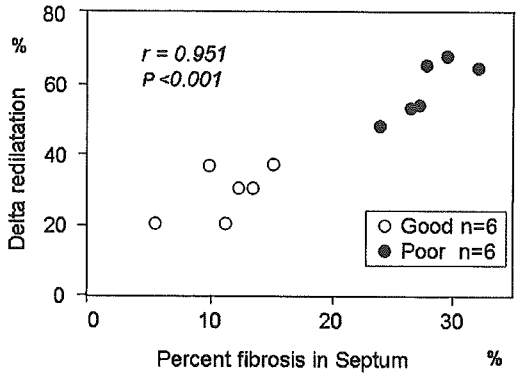

Fig. 3. The relationship between the ratio of LV redilatation and the fractional fibrosis in the septum. The more dilated LV dimension became after surgery, the more fibrotic the septum retained by surgery was. Delta redilatation was explained in the text.

In the present study, we adopted a DCM model created by means of autoimmunization with cardiac myosin because this model type is mimicked with humane DCM without hypertension [7,8]. Histological study showed that localized fibrosis scattered in the left ventricle and location of fibrosis differed individually. This means that the LV wall in this DCM model has inhomogeneous histology, and that the model is suitable for evaluating the relationship between LV wall property and the surgical result.

The findings of the present study are summarized as follows. Echocardiography and cardiac catheterization confirmed the efficacy of the surgery, but LV diameter which had been reduced right after the surgery had gradually enlarged as time passed. The ratio of LV redilatation differed in two groups. Half of animals remained in good shape with smaller LV dimension than baseline, and the other half deteriorated in bad shape with larger LV dimension. Histological study showed the fraction of residual fibrosis in Good result group was significantly less than that in Bad result group. There was a strong correlation between the ratio of LV redilatation and the relative amount of residual fibrosis.

This study infers that localization of fibrosis affects the long-term result of PLV. Decreasing number of heart transplantation around the world, there seeks a possibility of non-transplant surgery for end-stage heart failure. Initial experience of PLV has left a fact that PLV could salvage some candidates for heart transplantation to a considerable extent in spite of denying the almightiness of PLV [2]. We must consider not only resecting the posterolateral wall like PLV but also some intervention to the anteroseptal wall such as SAVE operation depending on the patients' LV wall property. Such a strategic approach as a combination of resecting damaged myocardium and retaining sound muscle can improve a long-term result of LV restoration surgery for DCM.

In conclusion, a long-term result of LV volume reduction surgery for DCM rats was affected by the amount of residual fibrosis. This information suggests that proper selection of the surgical site is important to achieve a good result of LV restoration surgery for DCM.

5. Study limitations

This study was conducted as a retrospective analysis to examine the result of the surgery. We can conduct the study prospectively if we recognized the LV wall property exactly before surgery. In the present time we have no reliable tool to examine the interstitial fibrosis of the whole heart without excising the heart for pathological examinations. Alternatively we sacrificed 6 DCM rats before surgery for histological study, which confirmed that they had wide variety of LV wall property in terms of myocardial fibrosis.

Further development of the technology may help to detect the LV wall property exactly and predict the result of the surgery prospectively.

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Appendix A. Conference discussion

Dr R. Poston *(Baltimore, MD, USA)*: The hearts that did bad, did they have more overall inflammation, or was it localized to the septum?

Dr Horii: I don't follow you.

Dr Poston: It is possible that there is just overall more myocardial inflammation and fibrosis that also included the septum in the hearts that did bad which was not necessarily specific to the septum. If that's the case, it could just be that in your model a bad heart outcome is due mainly to a more intense inflammatory response from the autoimmune stimulus. Irregardless of the ventricular surgery, the inflammatory response led to the hearts dilating back out.

Dr Horii: Unfortunately, I don't have a good answer for you, because I cannot tell you if such a fibrosis is the reason or is the cause.

Regarding myocardial fibrosis, the septum was the least damaged by surgery itself and the least fibrotic portion as shown in my presentation. And even in the clinical setting and also in this experimental setting, there was localized fibrosis and heterogeneity of LV wall in DCM hearts. And so, LV surgery can be performed in an appropriate way to treat DCM hearts.

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Surgical Ventricular Restoration in the Treatment of Congestive Heart Failure Due to Post-Infarction Ventricular Dilation

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Birmingham, Alabama; Los Angeles, California; Monte Carlo, Monaco; Florence and Milan, Italy; Sao Paulo, Brazil; Freiburg, Germany; Charlottesville, Virginia; Kanagawa, Japan; St. Louis, Missouri; Orlando, Florida; Cleveland, Ohio; New York, New York; and Bordeaux, France

OBJECTIVES The purpose of this study was to test how surgical ventricular restoration (SVR) affects early and late survival in a registry of 1,198 post-anterior infarction congestive heart failure (CHF) patients treated by the international Reconstructive Endoventricular Surgery returning Torsion Original Radius Elliptical shape to the left ventricle (RESTORE) team.

