Targeting Phospholamban by Gene Transfer in Human Heart Failure

Federica del Monte, MD, PhD; Sian E. Harding, PhD; G. William Dec, MD; Judith K. Gwathmey, VMD, PhD; Roger J. Hajjar, MD

**Background**—Myocardial cells from failing human hearts are characterized by abnormal calcium handling, a negative force-frequency relationship, and decreased sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA2a) activity. In this study, we tested whether contractile function can be improved by decreasing the inhibitory effects of phospholamban on SERCA2a with adenoviral gene transfer of antisense phospholamban (asPL).

**Methods and Results**—Myocardial cells isolated from 9 patients with end-stage heart failure and 18 donor nonfailing hearts were infected with adenoviruses encoding for either the antisense of phospholamban (Ad.asPL), the SERCA2a gene (Ad.SERCA2a), or the reporter genes β-galactosidase and green fluorescent protein (Ad.βgal-GFP). Adenoviral gene transfer with Ad.asPL decreased phospholamban expression over 48 hours, increasing the velocity of both contraction and relaxation. Compared with cardiomyocytes infected with Ad.asPL (n=13), human myocytes infected with Ad.βgal-GFP (n=8) had enhanced contraction velocity (20.3±3.9% versus 8.7±2.6% shortening/second; P<0.01) and relaxation velocity (26.0±6.2% versus 8.6±4.3% shortening/second; P<0.01). The improvement in contraction and relaxation velocities was comparable to cardiomyocytes infected with Ad.SERCA2a. Failing human cardiomyocytes had decreased contraction and Ca\(^{2+}\) release with increasing frequency (0.1 to 2 Hz). Phospholamban ablation restored the frequency response in the failing cardiomyocytes to normal; increasing frequency resulted in enhanced sarcoplasmic reticulum Ca\(^{2+}\) release and contraction.

**Conclusion**—These results show that gene transfer of asPL can improve the contractile function in failing human myocardium. Targeting phospholamban may provide therapeutic benefits in human heart failure. (*Circulation. 2002; 105:904-907.)*

**Key Words:** calcium ■ heart failure ■ gene therapy

Congestive heart failure has been intimately associated with abnormalities in intracellular [Ca\(^{2+}\)] handling.\(^1\) In the failing heart, resting [Ca\(^{2+}\)] is elevated, the amplitude of the [Ca\(^{2+}\)] transient is decreased, and its duration is prolonged.\(^2\) These changes are the direct result of an abnormal sarcoplasmic reticulum (SR) Ca\(^{2+}\) ATPase pump (SERCA2a), the activity of which is reduced in heart failure.\(^3–5\) SERCA2a is regulated by phospholamban, the phosphorylation of which relieves the inhibition of SERCA2a. The phospholamban/SERCA2a interaction controls the calcium content of the SR and ultimately controls cardiac contractility.\(^6–7\) A decrease in the phosphorylation of phospholamban, along with an increase in the phospholamban/SERCA2a ratio, contributes to contractile dysfunction in heart failure.\(^3–5\) Contractility, calcium handling, and the frequency response were restored in isolated failing human cardiomyocytes by restoring this ratio by means of a gene transfer that caused the overexpression of SERCA2a.\(^8\) Another approach to restoring the phospholamban/SERCA2a ratio in failing hearts to normal would be to decrease levels of phospholamban through the use of antisense strategies.

In the present study, we have used such a strategy by generating adenoviral vectors expressing antisense of phospholamban and examining their effects on phospholamban expression, SERCA2a activity, and myocyte function in failing human cardiomyocytes.

**Methods**

Failing human ventricular myocardial tissue was obtained from 9 explanted hearts (5 ischemic and 4 with dilated cardiomyopathy), and nonfailing tissue was obtained from 18 donor hearts. Myocytes were isolated from 1 g of myocardial tissue removed from the free wall of the left ventricle by enzymatic digestion, as described previously.\(^4\) The proportion of rod-shaped viable cells at the time of isolation was 30% to 50% (n=18) for failing and 40% to 60% (n=18) for nonfailing cardiomyocytes. After isolation, the cells were resuspended in M199, 50 U/mL penicillin, and 50 U/mL streptomycin; equilibrated to pH 7.4; and infected with the adenoviruses at a multiplicity of infection of 100.

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The antisense cDNA of phospholamban was first cloned into a shuttle vector, pAdTrack-CMV. The resultant plasmid was linearized with restriction endonuclease Pmel and subsequently was cotransformed into E.coli BJ5183 cells with an adenoviral backbone plasmid, pAdEasy-1. Recombinants were selected for kanamycin resistance, and recombination was confirmed by multiple-restriction endonuclease analyses. The linearized recombinant plasmid was transfected into adenovirus-packaging cell lines (293 cells). Ad., endonuclease analyses. The linearized recombinant plasmid was transfected into adenovirus-packaging cell lines (293 cells). Ad., which contains both β-galactosidase and green fluorescent protein (GFP) controlled by separate cytomegalovirus promoters, was used as control. Ad.SERCA2a, which carries both the SERCA2a protein (GFP) controlled by separate cytomegalovirus promoters, was used as control. Ad.SERCA2a, which carries both the SERCA2a protein (GFP) controlled by separate cytomegalovirus promoters, was used as control. Ad.asPL showed a 50% decrease in the expression of phospholamban, as shown in Figure 1A. SERCA2a measured from failing cardiomyocytes showed a decrease compared with nonfailing cardiomyocytes. Both ablation of phospholamban and increased SERCA2a overexpression enhanced ATPase activities. However, the increase in ATPase activity was larger in cells overexpressing SERCA2a, as shown in Figure 1B. Figure 1C shows tracings from representative cardiomyocytes isolated from a donor nonfailing heart and from a failing heart infected with an adenovirus expressing either Ad.asPL, Ad.$\beta$gal-GFP, Ad.$\beta$gal-GFP, or Ad.SERCA2a, stimulated at 1 Hz at 37°C. The failing cells had a characteristic decrease in contraction and prolonged relaxation, along with a prolonged Ca2+ transient. Ablation of phospholamban in the failing cardiomyocytes normalized these parameters.

