β-Adrenergic Receptor Number and Adenylate Cyclase Function in Denervated Transplanted and Cardiomyopathic Human Hearts

A. Robert Denniss, MD, James D. Marsh, MD, Rebecca J. Quigg, MD, John B. Gordon, MD, and Wilson S. Colucci, MD

To test the hypothesis that there is up-regulation of β-adrenergic receptor density or supersensitivities of β-adrenergic receptor–stimulated adenylate cyclase in the denervated transplanted human heart, we studied myocardium from transplanted, normal, and failing hearts. Myocardium was obtained from 10 patients 9±3 months after cardiac transplantation, from 10 patients without cardiac disease, and from eight patients with symptomatic congestive heart failure due to idiopathic cardiomyopathy. β-Adrenergic receptor density in transplanted myocardium (15±3 fmol/mg protein, 1.20±0.14 fmol/mg DNA) was not different from that in normal myocardium (22±3 fmol/mg protein, 1.46±0.13 fmol/mg DNA; p=NS for both). In myocardium from cardiomyopathic hearts, β-adrenergic receptor density was markedly reduced (8±2 fmol/mg protein, 0.84±0.13 fmol/mg DNA; p<0.05 and p<0.01 vs. normal myocardium, respectively). Likewise, the response of adenylate cyclase to isoproterenol in transplanted myocardium was not significantly different from that in normal myocardium, but the response was markedly depressed in cardiomyopathic myocardium. Although forskolin-stimulated adenylate cyclase activity was similar in all three groups, guanine nucleotide–stimulated adenylate cyclase activity was markedly reduced in transplanted myocardium (20±17 vs. 78±13 pmol/mg/min for normal myocardium, p<0.01) and to a lesser degree in cardiomyopathic myocardium (39±14 pmol/mg/min, p<0.03 vs. normal myocardium). Thus, there is no evidence of β-adrenergic receptor up-regulation or supersensitivity in denervated transplanted human myocardium. Moreover, the depressed adenylate cyclase response to guanine nucleotide stimulation in transplanted and cardiomyopathic myocardium indicates that in these conditions there may be an alteration in the expression or function of one or more guanine nucleotide regulatory proteins. (Circulation 1989;79:1028–1034)

Numerous in vitro studies suggest that there is an inverse relation between the level of adrenergic stimulation and the density of β-adrenergic receptors in a variety of target tissues, including myocardium.1 The availability of myocardium from patients with severe congestive heart failure, a condition associated with elevated levels of circulating catecholamines and increased sympathetic nerve activity,2 has allowed demonstration of a substantial decrease in the density of myocardial β-adrenergic receptors that is associated with significant decreases in the adenylate cyclase and contractile responses to β-adrenergic receptor stimulation.3–5 Conversely, because of denervation, the transplanted human heart may exhibit “up-regulation” of β-adrenergic receptor density6,7 and therefore would be supersensitive to β-adrenergic receptor stimulation. Studies in animal models of cardiac transplantation or denervation have shown an increase in myocardial β-adrenergic receptor density consistent with this hypothesis.8,9 At least two studies in patients7,10 have suggested that there is increased chronotropic responsiveness to β-adrenergic receptor stimulation after cardiac transplantation. However, tissue from human heart has not been examined to test directly the hypothesis that the denervated...
transplanted human heart exhibits up-regulation of β-adrenergic receptor density associated with increased activation of adenylate cyclase in response to β-adrenergic receptor stimulation.

The purpose of this study was to determine directly whether or not denervated transplanted human myocardium exhibits up-regulation of β-adrenergic receptor density or increased responsiveness of adenylate cyclase to β-adrenergic stimulation. β-Adrenergic receptor density and adenylate cyclase activation by isoproterenol, guanine nucleotides, and forskolin were determined in myocardial samples obtained from 10 clinically stable patients an average of 9±3 months after cardiac transplantation and from 10 patients without known cardiac disease. To establish further the ability of these methods to distinguish alterations in β-adrenergic receptor density or adenylate cyclase responsiveness, we also evaluated eight patients with end-stage congestive heart failure and elevated circulating catecholamines.

β-Adrenergic receptor density and isoproterenol-stimulated adenylate cyclase activity were not increased in transplanted myocardium. Surprisingly, guanine nucleotide-stimulated adenylate cyclase activation was substantially depressed in the transplanted myocardium. These data do not support the hypothesis that denervation leads to myocardial β-adrenergic receptor up-regulation or supersensitivity, and they suggest an alteration in adenylate cyclase function in transplanted myocardium.