BACKGROUND Congestive heart failure may be caused by late left ventricular (LV) dilation after anterior infarction. The infarcted segment is often akinetic rather than dyskinetic because early reperfusion prevents transmural necrosis. Previously, only dyskinetic areas were treated by operation. Surgical ventricular restoration reduces LV volume and creates a more elliptical chamber by excluding scar in either akinetic or dyskinetic segments.

METHODS The RESTORE group applied SVR to 1,198 post-infarction patients between 1998 and 2003. Early and late outcomes were examined, and risk factors were identified.

RESULTS Concomitant procedures included coronary artery bypass grafting in 95%, mitral valve repair in 22%, and mitral valve replacement in 1%. Overall 30-day mortality after SVR was 5.3% (8.7% with mitral repair vs. 4.0% without repair; $p < 0.001$). Perioperative mechanical support was uncommon (<9%). Global systolic function improved postoperatively. Ejection fraction (EF) increased from 29.6 ± 11.0% preoperatively to 39.5 ± 12.3% postoperatively ($p < 0.001$). The left ventricular end-systolic volume index (LVESVI) decreased from 80.4 ± 51.4 ml/m^2 preoperatively to 56.6 ± 34.3 ml/m^2 postoperatively ($p < 0.001$). Overall five-year survival was 68.6 ± 2.8%. Logistic regression analysis identified EF ≤30%, LVESVI ≥80 ml/m^2, advanced New York Heart Association (NYHA) functional class, and age ≥75 years as risk factors for death. Five-year freedom from hospital readmission for CHF was 78%. Preoperatively, 67% of patients were NYHA functional class III or IV and postoperatively, 85% were class I or II.

CONCLUSIONS Surgical ventricular restoration improves ventricular function and is highly effective therapy in the treatment of ischemic cardiomyopathy with excellent five-year outcome. (J Am Coll Cardiol 2004;44:1439–45) © 2004 by the American College of Cardiology Foundation

The etiology of congestive heart failure (CHF) is coronary artery disease in approximately two-thirds of cases. The majority of these patients have experienced myocardial infarction (1). Despite successful early reperfusion, late left ventricular (LV) dilation develops in 20% of patients and leads to CHF (2,3). Myocardial necrosis progresses sequentially in the untreated transmural infarction from endocardium to epicardium (4). Early reperfusion alters the infarction process by sparing the epicardial layer and preventing thin-walled dyskinetic aneurysm formation. The reperfused infarcted myocardium retains its thickness and normal epicardial appearance, resulting in an akinetic segment with varying degrees of mid-myocardial and epicardial fibrosis. The remote non-infarcted myocardium undergoes changes in volume and shape during the course of "ventricular remodeling." As the ventricle enlarges, its normal elliptical shape becomes spherical and global systolic function worsens, resulting in CHF (5). The prognosis of patients with

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Abbreviations and Acronyms

ACE	= angiotensin-converting enzyme
CABG	= coronary artery bypass grafting
CHF	= congestive heart failure
EF	= ejection fraction
LV	= left ventricular
LVAD	= left ventricular assist device
LVESVI	= left ventricular end-systolic volume index
NYHA	= New York Heart Association
SVR	= surgical ventricular restoration

ischemic cardiomyopathy is more closely related to LV volume rather than to ejection fraction (EF) (6).

The new term "surgical ventricular restoration" (SVR) includes operative methods that reduce LV volume and "restore" ventricular elliptical shape (7–9). Excision of a thin-walled aneurysm with direct closure is an early method of SVR first described by Cooley et al. (10,11) and modified over the years. This operation is rarely performed currently because early reperfusion spares epicardial muscle, resulting in regional thick-walled akinesia rather than thin-walled dyskinesia. Dor (12) recognized that the adverse effects of remodeling on the remote non-infarcted myocardium were similar for akinesia and dyskinesia and was the first to utilize the endocardial patch plasty procedure for both morphologies. Dor's (12) operation improves systolic function and New York Heart Association (NYHA) functional class (13). However, the operation is not widespread because surgeons have been unwilling to exclude the akinetic normal-appearing segments often encountered after early reperfusion. Instead, coronary artery bypass grafting (CABG) is performed and the non-functioning akinetic muscle segment containing deeper scar is left undisturbed.

The Reconstructive Endoventricular Surgery returning Torsion Original Radius Elliptical shape to the left ventricle (RESTORE) group is a team of cardiologists and surgeons from 12 centers on four continents: six in the U.S., four in Europe, one in South America, and one in Asia (Appendix). The following sections report on the RESTORE SVR registry with five-year follow-up and provide an update of our previous three-year findings in 439 patients (14).