4 mmol/L. However, cells overexpressing SERCA2a were more tolerant to the higher calcium concentrations than were the cells infected with Ad.asPL.

Phospholamban plays an important role in modulating the response to β-adrenergic stimulus because its phosphorylation leads to increased ATPase activity10 and acceleration of both contraction and relaxation. Addition of isoproterenol in nonfailing cardiomyocytes enhanced contraction but also was associated with aftercontractions (data not shown). Ablation of phospholamban increased contraction and significantly decreased the time of contraction, as depicted in Figure 2B. With the substantial decrease in the time course of contraction in failing human cardiomyocytes infected with Ad.asPL, the addition of isoproterenol still had additive effects on the time course of contractions, as shown in Figure 2B.
Discussion

The present study revealed important findings with regard to targeting calcium cycling proteins in failing human cardiomyocytes: (1) Decreasing phospholamban expression restores contractility in failing ventricular cells of different etiologies; and (2) ablation of phospholamban results in improvement in contractility similar to that of SERCA2a overexpression.

Studies by Kranias et al.\(^6,7\) have shown clearly that murine models of phospholamban knockout have enhanced contractility. Furthermore, ablation of phospholamban prevented the development of heart failure and restored cardiac function in several murine models of heart failure, including muscle lim protein–knockout (MLP\(^{-/-}\)) mice.\(^{11}\) Our results show that improving calcium cycling by decreasing phospholamban inhibition to SERCA2a restores contractility in failing human ventricular cardiomyocytes. These findings also extend previous results showing that overexpression of SERCA2a improves contractile function in human failing cardiac myocytes.

Even though the two strategies—phospholamban decrease and overexpression of SERCA2a—improved contractile function in the failing human cardiomyocytes to the same extent, there were certain differences. SERCA2a activity increased to a greater degree in cardiomyocytes overexpressing SERCA2a. In failing cardiomyocytes, relieving inhibition to SERCA2a pumps, which may be impaired because of oxidative stresses,\(^{12}\) may not restore ATPase activity to normal.

<table>
<thead>
<tr>
<th></th>
<th>% Contraction</th>
<th>Contraction Velocity, %/s</th>
<th>Relaxation Velocity, %/s</th>
<th>(\tau), s Cells,</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad.(\beta)gal-GFP</td>
<td>2.7 ± 1.0</td>
<td>8.7 ± 2.6</td>
<td>8.6 ± 4.3</td>
<td>0.6 ± 0.13</td>
<td>8</td>
</tr>
<tr>
<td>Ad.asPL</td>
<td>5.0 ± 0.9*</td>
<td>20.3 ± 3.9*</td>
<td>26.0 ± 6.2*</td>
<td>0.29 ± 0.05*</td>
<td>13</td>
</tr>
<tr>
<td>Ad.SERCA2a</td>
<td>4.9 ± 1.8*</td>
<td>25.4 ± 8.8*</td>
<td>27.4 ± 10.0*</td>
<td>0.2 ± 0.04*</td>
<td>9</td>
</tr>
</tbody>
</table>

\(\tau\) indicates time course of relaxation as a function of increasing extracellular calcium.

*\(P<0.01\) compared with Ad.\(\beta\)gal-GFP.

Figure 2. A, Contractile parameters, including percent shortening, velocity of contraction, velocity of relaxation, and \(\tau\) (time course of relaxation as a function of increasing extracellular calcium), in human cardiomyocytes infected with Ad.asPL (n = 6), Ad.SERCA2a (n = 6) or Ad.\(\beta\)gal-GFP (control, n = 8). B, Tabulated effects of isoproterenol in human cardiomyocytes infected with either Ad.\(\beta\)gal-GFP or Ad.asPL (control, n = 5; +isoproterenol, n = 14). *\(P<0.01\) compared with Ad.\(\beta\)gal-GFP.
One concern about the strategy of increasing SERCA2a by diminishing phospholamban inhibition is the energy cost, which would be anticipated to increase ATP hydrolysis. Our group has recently shown that overexpression of SERCA2a in a rat model of heart failure enhances contractility without energetic compromise. In fact, in this model of heart failure, overexpression of SERCA2a restored the balance between ATP and creatine phosphate.

Another concern with the antisense approach is that it uncouples the β-receptors from one of their downstream targets, phospholamban, rendering them “spare receptors.” This would decrease the modulatory effects of isoproterenol. Interestingly, though, with phospholamban ablation, the contractile state of the cardiomyocyte at baseline is high. Furthermore, the human heart has only a small receptor reserve (if any) for β-adrenoceptors. It is unclear from our study what the long-term effects of functionally increasing these spare receptors will be. Also, the ratio of the Na/Ca exchanger to SERCA2a has been shown to be increased in failing hearts and to be predictive of diastolic function in these hearts. Even though ablation of phospholamban would not affect the ratio of SERCA2a to Na/Ca, the resultant enhancement of SERCA2a activity would contribute to restoring diastolic function.

Our results demonstrate that targeting calcium regulation by ablation of phospholamban improves contractile function in failing human cardiomyocytes. This study validates the feasibility of cardiac gene transfer in failing hearts as a therapeutic modality.

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References

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