**Methods**

**Patient Characteristics**

Specimens of right ventricular endomyocardium were obtained from 28 patients representing three groups: 1) postorthotopic cardiac transplantation (n=10), 2) idiopathic cardiomyopathy (n=8), and 3) normal cardiac function (n=10).

Patients who were studied after cardiac transplantation ranged in age from 14 to 58 years (mean, 41±5 years), and all were stable clinically without histologic evidence of significant rejection at the time of the study. Transplantation occurred from 1 to 21 months (mean, 9±3 months) before study. Immunosuppressive therapy consisted of corticosteroids in 10 patients, azathioprine in seven, and cyclosporin-A in 10. Resting hemodynamic and plasma catecholamine levels (Table 1) were within normal limits except for a mild tachycardia, which is consistent with vagal denervation.

Patients with idiopathic cardiomyopathy ranged in age from 18 to 75 years (mean, 44±5 years). All had symptomatic New York Heart Association functional Class III or IV congestive heart failure despite therapy with digitalis, diuretics, and vasodilators. No patient had received a β-adrenergic agonist or antagonist before study. Ischemic heart disease and active myocarditis were excluded in all cases by the results from coronary angiography and myocardial biopsy. Resting hemodynamic indexes were markedly abnormal: tachycardia at rest, reduced cardiac index, and elevated right and left heart filling pressures (Table 1). Resting plasma norepinephrine and epinephrine were significantly elevated, which is consistent with symptomatic congestive heart failure.

Patients with normal cardiac function ranged in age from 22 to 58 years (mean, 34±3 years), and all had died of noncardiac causes within 1–4 days after institution of life support measures. Patients were brain-dead organ donors from whom kidney and heart valves were being collected. Absence of significant anatomic coronary or myocardial disease was confirmed by detailed pathologic study in all cases.

**Tissue Procurement**

Right ventricular endomyocardium (weight, 3–25 mg) from 18 patients was obtained by endomyocardial biopsy from the right ventricle as described by Fowler et al. Briefly, a 9F sheath was inserted percutaneously into the right internal jugular vein under local anesthesia. After right heart and pulmonary artery wedge pressures were measured with a balloon-tipped catheter, a 50-cm right ventricular
bioptome was inserted through the sheath to the right ventricle and positioned against the interventricular septum. In this study, two or three specimens were obtained for β-adrenergic receptor analysis. For patients with cardiomyopathy, ventricular tissue was obtained from the explanted heart at the time of cardiac transplantation. Ventricular tissue was frozen in liquid nitrogen within 5 minutes of explanting the heart. Similarly, normal cardiac tissue was collected and frozen within 5 minutes of terminating circulatory support in noncardiac organ donors. Control experiments showed that receptor-binding and adenylate cyclase properties were indistinguishable between membranes from fresh tissue and membranes from tissue frozen in liquid nitrogen.

Receptor-Binding Studies

β-Adrenergic density was assessed with the β-adrenergic receptor ligand 125I-iodopindolol. 125I-Iodopindolol binding was characterized previously in this laboratory for chick and human myocardium. Specimens of right ventricular endomyocardium were warmed to 4°C immediately before membrane preparation. Tissue was then immersed in ice-cold buffer (10 mM NaHCO3 plus 10 mM histidine, pH 7.5) and homogenized with three 10-second bursts at full speed on a Polytron homogenizer (Brinkmann Instruments, Westbury, New York). The homogenate was centrifuged at 40,000g (Servall RC-5, Wilmington, Delaware) for 30 minutes. The pellets containing sarcosomal membranes were resuspended in fresh ice-cold assay buffer (150 mM NaCl; 10 mM Tris; pH, 7.5) by 20 strokes of a tight-fitting Dounce homogenizer (Sigma Chemical, St. Louis, Missouri). The entire preparation was performed at 4°C.

The membrane preparations (300 μl) were added to tubes containing assay buffer and 200 pM I-iodopindolol with or without 10−4 M (-) isoproterenol in a final volume of 450 μl. Ascorbic acid (10−4 M) was present in all assay tubes to prevent isoproterenol oxidation. The assay mixture was incubated with shaking at 37°C for 120 minutes, and the reaction was stopped by addition of 3 ml ice-cold buffer (10 mM Tris, 150 mM NaCl), followed by rapid filtering under controlled vacuum through glass fiber filters presoaked in 0.5% aqueous polyethyleneimine solution (Whatman GF/B, Clifton, New Jersey) with a multiple port cell harvester (Brandel M-24R). The tubes were washed three times with 5 ml ice-cold buffer, dried under high vacuum, and counted at 69% efficiency in a gamma counter (LKB Instruments, Gaithersburg, Maryland).