METHODS

The SVR was performed in 1,198 patients between 1998 and 2003. Inclusion criteria were previous anterior myocardial infarction, significant ventricular dilation (left ventricular end-systolic volume index [LVESVI] $\geq$60 ml/m^2), and a regional asynergic (non-contractile) LV circumference of $\geq$35%. Patients were in NYHA functional class I in 9%, class II in 22%, class III in 40%, and class IV in 29% of cases. The small percentage of patients in class I underwent SVR while undergoing CABG or mitral repair as the primary operative indication, because LV volume exceeded 60 ml/m^2. Echocardiography, ventriculography, or magnetic resonance imaging was used to confirm the asynergic segment and calculate EF. The LVESVI was determined by ventriculography or magnetic resonance imaging. Institutional review board approval was not obtained because the investigators considered the operation an established therapy based on the acceptance of endoventricular circular patch plasty and the reported outcomes of Dor et al. (15).

Anteroseptal, apical, and anterolateral LV scarred segments were identified and excluded by an intracardiac patch or direct closure. The operation is illustrated in Figure 1. Patients were placed on cardiopulmonary bypass with moderate hypothermia (approximately 34°C). Hearts were protected with warm and cold-blood cardioplegia during coronary grafting and/or mitral procedures. The ventricular restoration portion of the operation was performed during cardioplegia-arrested heart by about half of the surgeons, and in the open-beating heart by the others. Postoperative EF and volumes were obtained before hospital discharge. Follow-up NYHA functional class was obtained during physician visit or by telephone interview.

Statistics. Analysis of survival and readmission probabilities versus volume, EF, and age were carried out using Kaplan-Meier survival analysis to properly account for patients lost to follow-up. A similar analysis was used to determine the effect of mitral valve repair, replacement, and readmission. Times were taken as times to first readmission for readmitted patients, time to death for non-readmitted patients who died, and time to last follow-up for all others. Patients lost to follow-up were removed from the study (censored) for Kaplan-Meier survival analysis as of the date of last follow-up. Data comparisons used the general linear model for numeric data and logistic regression for categorical data. The SAS Institute JMP 4.0 statistical package was used for all tests.

RESULTS

Baseline characteristics. Patient age ranged from 25 to 89 years with a mean of 63 $\pm$ 11 years. The interval between anterior infarction and SVR procedure averaged 4.4 years. Mean NYHA functional class was 2.9 preoperatively with 9% of patients in class I, 22% in class II, 40% in class III, and 29% in class IV. Akinesia was present in 66% of cases and dyskinesia in 34%. Larger ventricular volumes were noted in patients with akinetic segments. Among the ventricles with LVESVI $\geq$80 ml/m^2, akinesia was present in 73.3% and dyskinesia in 26.7%.

Concomitant procedures included CABG in 95%, mitral valve repair in 22%, and mitral valve replacement in 1%. Patients undergoing mitral valve procedures had reduced EF and larger ventricles compared with patients in whom no mitral procedure was performed. If EF was $\leq$30%, mitral valve procedures were performed in 33.3% versus 15.0% in patients whose EF was >30% ($p < 0.0001$). Among patients with LVESVI $\geq$80 ml/m^2, mitral procedures were more common as compared with patients with smaller volumes (34.1% vs. 21.1%, $p < 0.0001$).