In preliminary experiments, β-adrenergic receptor density was determined by a saturation point assay for normal and cardiomyopathic heart and was related to values determined from 18-point binding curves. For competition-binding curves, membranes were incubated with 18 graded concentrations of isoproterenol (from 1×10−4 to 1×10−10 M) and 125I-iodopindolol (from 20 to 50 pM). For these assays, pieces of tissue (50–100 mg) from normal and cardiomyopathic heart were used. A good correlation was found between β-receptor number determined from the binding curves and determinations obtained by assaying with a saturating concentration of 125I-iodopindolol with and without isoproterenol (equation of best fit: y=0.92±1.31; r=0.87, p<0.001). Subsequently, and as reported in this paper, β-receptor density was determined by saturation point assay for normal, transplanted, and cardiomyopathic tissue.

Adenylate Cyclase Assay

Adenylate cyclase activity was assayed from samples of frozen tissue homogenates by a modification of the method of Krishna et al as described by Neer. Tissue preparation was identical to that for the binding assay. The concentrations of the reactants were 7 mM 3H-ATP, 50 units creatine kinase, 1 μM creatinine phosphate, 0.08% bovine serum albumin, 5.7 mM MgCl2, 1×10−4 M Tris. The tubes were incubated at 37°C in a shaking water bath for 10 minutes, and the reaction was stopped by adding a 100-μl solution containing 50 mM Tris, 223 mM ATP, and 3.4 mM carrier cAMP. 14C-cAMP was added (800–1,000 cpm) to each assay tube to permit calculation of recovery of cAMP, which was 40–60%. The mixture was diluted with 300 μl water and was chromatographed on Dowex AG50W-X2. The columns were eluted with water, and a 1.5-ml fraction was collected. After precipitation twice with 5% ZnSO4 and saturated with BaOH to remove noncyclic nucleotide, the supernatant was assayed for radioactivity in a liquid scintillation spectrometer (LKB Instruments). cAMP production was assayed under basal conditions and with the addition of the following activators of components of the cyclase cascade: 2×10−5 M 5-guanylylimidodiphosphate [Gpp(NH)p], Gpp(NH)p plus 2×10−5 M isoproterenol, and 1×10−2 M MnCl2 plus 2×10−5 M forskolin. All assays were performed in triplicate. This assay method permits excellent separation (99%) of 3H-cAMP from other 3H products.

Protein and DNA Measurements

Protein concentrations were measured by the micro-Bradford method (Bio-Rad Labs, Cambridge, Massachusetts) with bovine serum albumin as standard. DNA concentrations were measured by fluorescence spectrometry with Hoechst dye 33258 with human placental DNA as standard.

Reagents

125I-Iodopindolol was from New England Nuclear (Boston, Massachusetts). 3H-ATP and 14C-cAMP were from Amersham (Arlington Heights, Illinois). Isoproterenol, Gpp(NH)p, and forskolin were from Sigma Chemical (St. Louis, Missouri). Creatine kinase and creatine phosphate were from Boehringer (New York, New York).
Statistical Analysis

Student’s t test was used for comparisons when data were normally distributed. The Mann-Whitney U test was used for nonparametric data. Results were considered statistically significant when p was less than 0.05 in a two-tailed t test. Results are reported as the mean±SEM.

Results

β-Adrenergic Receptor Number

Figure 1 and Table 2 show the β-adrenergic receptor densities for the myocardium from normal, post-transplantation, and cardiomyopathic hearts. The cardiomyopathic hearts, but not the transplanted hearts, had a significantly lower density of β-adrenergic receptors than did the normal control hearts. It is evident from Figure 1 that β-adrenergic receptor density in normal and transplanted hearts varied over a considerable range, whereas β-adrenergic receptor density in cardiomyopathic hearts was generally uniform and substantially reduced.

There was no apparent correlation between β-adrenergic receptor density and the time after transplantation in the transplanted hearts. The β-adrenergic receptor density of the five most recently transplanted hearts (17±4 fmol/mg) was not significantly different from that of the five most remotely transplanted hearts (13±3 fmol/mg, p=0.50). Likewise, there was no significant relation between β-adrenergic receptor density and age of the patient or between β-adrenergic receptor density and the age of the donor heart for transplant patients. To determine whether or not immunosuppressive therapy modifies receptor number, receptor properties of subgroups of patients were examined. All transplant patients were receiving cyclosporine and corticosteroids at time of study; seven were receiving azathioprine, and three were not. For those receiving azathioprine, receptor number was 13.3±3 fmol/mg. The three patients not receiving azathioprine had receptor numbers of 15.4, 31.1, and 12.0 fmol/mg.