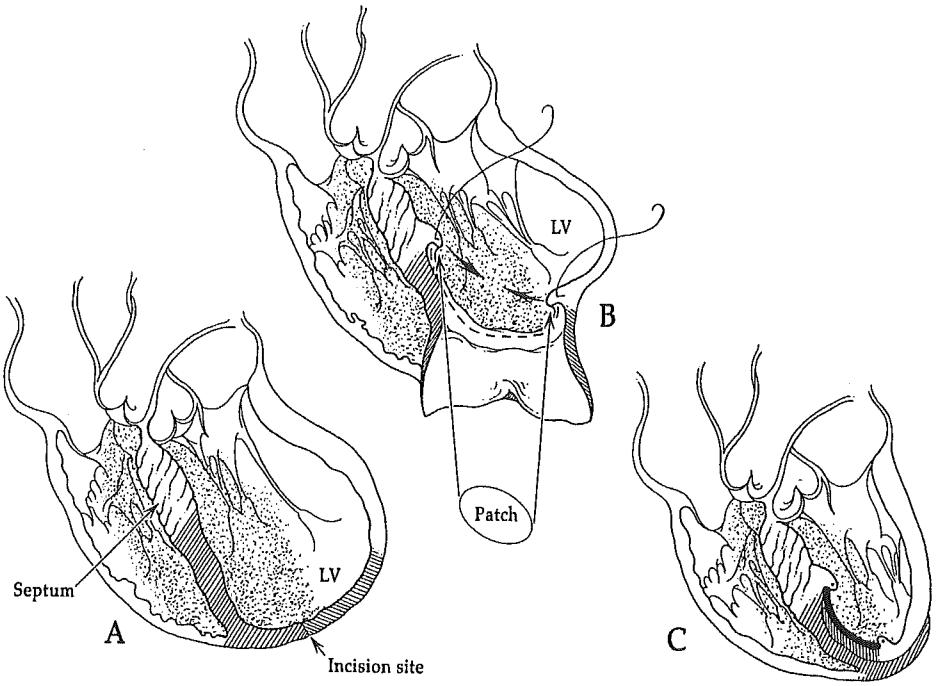

Figure 1. **(A)** Incision into the scar of the dilated ventricle. **(B)** Placement of a suture to exclude the scarred segment. **(C)** Completed repair with endocardial patch. LV = left ventricle.

Early outcome. Global systolic function improved postoperatively. The EF, measured in 1,118 patients before discharge from the hospital, increased from 29.6 ± 11.0% preoperatively to 39.5 ± 12.3% postoperatively ($p < 0.001$). Both preoperative and postoperative EF were significantly less for patients undergoing mitral valve repair. There was no difference between the groups in improvement in EF (Table 1).

The LVESVI, obtained in 671 patients, was reduced from 80.4 ± 51.4 ml/m^2 preoperatively to 56.6 ± 34.3 ml/m^2 postoperatively ($p < 0.001$). Normal LVESVI is 24 ± 10 ml/m^2 (16). Patients undergoing mitral valve repair had significantly larger hearts preoperatively than those with no mitral valve repair. There was no difference in postoperative LVESVI between the groups. Improvement in LVESVI was significantly greater in patients undergoing mitral valve repair (Table 2).

Thirty-day mortality after SVR was 5.3%, and it was higher among patients undergoing concomitant mitral valve repair (8.7%) versus patients in whom no mitral valve procedure was required (4.0%, $p < 0.001$). Perioperative mechanical support was uncommon; intra-aortic balloon pumping was used in 8.2%, left ventricular assist device (LVAD) in 0.7%, and extracorporeal membrane oxygenation in 0.3%.

To assess inter-institutional differences in outcome, the centers were arranged in order of increasing preoperative risk, as determined by logistic regression of a combination of preoperative NYHA functional class, EF, LVESVI, and age on survival. Incomplete data excluded some patients from this analysis. There was a nearly linear relationship between risk and five-year survival for all centers for which meaningful risks could be calculated.

Late outcome. Overall five-year survival was 68.6 ± 2.8%, calculated by the Kaplan-Meier product-limit method (Fig. 2). Mean time to death or loss to follow-up was 1.85 ± 1.45 years; at the end of five years, 22 patients remained in the study. Survival at five years was better in the group of patients that had dyskinetic as compared with akinetic morphology (80% vs. 65%; $p < 0.001$) (Fig. 3). Logistic regression analysis identified risk factors for death at any time after surgery. These included preoperative EF ≤30%, LVESVI ≥80 ml/m^2, advanced NYHA functional class, and age ≥75 years. Patients with EF ≥30% had survival 76.7 ± 3.2% as compared with 63.8 ± 3.9% for those with EF ≤30% (Fig. 4). Patients with EF >40% had survival

Table 1. LVESVI (ml/m^2) and Mitral Valve Repair

	No Mitral Repair	Mitral Repair	p Value
Preoperative	76.3	89.4	< 0.006
Postoperative	56.0	55.8	NS
Change	20.3	33.6	< 0.002

LVESVI = left ventricular end-systolic volume index.

Table 2. EF (%) and Mitral Valve Repair

	No Mitral Repair	Mitral Repair	p Value
Preoperative	31.0	25.4	< 0.0001
Postoperative	41.3	34.0	< 0.0001
Change	10.3	9.3	NS

EF = ejection fraction.

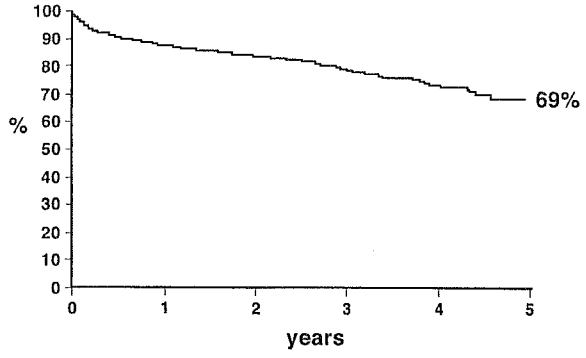

Figure 2. Overall five-year survival.

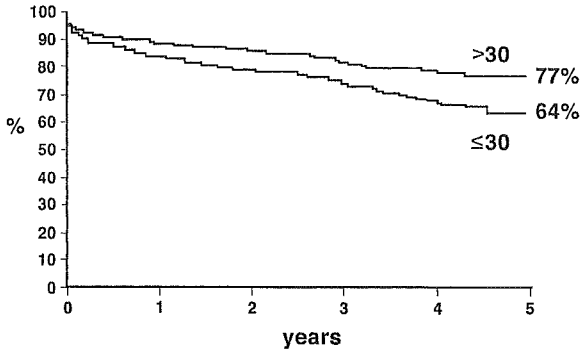

Figure 4. Survival based upon preoperative ejection fraction.

83.0 ± 4.0% as compared with 67.4 ± 3.0% for those with EF ≤40% ($p < 0.001$). Patients with LVESVI <80 ml/m^2 had survival 79.4 ± 3.3% as compared with 67.2 ± 3.2% for those with hearts >120 ml/m^2 ($p < 0.001$) (Fig. 5). Preoperative NYHA functional class was associated with long-term survival. At five years, survival was 94.5 ± 2.7% in NYHA functional class I, 87.2 ± 3.3% in class II, 69.9 ± 4.7% in class III, and 49.7 ± 5.8% in class IV ($p < 0.001$) (Fig. 6).

Mitral valve procedures were performed more commonly in patients with larger ventricles and reduced EF. Mortality at 30 days was higher in patients who underwent concomitant mitral repair (9.1%) as compared with those in whom no mitral valve procedure was performed (4%; $p < 0.001$). However, at five years, the survival curves were not different between patients who underwent repair and those who did not (68.7 ± 3.9% vs. 70.8 ± 3.3%). Mitral valve replacement was rare (30 patients, <1%) and was performed early in the registry. These patients had extensive areas of remote muscle scar precluding simple annuloplasty to correct mitral regurgitation.

Freedom from readmission to the hospital for CHF was 78%. The NYHA functional class improved from a mean of 2.9 preoperatively to 1.7 postoperatively. Preoperatively, 67% of patients had NYHA functional class III or IV symptoms (39% class III, 28% class IV). Postoperatively, 85% were functional class I or II (48% class I, 37% class II).

SVR in the elderly. Age was also a risk factor. Among all patients operated on, 12.4% (149) were ≥75 years old.

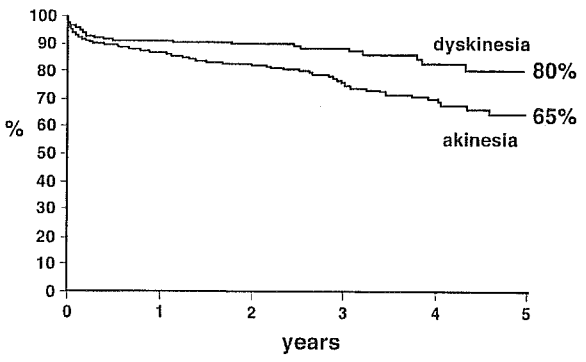

Figure 3. Survival based upon chamber morphology: dyskinesia versus akinesia.

Concomitant procedures included coronary bypass in 98%, mitral valve repair in 17%, and replacement in 4%. Mortality 30 days after operation was 13% and higher than in younger patients. Postoperative hemodynamic support with intra-aortic balloon pumping was required in 13%. The EF improved from 31% preoperatively to 39% postoperatively ($p < 0.001$). The LVESVI was reduced from 88 ml/m^2 to 60 ml/m^2 ($p < 0.001$). Overall five-year survival in the elderly was 63% and related to preoperative EF (50% if preoperative EF <30% and 73% if EF ≥30%; $p < 0.001$). Among elderly patients undergoing concomitant CABG, the five-year survival was 71% compared with 55% among patients undergoing simultaneous mitral valve procedures. The NYHA functional class III and IV was present in 70% of patients preoperatively and in 20% postoperatively. Mean NYHA functional class improved from 3.0 to 1.9. Hospital readmission for CHF at five years was 15%.

DISCUSSION

Remodeling after infarction enlarges chamber diameter and increases wall tension by Laplace's law. The augmented wall stress results in increased oxygen consumption, decreased subendocardial blood flow, and reduced systolic shortening. White et al. reported that LV volume was more predictive of survival than EF after infarction (6). Investigators in the Global Utilization of Streptokinase and t-PA for Occluded Coronary Arteries (GUSTO I) trial confirmed this and showed that LVESVI ≥40 ml/m^2 after infarction was associated with high CHF rates and poor long-term survival (3). The SVR reshapes the remodeled LV and significantly reduces chamber volume.