Adenylate Cyclase Activity

To probe the adenylate cyclase pathway, we examined cAMP stimulation by Gpp(NH)p (G-protein function), the incremental stimulation of Gpp(NH)p plus isoproterenol compared with Gpp(NH)p alone (β-adrenergic receptor function), and the response to manganese plus forskolin (catalytic unit function).

Basal adenylate cyclase activities were similar in the three groups (normal, 8±5; transplanted, 9±1; cardiomyopathic, 8±5 pmol/cAMP/mg/min). Gpp(NH)p stimulated cAMP production in normal hearts and, to a significantly lesser extent, in cardio-
myopathic hearts. In myocardium from transplant patients, Gpp(NH)p did not cause significant stimulation above basal values. The response to Gpp(NH)p was less for the transplanted tissue than for normal tissue (p = 0.009). The incremental stimulation over basal activity caused by addition of isoproterenol to Gpp(NH)p was significant in all three groups. When compared with the response in normal tissue, transplanted and cardiomypathic tissue tended toward a smaller response to isoproterenol plus Gpp(NH)p (p = 0.11 and 0.13, respectively). Likewise, although not statistically different, the response to forskolin plus manganese tended to be smaller in transplanted and cardiomyopathic myocardium.

The role of differences in immunosuppressive therapy was examined for adenylate cyclase activities. For patients receiving azathioprine, the adenylate cyclase activities in response to Gpp(NH)p and Gpp(NH)p plus isoproterenol were 25 ± 16 and 100 ± 45 pmol/mg/min, respectively; for the three patients not receiving azathioprine, adenylate cyclase activities in response to Gpp(NH)p and Gpp(NH)p plus isoproterenol were 25 ± 12 and 59 ± 35 pmol/mg/min, respectively (p = NS between patients receiving and not receiving azathioprine).

**Discussion**

The major new findings of this study are that β-adrenergic receptor density and isoproterenol-stimulated adenylate cyclase activation are not significantly increased in myocardium from patients after undergoing transplantation. Based on prior animal studies, studies of the chronotropic response to β-adrenergic stimulation in patients after heart transplantation, and a substantial number of in vitro studies, investigators have hypothesized that in humans the denervated transplanted myocardium would exhibit end-organ β-adrenergic supersensitivity associated with an increase in β-adrenergic receptor density. The data in this study clearly do not support this hypothesis, and if anything, they suggest a slight decrease in β-adrenergic receptor density in transplanted myocardium. A similar conclusion was reached by Gilbert et al., who also found a slight decrease in β-adrenergic receptor density in transplanted myocardium.

A potential limitation of this study is that the “normal” myocardium was obtained from patients, many of whom were exposed to states of stress associated with increased sympathetic nervous system activation or infusion of sympathomimetic agents before recovery of the myocardium or both. However, if such exposure affected the β-adrenergic receptor pathway, the anticipated effect would be a decreased β-adrenergic receptor density or adenylate cyclase responsiveness or both. That is, the normal tissue might tend to have a relatively low β-adrenergic receptor number and thus accentuate any up-regulation of receptors in transplanted myocardium. Despite this potential bias toward showing up-regulation in transplants, we found the opposite. Therefore, these data may underestimate the decrease in β-adrenergic receptor density or responsiveness in transplanted compared with normal hearts. Of note, compared with normal hearts, β-adrenergic receptor down-regulation and desensitization were clearly evident in the cardiomyopathic myocardium, suggesting that any desensitization existing in our normal myocardium was less than that in hearts from patients with severe congestive heart failure.

All cardiac transplant recipients in this study were receiving corticosteroids as part of their immunosuppressive regimen. Corticosteroids have no effect on the binding affinity of an antagonist ligand and tend to increase the number of β-adrenergic receptors. Although corticosteroids might be expected to amplify denervation-induced receptor up-regulation, no evidence of such an effect was found.

Of necessity, the amount of tissue obtained by right ventricular biopsy is limited (typically <12 mg wet wt/patient). This precluded the determination of full binding curves for biopsy tissue from transplanted hearts, and thus, it is not possible to exclude absolutely a change in Kd for antagonist binding. Corticosteroid treatment that the transplant patients received does not change antagonist Kd and antagonist Kd is not changed in normal compared with cardiomyopathic hearts (Denniss et al, manuscript submitted). For these reasons, we believe that there is probably not a major change in antagonist Kd or a major error in our estimation of receptor number. Finally, it is reassuring that the relative densities of β-adrenergic receptor in normal and transplanted myocardium in this study are very similar to those recently reported by Gilbert et al.