Ventricular shape in dilated cardiomyopathy is also an important determinant of function. As the enlarging LV changes from elliptical to spherical, normal systolic torsion is reduced. The myofibrils of the spherical LV are shifted away from their normal oblique axis toward a more transverse direction. The normal myofibril shortening of 15% generates a global EF of only 30% in spherical ventricles, as compared with an EF of 60% in elliptical ventricles with natural torsion (17). The circumferential radius of curvature increases after infarction with loss of regional EF in the remote non-infarcted myocardium (18). The Dor procedure

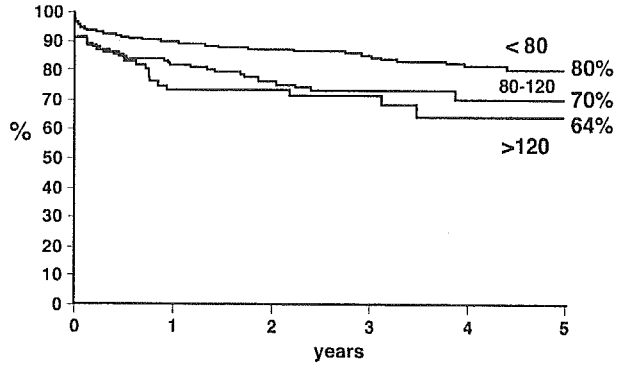

Figure 5. Survival based upon preoperative left ventricular end-systolic volume index.

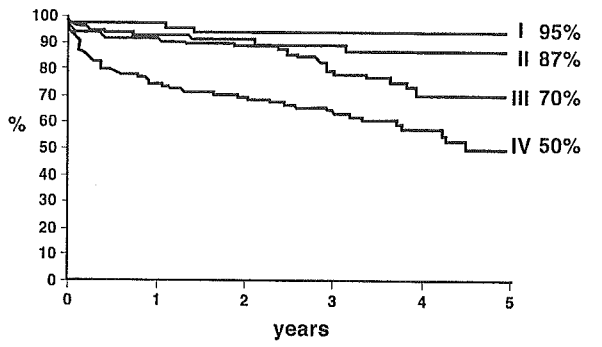

Figure 6. Survival based upon preoperative New York Heart Association functional class.

improves global systolic function by increasing regional function in remote non-infarcted segments (19). Postoperatively, LV shape becomes more elliptical in systole than it was in diastole (20).

In this RESTORE registry, SVR was used to correct LV geometry in all cases. Concomitant procedures included CABG in 95% and mitral valve intervention in 23%. Thus, the three pathologic components contributing to CHF—the ventricle, vessel, and valve—were all surgically corrected. Our integrated approach resulted in an overall five-year survival of approximately 70% and a rehospitalization for CHF of 22%. These results can be contrasted to current approaches including medical therapy, CABG alone, CABG with mitral intervention, ventricular assist devices, and transplantation.

Medical therapy. Angiotensin-converting enzyme (ACE) inhibitors increase survival among NYHA functional class IV CHF patients as shown in the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS) trial; however, only 64% survived to one year (21). Although the Carvedilol Prospective Randomized Cumulative Survival (COPERNICUS) trial demonstrated an advantage of adding carvedilol to ACE drugs in patients with EF <25%, survival at 28 months was 72% (22). Recently, the Carvedilol or Metoprolol European Trial (COMET) trial examined the efficacy of carvedilol in NYHA functional class III (48%) and IV (3%) patients with a mean EF of 26%. Survival at five years was 66% in the carvedilol treated group (23). In the Carvedilol and ACE Inhibitor Remodeling Mild Heart Failure EvaluatioN (CARMEN) trial, the majority of patients were NYHA functional class II (60% to 70%) and there were none in class IV. Event-free mortality or hospitalization was approximately 75% at two years with marginal reduction of LVESVI (7 ml/m^2) and EF improvement of 3.5% (24). Recently, spironolactone was added to ACE and beta-blocker therapy in the Randomized Aldactone Evaluation Study (RALES) trial among patients with EF ≤35%. Pre-treatment class was not reported, but the two-year survival was 65% in the treated group versus 54% in the placebo group (25).

In contrast to the above-cited trials, our RESTORE registry provides five-year follow-up in patients whose NYHA functional class was III or IV in 67% of cases. Systolic function was measured by EF and ventricular volume. Both showed dramatic improvement. Rehospitalization was very low in a high-risk patient population.