Our data do not explain the finding of Yusuf et al that the heart rate response to isoproterenol infusion is increased in transplanted hearts. At least two mechanisms should be considered. First, we studied only ventricular myocardium, and thus, our data do not exclude the possibility that denervation may result in a localized up-regulation of β-adrenergic receptor in atrial or sinoatrial tissue. Differential regulation of β-adrenergic receptor in atrial and ventricular tissue has been reported. Because atrial sympathetic innervation is more dense than that of the ventricular myocardium, atrial β-adrenergic receptor density may be tonically down-regulated relative to that in ventricular myocardium.

An alternative explanation is that the increased heart rate response to isoproterenol observed by Yusuf et al was due to lack of vagal tone in their transplanted, but not normal, hearts. In the study by Yusuf et al, the normal subjects had intact cardiac vagal efferents and did not receive atropine, and therefore, they may have been more sensitive to a baroreceptor-mediated negative chronotropic action in response to the isoproterenol-stimulated increase in pulse pressure. Gilbert et al and we have recently observed that when normal control
subjects are pretreated with atropine the heart rate responsiveness to isoproterenol in the normal and transplanted subjects is the same. The biochemical findings in our present study are consistent with the human physiologic findings of Gilbert et al. and Quigg et al., both of whom found no evidence of postsynaptic chronotropic supersensitivity to \( \beta \)-adrenergic receptor stimulation in transplanted hearts.

There is evidence that \( G_s \) or \( G_i \) or both in myocardium may be quantitatively or qualitatively altered in heart failure. Recently, Feldman et al. found that basal, guanine nucleotide–stimulated, and forskolin-stimulated adenylate cyclase activities were reduced approximately 30% in failing compared with nonfailing human myocardium. This reduction in adenylate cyclase activity was associated with a 36% increase in the 40,000 molecular weight pertussis toxin substrate (\( G_\beta \)), but no alteration in \( G_s \), and was corrected by pretreatment with pertussis toxin. Our finding that guanine nucleotide–stimulated adenylate cyclase activation is significantly reduced in failing hearts confirms that of Feldman et al. Two aspects of our data differ from those of Feldman et al. First, basal and forskolin-stimulated adenylate cyclase activities were not reduced in our failing hearts. Second, despite similar basal and forskolin-stimulated activities, the magnitude of guanine nucleotide stimulation was substantially higher in our study. These differences may relate to several potential factors such as patient selection, tissue handling, and assay conditions.

In myocardium from the patients with cardiomyopathy, there was a significant decrease in \( \beta \)-adrenergic receptor density and a substantial reduction in isoproterenol-stimulated adenylate cyclase activation. The latter was not statistically significant \(( p = 0.13)\) because of the large variance. These observations are consistent with several prior studies and support the proposition that if there was a substantial change in \( \beta \)-adrenergic receptor number in transplanted hearts, we would have detected it.

An unexpected finding of this study is that the adenylate cyclase response to Gpp(NH)p is significantly reduced in transplanted myocardium, and in fact, the response is more profoundly attenuated than in the failing hearts. The relatively normal response to forskolin in transplanted myocardium suggests that the defect may be at the level of one or more of the guanine nucleotide–sensitive proteins that regulate adenylate cyclase activity. Because the response to a guanine nucleotide is due to its action on both stimulatory \(( G_s \) and inhibitory \(( G_i \) regulatory proteins, these data do not allow conclusions regarding the precise molecular basis of this abnormal functional response. However, an intriguing possibility raised by these data is that normal guanine nucleotide regulatory protein expression depends on cardiac innervation. This thesis was suggested by Morris and Bilezikian, who showed that appearance of \( G_s \) function was coincident with cardiac innervation in the rat, and this is supported by the data of Liang et al., who showed that expression and function of \( G_i \) is modulated by cardiac parasympathetic innervation.

Taken together, these observations on the density of \( \beta \)-adrenergic receptor and the adenylate cyclase response to isoproterenol in transplanted and cardiomyopathic myocardium suggest that although the \( \beta \)-adrenergic pathway can be significantly desensitized in the setting of increased sympathetic nervous system activation, the converse is not necessarily true. Our data do not support the postulate that in humans, denervation results in increased myocardial \( \beta \)-adrenergic receptor density or responsiveness. Alternatively, the data raise the possibility that cardiac \( \beta \)-adrenergic receptor density is regulated by circulating catecholamine levels. These data further raise the interesting possibility that cardiac autonomic innervation regulates adenylate cyclase function in adult myocardium.

References


**Key Words**: β-adrenergic receptors • adenylate cyclase • cardiac transplantation • cardiomyopathy
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