Coronary revascularization. Coronary artery bypass grafting can be safely carried out in patients with reduced systolic function because of improved methods of myocardial protection; however, the five-year survival of patients with EF ≤35% is 50% to 65% (26–29). The CABG alone is not very effective when ventricular dilation occurs. In one study, CABG mortality was 27% if LV end-diastolic diameter was ≥81 mm (30). Moreover, CHF symptoms are common after CABG for ischemic cardiomyopathy. Yamaguchi's analysis of CABG for patients with EF ≤30% showed that outcome correlated with preoperative LVESVI. The five-year survival was 54% if the preoperative LVESVI was ≥100 ml/m^2 compared with 85% if LVESVI was ≤100 ml/m^2. Congestive heart failure at five years was seen in 69% of patients with the larger hearts versus 15% with the smaller ones (31). Luciani et al. reviewed 167 patients who underwent CABG with a mean EF of 28%. Among these patients, 40% were functional class III or IV. At five years, 60% of patients continued to have signs and symptoms of CHF, demonstrating the limitations of CABG surgery alone (32). Another study of CABG for ischemic cardiomyopathy confirmed that recurrent CHF was the most common cause of death postoperatively (27).

Mitral valve repair or replacement. Functional mitral regurgitation often accompanies ventricular dilation (33). Patients with ischemic cardiomyopathy undergoing mitral procedures have a five-year mortality of approximately 50% (27,34). Recurrence of CHF occurs in one-third of patients by five years and is the most common cause of death, presumably related to continuing dysfunction of the unmodified ventricle (27,35). Mitral valve repair is an integral part of the SVR procedure in addition to volume reduction and revascularization.

Ventricular assist or replacement. Other surgical approaches in the treatment of ischemic cardiomyopathy include LVAD and transplantation. The Randomized Evaluation of Mechanical Assistance for the Treatment of

Congestive Heart Failure (REMATCH) trial examined LVAD use as long-term myocardial replacement therapy in patients who were ineligible for cardiac transplantation. Less than 10% of patients survived to three years in the LVAD group, compared with no survivors among patients treated medically (36). The five-year survival after cardiac transplantation is 70%, but few patients receive a heart owing to donor shortage (37). The five-year survival of NYHA functional class IV patients undergoing SVR in our registry was approximately 50%. Surgical ventricular restoration should be considered as a therapeutic option because it can be more readily applied compared with LVAD or transplantation.

SVR. The SVR was applied to patients with previous infarctions primarily as therapy for advanced heart failure. The few patients with less advanced symptoms of CHF, namely NYHA functional class I or II, underwent SVR as a concomitant procedure to CABG or mitral valve repair based on the important recognition that volume, not EF, is the major determinant of survival after myocardial infarction. White et al. showed that patients with LVESVI >60 ml/m^2 have approximately a fivefold increase in mortality compared with those with normal volumes after infarction (6). The GUSTO I trial similarly demonstrated the importance of ventricular volume on outcome. Among infarction patients with successful thrombolysis, 17% had progressive LV enlargement above 40 ml/m^2. Mortality at one year was 16% among those with LVESVI 40 to 50 ml/m^2, 21% with LVESVI 50 to 60 ml/m^2, and 33% with LVESVI >60 ml/m^2 (3). A small number of patients (9%) in functional class I underwent restoration as an adjunct to CABG because ventricular dilation (LVESVI >60 ml/m^2) is a precursor of late development of CHF and early death (3). More importantly, the low five-year mortality of patients with NYHA functional class II and III symptoms of CHF, and LVESVI <120 ml/m^2 further define the importance of using volume measurement to gauge the progression of CHF and how LVESVI helps determine prognosis.

Our multivariate analysis of SVR shows that major risk factors were age, preoperative EF, LVESVI, and NYHA functional class. The five-year survival of 63% among patients ≥75 years old is a gratifying result in view of the fact that CHF is common in the elderly. Mitral valve procedures were performed in over 20% of patients in this series and were more common in patients with severely depressed systolic function and extensive ventricular dilation. Regurgitation was usually central and thus amenable to ring annuloplasty. Subgroup analysis showed patients with mitral repair had larger hearts from secondary mitral regurgitation, and sustained greater improvement in LVESVI when valve repair was added to CABG and SVR. The rare use (<1%) of mitral valve replacement was indicated because of previous infarcted segments in sites other than the antero-apical or antero-septal regions. These patients were a high-risk group, and our findings are consistent with the report by Di Donato et al. (38) of decreased survival at two years (52%) if the muscle remote from the anterior scar was asynergic. These findings illustrate the importance of preoperative assessment of the non-infarcted segments supplied by the right and circumflex arteries. Imaging of the remote muscle is crucial, and SVR should be avoided if the inferior and lateral wall segments are infarcted and asynergic (19). Hypokinesia, however, is not a contraindication to operation, because local contractility may improve with revascularization. Viability of remote segments is helpful in determining operability.

Study limitations. This was a non-randomized registry of patients who underwent the SVR procedure. Drug regimens were not standardized or reported. Quality of life was based on NYHA functional class. Criteria for CHF hospital admission may have varied among centers.

Although the overall geometric objective was similar, some centers used direct closure in selected cases, and others placed an endocardial patch in all patients. Myocardial protection varied. The indication for intra-aortic balloon pumping use was not specified. Measurements of EF and LVESVI were performed by the same method in each patient before hospital discharge; however, the interval between operation and these measurements was not standardized. Implantable defibrillators were not routinely placed in these patients, and arrhythmia management in the RESTORE registry varied. In most instances, preoperative electrophysiologic testing was not performed.

Conclusions. Surgical ventricular restoration reduces volume and "restores" a more normal elliptical ventricular shape in dilated hearts after anterior infarction. Our data demonstrate a low operative mortality, improved systolic function, a gratifying five-year survival, and a low rate of rehospitalization for CHF.

Our previous results were instrumental in the design of the current Surgical Treatment for Ischemic Heart Failure (STICH) trial that randomizes patients with ischemic cardiomyopathy to medical therapy, CABG alone, or CABG with SVR. The RESTORE groups' outcomes serve as a benchmark for this trial, which should further define the optimal indications for ventricular restoration.

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APPENDIX

For a list of the RESTORE group members, please see the October 6, 2004, issue of *JACC* at http://www.cardiosource.com/jacc.html.

Evidence of Viral Infection in the Myocardium of American and Japanese Patients With Idiopathic Dilated Cardiomyopathy

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Enteroviruses have been implicated in the pathogenesis of idiopathic dilated cardiomyopathy (IDC). Recently, the association of adenovirus or parvovirus with IDC has been reported. Viral infection in the myocardium of American and Japanese patients with end-stage IDC was evaluated. Myocardial specimens from 30 American patients with IDC and 47 Japanese patients with IDC were analyzed for the presence of cardiotropic viruses. The strand-specific detection of enteroviral ribonucleic acid (RNA) was performed to determine viral activity in hearts with IDC. Established reverse transcription–polymerase chain reaction (PCR) or PCR techniques were used to detect genomic sequences of influenza viruses, mumps virus, adenovirus, parvovirus, herpes simplex viruses, varicella-zoster virus, and Epstein-Barr virus. Enteroviral RNA was detected in 7 of the 30 American patients (23%) and in 15 of the 47 Japanese patients (32%). Minus-strand enteroviral RNA, an indicator of active enteroviral RNA replication, was demonstrated in 5 of 7 plus-strand-positive American patients (71%) and in 12 of 15 plus-strand-positive Japanese patients (80%). Sequence analysis revealed that the viruses detected were Coxsackie B viruses. No genomic sequences of other viruses were detected in the myocardium of either American or Japanese patients with IDC. Therefore, active group B Coxsackie virus RNA replication in the myocardium was demonstrated in a significant proportion of American and Japanese patients with end-stage IDC. There was no evidence of persistent infection by other viruses in hearts with IDC. Specific therapy should be designed for Coxsackie virus positive patients with IDC. ©2004 by Excerpta Medica, Inc.

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Enteroviruses have been implicated in the pathogenesis of idiopathic dilated cardiomyopathy (IDC).[1,2] This concept was originally supported by the long-term outcomes of some patients with acute myocarditis,[3] serologic analysis for viral antibodies,[4,5] and studies of animal models.[6] For the past decade, enteroviral ribonucleic acid (RNA)[7–11] and its antigen[12] have been demonstrated in the myocardium of patients at all stages of IDC. Recently, more studies have revealed a significant role for enteroviruses in the pathogenesis of IDC.[13] Specifically, transgenic mouse experiments clearly demonstrate that the low-level expression of the Coxsackie viral genome in the heart can cause dilated cardiomyopathy with characteristics that are similar to those seen with IDC in humans.[14] However, little is known about the character and activity of enteroviruses detected in the myocardium of patients with IDC. More recently, adenovirus[15] and parvovirus[16] have been proposed as causative agents of IDC. Because other viruses can also induce myocarditis,[17] there is a possibility that other potentially cardiotropic viruses are associated with the development of IDC. However, the worldwide prevalence of viral infection in hearts with IDC has not been investigated. In this study, we evaluated infection with a variety of viruses in the myocardium of American and Japanese patients with end-stage IDC.

METHODS

Patient population: Thirty patients with IDC who received heart transplants at Henry Ford Hospital and 47 patients with IDC who underwent partial left ventriculectomy (PLV) at Osaka Medical College Hospital or Shonan Kamakura General Hospital were included in this study. The clinical diagnosis of IDC was made according to World Health Organization and International Society and Federation of Cardiology criteria.[18] All patients underwent noninvasive and invasive evaluation, including echocardiography and cardiac catheterization with coronary angiography. All myocardial specimens resected during heart transplantation and PLV were subjected to histopathologic examination. All samples were immediately frozen in

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